STUDIES ON CAMPYLOBACTER SPUTORUM SUBSPECIES MUCOSALIS INFECTION IN PIGS.

VOLUME I

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"A science is any discipline in which the fool of this generation can go beyond the point reached by the genius of the last generation."

-Max Gluckman.

"I know why there are so many people who love chopping wood. In this activity one immediately sees the results."

-Albert Einstein.

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SUMMARY

The primary aim of the work reported in this thesis was to transmit intestinal adenomatosis to experimental piglets, thus enabling a study of the role of *Campylobacter sputorum* subspecies *mucosalis* (*mucosalis*) in the pathogenesis of the disease.

Field cases of porcine intestinal adenomatosis (PIA) were studied, initially to provide experience for the author in the techniques used, and later to obtain infective mucosa and strains of *mucosalis* with which to orally dose experimental piglets.

Naturally-farrowed, cross-suckled piglets were exposed to adenomatous mucosa and cultures of *mucosalis*. *Mucosalis* was not isolated from the enteric mucosa at necropsy and no lesions of adenomatosis were found, hence alternative approaches to the problem were tried.

Naturally-farrowed, colostrum-deprived piglets were exposed to adenomatous mucosa, but did not survive for long enough to assess whether adenomatous change would have occurred. The absence of maternally-acquired immunity did not appear to enhance greatly the establishment of *mucosalis* in the gut.

Gnotobiotic piglets were exposed to cultures of *mucosalis* alone or in combination with rotavirus. *Mucosalis* established and persisted in the lumen

of the gut in both groups but lesions of adenomatosis did not develop.

Weaned pigs, exposed to either dual *mucosalis* and rotavirus, or dual *mucosalis* and enterotoxigenic *Escherichia coli* infections, did not develop adenomatosis and *mucosalis* was not isolated from the gut at necropsy.

Finally a series of experiments was carried out, on both milk-fed and creep-fed piglets, to investigate whether cryptosporidia, protozoan parasites of the surface of epithelial cells, could promote parasitism of pig enterocytes by *mucosalis*. There was no evidence that cryptosporidia enhanced the establishment of *mucosalis* in the gut, and adenomatous change was not observed.

CHAPTER I.

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The pig industry is primarily concerned with producing pig meat and its by-products for human consumption. As my grandmother used to say, the only part of a pig that cannot be used is its squeal. Pork has long been enjoyed by man, illustrated by the following quotation from "The Good Cook - Pork" (Time-Life Books, 1981).

"Few animals have been as useful to man as the pig. Easily fed, cared for and housed, this omnivorous and prolific creature has been one of the main dietary props of civilization, both in the Western world and the Orient. For centuries, the pig provided Europe's peasantry with practically the only meat they ever ate. In China, pork was so universally prized that a symbol representing a pig beneath a roof became the ideogram for "home". Despite the wide range of meats available to most consumers today, pork remains popular world-wide. Recent estimates show the global pig population at between 400 and 500 million - roughly one pig for every 10 people on Earth."

The age old love of eating pork fostered the tradition of hunting wild swine. Domestication of the pig soon followed and a cottage industry was born. Initially pigs were reared for home-consumption, hence the custom of killing fat pigs at the onset of winter when fodder supplies were insufficient for winter feeding. The meat was preserved in a variety of ways

and became a staple of the winter diet.

In the 18th century wealthy farmers sought by supervised feeding and breeding to improve the quality of their pigs. They left a legacy of mixed farming systems; pigs, other livestock and crops being sold for commercial gain.

As with the production of other farm livestock, notably poultry, intensification, specialisation and sophistication is the modern trend. On a world-wide scale the pig industry has mushroomed over the last few decades (Alexander, 1981), and although in the United Kingdom the total numbers of pigs are declining, the average herd size is increasing, in keeping with tendencies elsewhere (Taylor, 1981).

Intensification of the business of producing pig meat means in effect that very large numbers of pigs are reared in relatively small areas. Hence the opportunity for multiplication and spread of pathogens within these large populations has greatly increased. The efficacy of disease control in large units is therefore a major factor in determining the success (in terms of profit) or failure (in terms of loss) of the unit, since there is little doubt that the presence of infectious disease within a herd causes economic loss (Beynon, 1978; Bolitho, 1978; Govier, 1978; Muirhead, 1978).

This thesis deals with a group of enteric diseases of the pig, recently termed the Intestinal

Adenomatosis Complex (Rowland and Lawson, 1981), which are believed to be infectious in nature and which are undoubtedly a cause of economic loss to the pig producer.

REVIEW OF LITERATURE

(i) Intestinal Adenomatosis Complex:

(a) Terminology and Definitions.

Although of widely differing gross appearance the enteric disorders which constitute the adenomatosis complex have a common underlying abnormality, that of a thickening of the mucous membrane of the affected portion of gut, which may be small intestine or large bowel. Histologically the fundamental pathological change in affected tissues is proliferation of undifferentiated crypt epithelial cells, and this lesion may be manifested by a variety of clinical symptoms, depending on the age of the animal affected and whether or not destructive changes have occurred in the altered mucosa.

Porcine intestinal adenomatosis (PIA) is the name given to the condition resulting from uncomplicated proliferation; necrotic enteritis (NE), regional ileitis (RI), and proliferative haemorrhagic enteropathy (PHE) are terms used to describe the other

members of the complex, in which additional changes are superimposed on the basic abnormality.

The use of specialised histological techniques and transmission electron microscopy has shown that the proliferating (adenomatous) cells contain bacteria, which lie mainly in the apical cytoplasm of host cells and which are morphologically indistinguishable from campylobacters. These bacteria can be cultured in large numbers from uncomplicated lesions and may be visualised intracytoplasmically by using immuno-fluorescence. This bacterium has been characterised and named Campylobacter sputorum subspecies mucosalis (Lawson, Rowland and Wooding, 1975).

The intestinal adenomatosis complex has been reviewed extensively by Roberts (1978) and recently by Rowland and Lawson (1981). This present review contains a brief summary of the historical and geographical aspects of the complex, and incorporates a brief description of the occurrence, clinical signs, and pathology of each member. Particular emphasis is given to recent contributions to the literature. Subsequent sections deal with previous attempts to reproduce the disease and possible reasons for the relative lack of success. Consideration is given to alternative approaches to the problem. Similar enteropathies in other species are compared and finally the role of campylobacters as pathogens in man and animals is discussed.

(b) Historical and Geographical Aspects

PIA was probably first recognised in America (Biester and Schwarte, 1931; Biester, Schwarte and Eveleth, 1939) as sporadic cases of ill-thrift in growing pigs which on post-mortem examination were found to have adenomatous proliferations of the mucosa of the terminal ileum, caecum, or spiral colon. A similar isolated case of PIA was reported in Canada (Moynihan and Gwatkin, 1941).

In 1951 the results were published of an abattoir survey of PIA in Danish pigs, mostly at bacon-weight (Emsbo, 1951). This author categorised his cases as either a muscular type or a mucous type, probably corresponding to regional ileitis and porcine intestinal adenomatosis respectively. Shortly after this description of the complex in Denmark, Field, Buntain and Jennings (1953) reported an outbreak of PIA/RI in a pig herd in Britain. Most of the cases they described occurred in post-weaned pigs of the 2-4 month age group, 12 of which died as a result of perforation of the thickened ileum. The remaining cases were detected when slaughtered at 7-8 months. Perforation of the ileum as a sequela to regional ileitis was also reported in Canada by Nielsen (1955).

Further reports of PIA in pigs in Hungary in the 1950's are cited by Roberts (1978).

In 1958 the complex was reported in Australia

(Pullar, 1958). This author described cases of porcine intestinal adenomatosis and necrotic enteritis observed at slaughter of pigs from a number of herds belonging to a scheme for the eradication of infectious pneumonia. He reported that the adenomatous change was sometimes but not always associated with necrotic enteritis and concluded that the two syndromes were endemic in eight herds.

There were several more reports of members of the PIA complex occurring in Europe and America in the next decade. Goodwin and Jennings (1959) described several cases of PIA/NE in Britain. Hoorens (1962) in Belgium and Westendorp (1965) in Holland carried out detailed studies of cases of PIA and RI, with some cases having features of both PIA and RI. Dodd (1968) in America described two cases in post-weaned pigs, one resembling PIA and the other NE. Proliferative enteropathies in pigs have also been reported from Mexico (Necoechea, Melgar and Guzman de Las Casas, 1969) and India (Rajan, Nair and Maryamma, 1975). Hence the adenomatosis complex would appear to be world-wide in distribution.

The haemorrhagic form of the PIA complex was probably first described in 1970 in Britain by O'Neill (O'Neill, 1970). This form affected pigs in a minimal disease herd ranging from 4½-34 months. This author described the characteristic clinical signs of PHE and the typical gross post-mortem finding of

massive haemorrhage into the posterior third of the small intestine but did not mention gross or microscopic thickenings of the mucosa underlying the haemorrhage, a feature possibly overlooked. A similar outbreak had occurred in 1965 in a Scottish minimal disease herd and this was reported by Rowland and Rowntree (1972), who observed mucosal thickening grossly and adenomatous change microscopically in animals dying of haemorrhage into the lower ileum. All ages of animals from 3 weeks upwards were affected by the haemorrhagic syndrome and PIA occurred in post-weaned animals subsequently, affecting animals in the 6 - 16 week age group.

Since 1972 PHE has emerged as a well-recognised clinical entity usually occurring in minimal disease or closed pig herds. In the United Kingdom it is most common as a problem in boar - testing stations (Allen and Saunders, 1976; Jackson, 1980), and has been reported, usually in minimal disease herds, from many areas of the world; in Australia (Kelly and Cameron, 1976; Love, Love and Edwards, 1977); in Japan (Kubo et al., 1979); in America (Kurtz, 1973); in New Zealand (O'Hara, 1972); in Taiwan (Redman Chu and Hong, 1973); and in Canada (Yates et al., 1979).

(c) Porcine Intestinal Adenomatosis.

Occurrence:

PIA is most commonly seen in the post-weaned

fattening pig between 6 and 20 weeks of age (Rowland and Rowntree, 1972; Rowland and Lawson, 1981), although cases have been reported in older animals up to 9 months of age (Biester and Schwarte, 1931; Moynihan and Gwatkin, 1941). The prevalence of PIA and the related enteropathies RI and NE are difficult to assess in individual herds since the conditions cannot be identified clinically with certainty and affected animals may recover, any lesions present resolving (Roberts, 1978; Rowan and Lawrence, 1982).

Emsbo (1951) found that less than 1.0% of pigs at slaughter had lesions whereas necropsy of 3-4 month old pigs demonstrated related lesions in 2.5%. These figures support the observation that some pigs recover and progress to slaughter. Roberts et al. (1979), using a cumulative sum technique to select fatteners for necropsy, estimated that 2.5% of the throughput of the herd studied were affected. Lawson et al. (1980) in a study of another herd found that 1.0% of the throughput died because of adenomatosis or the related conditions.

Clinical Signs:

In many cases the clinical signs are very slight but affected animals are usually thin, show poor growth rate and appetite, and may exhibit mild or intermittent diarrhoea (Biester, Schwarte and Eveleth, 1939; Rowland and Rowntree, 1972; Rowland and Lawson, 1974; 1981) (Figure 1.1). Recovery in

uncomplicated cases is marked by swift return of appetite and improved weight gains (Rowland and Lawson, 1981). The disease is afebrile and most animals recover within 6 weeks of initial signs (Rowland and Lawson, 1974).

Changes in the haematology of affected pigs are consistent with debility. Thus Biester, Schwarte and Eveleth (1939) found high inorganic phosphorus levels and concluded this was indicative of increased metabolism of the affected pig's own proteins. Other significant changes in blood serum include decreased alkaline phosphatase activity, decreased serum albumin and decreased zinc levels (Martinsson et al., 1974; Martinsson, Ekman and Jönsson, 1976). Blood samples of pigs with PIA showed significantly increased levels of cortisol and α1-antitrypsin and increased numbers of white blood cells, suggesting the presence of a low grade inflammatory response (Martinsson, Ekman and Jönsson, 1976). There is recent evidence that PIA may be associated with malabsorption of dietary amino acids (Rowan and Lawrence, 1982).

Pathology.

Gross Findings:

On post-mortem examination the carcases are in poor bodily condition, often emaciated, and there is little carcase fat. Lesions relating to PIA are

restricted to the alimentary tract (Biester, Schwarte and Eveleth, 1939; Rowland and Rowntree, 1972). The areas affected may extend caudally from the mid - small intestine and sometimes include the caecum and the spiral colon (Dodd, 1968; Rowland and Rowntree, 1972; Roberts, 1978). Lesions are more frequently observed in the terminal small intestine than elsewhere (Roberts, 1978). Grossly the most striking feature is the thickening of the gut wall in affected areas. The serosa may appear reticulate due to patchy areas of subserosal oedema (Rowland and Rowntree, 1972) and there is often marked oedema of the mesentery of the affected bowel. The drainage lymph glands are enlarged. On opening the portions of gut involved the increase in the width of the gut wall is seen to be due to thickening of the mucosal lining which is often thrown up into exaggerated folds or appears as diffuse polypoid growths, becoming less prominent towards the periphery of the lesion where there may be intervening areas of non-thickened mucosa (Figures 1.2 - 1.4). The mucosal surface appears granular, the tips of the corrugations are commonly reddened and there may be adherent grey-green flecks of necrotic debris (Roberts, 1978). There may also be oedema of the submucosa and hypertrophy to a greater or lesser degree of the inner circular and outer longitudinal muscle layers of the wall (Emsbo, 1951; Roberts, 1978).

Histopathology:

In the small intestine adenomatous areas are conspicuously devoid of villi, the mucosal surface being flat and at the periphery of the lesion in sharp contrast with the normal mucosal structure of conical villi overlying crypt glands abundant in goblet cells (Figures 1.5 - 1.6; Figures 1.9 - 1.13).

The mucosal corrugations observed grossly are seen to consist of vigorously proliferating immature epithelial cells forming enlarged, misshapen glands which are often tortuous or branched and in which goblet cells are conspicuous by their absence (Dodd, 1968; Rowland and Lawson, 1974; Roberts, 1978) (Figure 1.7). The proliferating epithelial cells appear crowded, mitotic figures are numerous, and the cells often overlap, giving a palisade appearance to the nuclei (Rowland and Rowntree, 1972) (Figure 1.8). Two types of cell are apparent, both have deeply eosinophilic cytoplasm but in one type the nuclei are large and vesicular while in the other the nuclei are elongated and deeply staining (Emsbo, 1951; Rowland and Rowntree, 1972; Roberts, 1978). Silver-staining techniques have regularly demonstrated the presence of irregularly curved bacterial forms mainly concentrated in the apical cytoplasm of these adenomatous cells (Roberts, Rowland and Lawson, 1977; Roberts, 1978; Rowland and Hutchings, 1978; Rowland,

Lawson and Roberts, 1978) (Figures 1.14 - 1.16).

In the lamina propria, lymphocytes, macrophages and plasma cells are present in reasonable numbers and in uncomplicated PIA there is a relative lack of cellularity of the interglandular areas (Rowland and Rowntree, 1972; Roberts, 1978). The lack of inflammatory response in uncomplicated lesions of PIA has resulted in the term "enteropathy" which is used in preference to "enteritis" by Rowland and Lawson (1981), when they offer Porcine Proliferative Enteropathies as an alternative title to the Intestinal Adenomatosis Complex.

Infiltrative growth of adenomatous cells has been observed, usually into the follicles of Peyer's patches (Nielsen, 1955), but sometimes also into the submucosa (Emsbo, 1951), penetrating lymphatics (Roberts, 1978) and from there metastasising to regional lymph glands (Emsbo, 1951; Roberts, 1978; Roberts, Rowland and Lawson, 1980a).

In uncomplicated PIA, oedema of the submucosa and a degree of hypertrophy of the muscle layers may also be observed (Emsbo, 1951; Roberts, 1978) but these layers are sometimes unaffected (Rowland and Rowntree, 1972; Roberts, 1978).

In recovering cases the mucosa gradually reverts to a more normal structure, adenomatous glands are fewer in number and there is an attempt at villous

formation. Glands at the base of the mucosa revert to normal before those at the surface. Goblet cells are more numerous and glands which are enlarged, misshapen and branched (i.e. of an 'adenomatous' shape) are seen with goblet cell hyperplasia. The lumina of remaining adenomatous glands contain inflammatory cell debris and apoptotic cells, the glands themselves are isolated within a more normal mucosa or seen in surface areas only (Roberts, 1978).

Ultrastructural Findings:

On electron microscopic examination of adenomatous areas, the cells bear all the features of immature crypt cells but in addition contain a variable number of bacterial bodies, of typical campylobacter morphology, free of host cell membranes and concentrated mainly in the apical cytoplasm (Figures 1.17 - 1.19). These bacterial profiles have been observed in the process of dividing within host cells (Rowland, Lawson and Maxwell, 1973; Rowland and Lawson, 1974; Roberts, 1978).

In recovering cases many of the intracellular bacteria show signs of degenerative change such as increased electron-density of the bacterial cell wall, shrinking of bacteria, condensation of bacterial cytoplasm, and clumping of these degenerate forms within polyphagolysomes. Some degenerate bacteria are swollen with electron-lucent cytoplasm. In addition degenerate bacteria are extruded into the

lamina propria where they are phagocytosed by macrophages and neutrophil polymorphs. Inflammatory cells containing dead or dying bacteria are common in crypt lumina as are extruded degenerate epithelial cells containing bacterial remnants. Electron-dense apoptotic bodies, composed of effete bacterial or host cell DNA, are numerous and found at the base of glands, or in the lamina propria or gland lumina, and are also engulfed by macrophages. Goblet cells and mature absorptive cells free of intracellular bacteria begin to appear in recovering glands, interspersed between adenomatous cells containing bacteria in various stages of degeneration (Roberts, 1978).

Bacteriology:

Culture of adenomatous mucosa using the methods of Lawson and Rowland (1974) has consistently resulted in the isolation of large numbers of a Gram-negative, catalase-negative, microaerophilic campylobacter, Campylobacter sputorum subspecies mucosalis (mucosalis) (Figure 1.20). This organism has been characterised (Lawson, Rowland and Wooding, 1975; Lawson et al., 1981) and there is considerable evidence from bacteriological, electron-microscopic and immunofluorescent studies that mucosalis is the bacterium observed within adenomatous cells (Lawson and Rowland, 1974; Rowland and Lawson, 1974; Roberts, 1978).

The majority of mucosalis strains recovered from PIA react with whole-cell antisera prepared in rabbits against any one of these strains; such strains are serotype A. Within these isolates a considerable number of heat-labile surface antigens exist that allow laboratory strains to be differentiated from one another. A minority of PIA cases yield strains which do not react in agglutination tests with serotype A antisera; such isolates are serotype B. Evidence for the existence of other distinct serotypes is incomplete (Rowland and Lawson, 1981).

Immunofluorescence:

Most cases of PIA show specific particulate fluorescence in the apical cytoplasm of adenomatous cells when stained for serotype A mucosalis. The fluorescence corresponds to the sites where the bacteria are concentrated in the host cell (Lawson and Rowland, 1974; Rowland and Lawson, 1974) (Figure 1.21). Most cases of PIA also exhibit particulate fluorescence in these sites when stained for swine IgA, indicating that the intracellular bacteria are coated with host IgA (Lawson et al., 1979).

Immune Response:

That pigs affected with PIA mount an immune response to intracellular parasitism by mucosalis is demonstrated by the presence of host antibody coating the bacteria (Lawson et al., 1979). There is also

evidence that a specific humoral response results from mucosalis infection (Lawson et al., 1982). However at present it is unclear to what extent the presence of serum antibodies to mucosalis reflects a previous episode of intracellular parasitism since a high proportion of pigs at slaughter have serum antibodies - a much higher proportion than the current awareness of the occurrence of PIA (Lawson et al., 1982).

(d) Necrotic Enteritis

Occurrence:

Necrotic enteritis, like PIA, occurs most commonly in the post-weaned age group of pigs (Rowland and Lawson, 1974; Roberts, 1978) although cases are seen in older pigs of slaughter-weight (Rowland and Hutchings, 1978).

Clinical Signs:

The initial signs are as for PIA i.e. in-appetence and ill-thrift, with periods of diarrhoea; sometimes the faeces contain flecks of necrotic material. Instead of recovery the affected animals may die suddenly or may continue to be ill-thriven up to slaughter.

Gross Findings:

At post-mortem examination there is the characteristic thickening of the affected areas of gut, usually the lower small intestine, which in necrotic

enteritis appears dark reddish-brown in colour (Rowland and Lawson, 1975b; Roberts, 1978). On exposure the thickened mucosa is grey-green and necrotic and the corrugations of the mucosal folds appear dry, cracked and fissured. The necrosis varies in extent from case to case sometimes occurring as an adherent membrane which can be peeled off revealing remnants of adenomatous mucosa beneath and sometimes involving the entire depth of the mucosa (Roberts, 1978).

Histological and Other Findings:

Histologically the changes in the mucosa are seen to be fundamentally proliferative with coagulative necrosis of the adenomatous tissue which varies in extent from case to case. Typical campylobacter-like organisms can be demonstrated in the surviving adenomatous tissue by light, electron and immunofluorescent microscopy and Campylobacter sputorum subspecies mucosalis can be isolated from the viable areas of adenomatous mucosa in numbers equivalent to those cultured from uncomplicated PIA cases (Rowland and Lawson, 1975b; Roberts, Rowland and Lawson, 1977; Roberts, 1978).

(e) Regional Ileitis

Occurrence and Clinical Signs:

Regional ileitis tends to occur in an older age-group of pigs than PIA and is often an incidental finding at slaughter (Emsbo, 1951; Pullar, 1958;

Westendorp, 1965; Rahko and Saloniemi, 1972; Rowland and Hutchings, 1978) with no previous history of ill-thrift. Occasionally the affected bowel may rupture, resulting in death of the animal or survival followed by chronic ill-thrift (Emsbo, 1951; Field, Buntain and Jennings, 1953; Nielsen, 1955; Westendorp, 1965).

Gross Findings:

At post-mortem there is considerable variation depending on whether rupture of the affected bowel has occurred. If rupture has occurred then there may be an acute fibrinous peritonitis or chronic fibrous peritonitis of varying extent and severity. The ileum is thickened and rigid and has been likened to a garden hose, hence the term 'hose-pipe gut' (Emsbo, 1951; Rowland and Lawson, 1975b). The mucosa shows a variable degree of thickening and ulceration, interspersed with a variable amount of scar tissue formation. In some cases the mucosal surface appears flat, with only isolated areas of nodular mucosal proliferation surrounded by scar tissue formation and/or ulceration. The fibrous tissue often extends into the submucosa to a greater or lesser extent and the muscle layers of the gut are massively hypertrophied (Roberts, 1978).

Histological and Other Findings:

Histologically it can be confirmed that only isolated remnants of the glandular mucosa remain and these are adenomatous in character. There is substantial

granulation tissue formation in the lamina propria and submucosa and the muscle coats are greatly hypertrophied (Roberts, 1978). As in the related enteropathies campylobacter-like organisms can be visualised in the adenomatous cells by light and electron microscopy and identified as mucosalis by immunofluorescent techniques (Roberts, 1978). Mucosalis can also be cultured from the areas where there is viable adenomatous mucosa (Roberts, 1978).

(f) Intermediate Forms of PIA, NE and RI.

The above categories are not absolute and intermediate forms occur. This has been stressed by several authors (Emsbo, 1951; Jönsson and Martinsson, 1976; Roberts, 1978). Necrotic enteritis and regional ileitis seem logical sequelae to PIA as pointed out by Rowland and Lawson (1975b),

"In PIA the epithelial cells are immature and deprived of their protective mucus coat. It seems reasonable to suggest that either or both these changes renders the cells more susceptible to damage on occasion by normal bacterial or protozoal inhabitants of the alimentary tract. Where this takes place, necrosis of the epithelium may develop with either the death of the pig, which in necropsy demonstrates as necrotic enteritis, or survival with the mucosal surface destroyed. In the latter instance, repair of the already altered mucosa may be less effective than when a healthy mucosa is damaged with consequent granulation tissue proliferation rather than mucosal regeneration. This

explanation provides a logical sequence of events for the development of NE or RI from PIA".

(g) Proliferative Haemorrhagic Enteropathy

Occurrence:

Proliferative haemorrhagic enteropathy occurs as a distinct clinical entity, usually in closed pig herds or minimal disease herds which are the progeny of stock originally Caesarean-derived (Rowland and Rowntree, 1972; Love, Love and Edwards, 1977). The disease often occurs initially as an explosive outbreak affecting all ages of animals in Caesarean-descended pigs from 3 weeks upwards, with a morbidity of 12% (Rowland and Rowntree, 1972; Love, Love and Edwards, 1977) and subsequently as a sporadic cause of loss in closed pig herds (Redman Chu and Hong, 1973), or boar-testing stations (Jackson, 1980).

Clinical Signs:

Clinical signs vary from sudden death with carcase pallor, to sudden anorexia followed by an acute episode of haemorrhagic diarrhoea. Affected animals occasionally vomit and usually pass profuse, watery, black and foul-smelling faeces sometimes containing fibrinous blood clots (Redman Chu and Hong, 1973; Love, Love and Edwards, 1977). Some pigs appear white or blanched prior to the onset of diarrhoea and temperatures

vary from 40°C to subnormal (Rowland and Rowntree, 1972; Redman Chu and Hong, 1973; Love, Love and Edwards, 1977). Pregnant sows may abort but this is not a constant feature (Love, Love and Edwards, 1977). Death usually occurs within 24 hours although 50% of affected animals recover.

Gross Findings:

Grossly at necropsy the carcase is in good condition but with marked pallor, and the perineum may be blood-stained. On opening the carcase the viscera appear anaemic and there may be petechial subepicardial or subserosal haemorrhages accompanied by congestion and oedema of the lungs (Yates et al., 1979), changes consistent with a haemorrhagic anaemia. Specific pathological abnormalities are observed in the alimentary tract (Rowland and Rowntree, 1972). The ileum may appear dilated and the wall is turgid and thickened with sharply defined areas of subserosal oedema. On opening, the lumen is filled with variable amounts of clotted and free blood. Fibrin casts are commonly seen closely applied to the mucosa which is thickened and corrugated. In some cases the haemorrhage is less massive and in others much of the blood has passed into the large bowel where it is mixed with ingesta (Rowland and Rowntree, 1972). The ileal mucosa is deeply congested, the mesenteric vessels engorged, and the drainage lymph glands congested and oedematous. Interestingly, despite the

massive haemorrhage, no site of bleeding is obvious (Rowland and Rowntree, 1972; Love, Love and Edwards, 1977; Yates et al., 1979).

Histological Findings:

The lumen of the affected portion of small intestine contains a variable amount of free red blood cells and fibrin while the underlying mucosa is extensively oedematous and essentially proliferative. Poorly formed villi overly the hyperplastic glands (Rowland and Rowntree, 1972). The lamina propria is congested and haemorrhagic (Love and Love, 1979).

Some of the histological features are similar to a recovering PIA case in that goblet cells are not infrequently seen at the base of the affected mucosa, the lamina of hyperplastic glands are packed with cellular debris and there are often poorly-formed villi overlying the adenomatous areas (Roberts, 1978). As in PIA, RI and NE, many campylobacter-like bacteria can be demonstrated by silver-staining in the apical cytoplasm of proliferating enterocytes (Roberts, 1978).

Ultrastructural Findings:

These are similar to those in a recovery PIA case in that many affected enterocytes and intracellular bacteria show degenerative changes (Roberts, 1978).

Other Findings:

Bacteriology: Unlike typical cases of PIA, in PHE it is often not possible to isolate mucosalis or where isolation is successful the numbers of viable

organisms are much reduced (Roberts, 1978; Lawson et al., 1979).

Immunofluorescence: As in PIA cases there is bright particulate apical fluorescence of affected enterocytes when PHE mucosa is stained for mucosalis antigens or for Swine IgA, indicating the presence of antibody-coated bacteria (Lawson et al., 1979).

(ii) Attempts to Reproduce the Intestinal Adenomatosis Complex.

There have been numerous attempts in the past to reproduce PIA or RI, and to determine their aetiology. Most of these have been unsuccessful.

Biester and Schwarte (1931) fed intestinal contents and scrapings from the large intestine of affected pigs and an acute dysentery was produced. Their cases had an underlying infective process, and although four cases examined histologically had epithelial proliferative changes, it is difficult to evaluate their results. Later attempts by this group to transmit PIA using material from an affected pig were unsuccessful (Biester, Schwarte and Eveleth, 1939).

Between 1929 - 1932 Adsersen (cited by Emsbo, 1951) attempted unsuccessfully to reproduce PIA using as his inocula bacterial cultures from affected intestines and minced organ material. He was also

unsuccessful in transmitting the disease by direct contact of experimental pigs with confirmed cases of PIA. Balo and Korpassy (1935, 1939; cited by Roberts, 1978) also reported unsuccessful transmission attempts about this time.

In 1951 Emsbo attempted but failed to reproduce PIA by direct contact and by inoculation of normal pigs with lesions of PIA. Neither did feeding diets deficient in vitamins or surgical intervention in the ileum result in development of adenomatous change.

Korpassy and Tiboldi (1957) used material from affected animals as inoculum but failed to transmit the disease. Hoorens (1962) in a study of RI described a number of experiments involving the feeding of talc and silica gel, surgical procedures such as ligation of lymph nodes and lymphatics, partial occlusion of the gut lumen and the injection of sclerosing substances into the ileal mucous membrane and drainage lymphatics. He produced changes consistent with such surgical intervention and introduction of irritants - oedema, connective tissue proliferation and a foreign body response. He concluded that although the primary cause of RI was still unknown, obstruction of the lymphatics of the gut wall and mesentery were important in the pathogenesis. Similar experiments with similar results were described by Rahko, Saloniemi and Kalima (1973),

Saloniemi, Rahko and Kalima (1974), and Kalima, Saloniemi and Rahko (1976).

Rowland and Rowntree (1972) studied an outbreak of PHE in a minimal disease herd. The outbreak was followed by sporadic cases of PIA. They attempted to reproduce lesions by feeding a group of experimental pigs with the same batch of feed in use at the time of the outbreak. They also fed adenomatous mucosa to both sucking and weaner pigs and in addition a day-old litter of pigs was dosed orally with faeces from an adult with PHE. None of these procedures resulted in transmission of the disease.

A similar series of experiments was conducted by Kurtz, Mirocha and Meade (1974) after a PHE outbreak in a herd of specific pathogen free (SPF) swine. They fed affected intestinal mucosa to 60lb pigs, and the ration in use at the time of the outbreak to conventional and to SPF pigs without producing clinical signs of disease.

Strenuous efforts were made by Roberts (1978) to reproduce PIA and the related enteropathies. Roberts studied the infectivity of Campylobacter sputorum ss mucosalis for neonatal and post-weaned pigs (Roberts, 1978; Roberts, Lawson and Rowland, 1980a, b, c) and attempted transmission of the disease in these 2 categories of pigs using cultures of mucosalis and adenomatous mucosa as inocula (Roberts, Rowland and Lawson, 1977; Roberts, 1978). Roberts'

experiments are summarised below:

I. Infectivity experiments (Roberts, 1978).

Roberts dosed neonatal piglets with cultures of mucosalis and found that the organism established readily in the oral cavity of dosed piglets for up to 8 weeks post-infection (p.i) but was isolated only in low numbers from a minority of sites sampled in the gut of some of these piglets. The organism persisted in the gut for up to 40 days p.i. and there was rapid spread between dosed and undosed littermates. When the drug benzetimide was given to reduce gastrointestinal peristalsis, reisolation of mucosalis from the gut after oral dosing occurred up to 48 days p.i. and in 2 pigs of this group isolated adenomatous glands were observed in the small intestine.

In contrast post-weaned pigs were resistant to infection with mucosalis as no isolations were made from these pigs even after an attempt to increase susceptibility by prior treatment with antibiotics. There was no apparent spread of infection between dosed and undosed pigs kept in the same room. Benzetimide apparently enhanced the establishment of infection in post-weaned pigs as mucosalis was recovered from the gut of 3 out of 4 pigs given both mucosalis and benzetimide.

II. Transmission experiments (Roberts, 1978).

Transmission experiments were attempted initially in post-weaned pigs. Roberts fed high levels of

antibiotics from birth in order to exclude the possibility of natural infection with mucosalis and hence development of immunity. Once antibiotic-feeding ceased Roberts dosed these weaned pigs with either cultures of mucosalis alone or with cultures of mucosalis and homogenised adenomatous mucosa. Both groups were given chalk beforehand to neutralise gastric acidity and benzetimide to reduce peristalsis. Mucosalis was not reisolated from either group and there was no gross or histological evidence of adenomatous change.

Limited but apparently successful transmission was achieved in sucking piglets dosed in the first few days of life. In 2 such litters some piglets were dosed with cultures of mucosalis, plus chalk and benzetimide, while littermates were given cultures of mucosalis and homogenised adenomatous mucosa, plus chalk and benzetimide. In a third litter 9 piglets were dosed with cultures of mucosalis, chalk, benzetimide and homogenised adenomatous mucosa. Two littermates were killed some hours prior to this exposure as "negative" controls. Eight of the 9 dosed piglets developed gross or microscopic evidence of PIA or NE after weaning in the period between 50-65 days post-infection.

Increasing numbers of mucosalis were isolated from the mucosa of some of the pigs killed serially during this time period and isolations of mucosalis

correlated well with the appearance of adenomatous change. Mucosalis was isolated, but in low numbers, from the mucosa of the 9th piglet, killed prior to the development of lesions in littermates.

In the other 2 litters there was less evidence of PIA - low numbers of mucosalis were isolated from the mucosa of some piglets and occasional adenomatous glands were found on histological examination.

The main criticism of Roberts' (1978) claim to have successfully transmitted PIA is that his experiments were poorly controlled. His "negative" controls were killed in the neonatal period and not surprisingly there was no evidence of adenomatosis. Adenomatous change has never been reported in neonatal piglets and the youngest age in which a member of the complex (PHE) has been described is 3 weeks (Rowland and Rowntree, 1972).

Thus his "negative" controls were only of use in demonstrating freedom from mucosalis infection in the neonatal period. The development of PIA and NE in the rest of the litter after weaning may have been unrelated to the experimental exposures in the neonatal period.

Nevertheless the experiment described above provides further evidence of the association between mucosalis and lesions of adenomatosis, since the appearance of lesions in this litter were preceded and

accompanied by increasing numbers of mucosalis in the enteric mucosae of the piglets. Some of the piglets killed later in the experiment had lesions compatible with recovery from PIA and lower numbers of mucosalis were recovered from the enteric mucosae of these piglets (Roberts, 1978).

AETIOLOGY OF THE COMPLEX.

Despite the constant association of Campylobacter sputorum ss mucosalis with lesions of PIA, NE, RI and PHE, the aetiology and pathogenesis of this group of enteropathies is poorly understood. Attempts at transmission using cultures of mucosalis and homogenised adenomatous mucosa have only once met with apparent success (Roberts, 1978).

Prior to the recognition of mucosalis in adenomatous epithelial cells (Rowland, Lawson and Maxwell, 1973; Lawson and Rowland, 1974; Rowland and Lawson, 1974) numerous aetiologies had been postulated and these will be briefly reviewed:

(i) Genetic Component:

A familial predisposition to the condition has been suggested (Adsersen, 1932; Emsbo, 1951; Hoorens, 1962) based on the observation that more than one pig from a litter may be affected. Although an

infectious aetiology could also explain this occurrence there is some evidence for a genetic component since the haemorrhagic form of the disease (PHE) is more common in Large White boars than in other breeds (Jackson, 1980).

The litter which developed lesions of the complex in Roberts' (1978) experiment may have been a particularly susceptible strain. It is interesting that in a study of PIA in a closed pig herd certain sows consistently produced litters of poorly-thriving pigs, which may have indicated the susceptibility of certain genotypes to the disease (Roberts et al., 1979). Whether any genetic factor operates at the level of the host cell e.g. in a similar way to K88 Escherichia coli infection in piglets (Sellwood et al., 1975; Sellwood, 1979), is unknown. Certain strains of hamsters are more susceptible to transmissible ileal hyperplasia, a disease with many similarities to the PIA complex in pigs (Frisk, 1976).

(ii) Viral Aetiology:

This has been considered by several authors (Biester, Schwarte and Eveleth, 1939; Korpassy and Tiboldi, 1957; Rowland and Rowntree, 1972) but none of these workers were able to demonstrate the presence of a viral agent in adenomatous lesions. Bergeland et al., (1975) did find reo-like viruses in cases of PIA and PHE but these were not demonstrable in all

their cases and were also found in a wide variety of other enteric disorders.

It is possible that the proliferative enteropathies of the pig are occasional sequelae to primary viral enteric infections and this has been mooted by Goodwin and Jennings (1959) who found cases of RI in a herd which had experienced a previous outbreak of transmissible gastro-enteritis (TGE). It is unlikely that TGE is a specific essential factor in the aetiology of PIA since the virus is unknown in Scotland and yet the PIA complex occurs. Enteric disorders of many kinds may theoretically predispose to PIA but specific factors have not yet been elucidated.

(iii) Allergic Component:

Ludvigsen (1958) suggested that RI was due to an allergic reaction in the terminal ileum of pigs after weaning. Affected pigs, diagnosed by laparotomy, recovered after corticosteroid treatment. It is difficult to evaluate such evidence as uncomplicated cases of PIA recover without treatment (Rowland and Rowntree, 1972; Rowland and Lawson, 1974; Roberts, 1978; Rowan and Lawrence, 1982). An allergic component in PHE has also been implied (O'Neill, 1970; Pill, 1971; Redman Chu and Hong, 1973; Love and Love, 1979) although the epidemiology of PHE suggests an infectious agent acting in a totally susceptible population (Love, Love and Edwards, 1977).

(iv) Other Possible Aetiologies:

These have been discussed in detail by Roberts (1978) and will be mentioned briefly here:

(a) Mycotoxins have been investigated as of aetiological importance in PHE but there is little evidence that they play a role (Van Ulsen, 1971; Kurtz, Mirocha and Meade, 1976) and this theory is no longer prevalent (Yates et al., 1979).

(b) Vitamin Deficiency: Nicotinic acid (Davis and Freeman, 1940; Runnels, Monlux and Monlux, 1965; Hungerford, 1975) and Pantothenic acid deficiency (Goodwin, 1962) have been suggested as important in the development of NE but in neither case do the histological changes described conform to the description of Rowland and Lawson (1975b) where all cases of NE have an underlying hyperplastic epithelium lacking goblet cells. Vitamin deficiency may be an effect of PIA rather than a cause, possibly due to impaired digestion in the lower bowel.

It is unknown whether specific vitamin deficiencies promote adherence to or penetration of host cells by mucosalis. The mechanisms whereby mucosalis enter host enterocytes are unknown but penetration of such cells logically seems important in the pathogenesis of the disease. The main function of Vitamin A is to maintain the structural and functional integrity of epithelium, particularly epithelial tissues with a secretory function (Penny and Muirhead, 1981).

Investigation of the role of Vitamin A in relation to mucosalis and the enteric mucosa may prove fruitful in the future.

(c) Zinc Deficiency: Martinsson and Ekman (1974) reported low zinc levels in pigs which on post-mortem examination had PIA. Other pigs with similar clinical signs of wasting responded well to zinc therapy, although improved weight gains could also be due to natural recovery from PIA (Rowland and Rowntree, 1972; Roberts, 1978). Martinsson, Ekman and Jönsson (1976) also reported low zinc levels in their cases of RI, but they noted that in most of the herds investigated there was no correlation between a low content of zinc in the feed and the occurrence of wasting pigs or pigs with RI. They concluded that low zinc levels were a result rather than a cause of the proliferative enteropathies. It is not known if low zinc levels may also contribute to the establishment of mucosalis in the intestinal mucosa (Roberts, 1978).

(v) Campylobacter sputorum subspecies mucosalis.

The strongest evidence yet for the aetiological role of mucosalis in the intestinal adenomatosis complex has been reported by Roberts, Rowland and Lawson (1977), Roberts (1978), Roberts, Rowland and Lawson (1980b), and Rowland et al., (1980).

Roberts, Rowland and Lawson (1977) observed lesions of PIA and NE after feeding cultures of mucosalis and

homogenised adenomatous mucosa to 9 sucking piglets in the neonatal period. These piglets gained weight poorly, this becoming clinically apparent from three weeks, and in addition their appetites were capricious.

Increasing numbers of mucosalis were isolated from the mucosa of piglets killed up to 53 days of age, reaching numbers equivalent to those obtained in naturally occurring PIA cases. Three piglets killed between 53 and 59 days of age had lesions of PIA while a piglet killed on day 51 had lesions of NE. Three pigs killed between 65 and 66 days of age had latterly shown improved weight gains. Mucosalis was not recovered from these last three animals and there was an absence of the clear cut proliferative lesions seen in the mucosa of pigs killed earlier in the experiment.

Bacteria resembling mucosalis were demonstrated by silver-staining techniques or by ultrastructural examination of the apical cytoplasm of proliferating enterocytes. Immunofluorescence using hyperimmune mucosalis antiserum also confirmed the presence of the organisms in the apical cytoplasm of the adenomatous cells.

In other experiments carried out by Roberts (1978) naturally-reared piglets were dosed in the neonatal period with cultures of mucosalis and with benzetimide to reduce gut motility. In 2 piglets killed 41 days

post-exposure there were a large number of isolated hyperplastic crypt glands in the mid-small intestine and terminal ileum. These isolated glands had many features of adenomatous change and were considered by Rowland et al., (1980) to represent early lesions of PIA. The glands were enlarged and lined by proliferating immature enterocytes. Goblet cells were reduced in number or absent. Silver-staining techniques demonstrated the presence of irregularly curved bacterial profiles in the apical cytoplasm of affected cells and immunofluorescence confirmed the identity of these as mucosalis.

The work described above links the degree of intracellular parasitism by mucosalis with the amount of adenomatous change and suggests that adenomatous proliferation occurs as a result of intracellular infection by mucosalis. No evidence has been found to date of the presence of adenomatosis in the absence of intracellular bacteria. It seems that the presence of intracellular bacteria, namely mucosalis, is inextricably associated with the lesions of PIA.

Despite the major steps taken over the last decade by Lawson, Roberts, Rowland and workers elsewhere, much concerning the aetiology and pathogenesis of intestinal adenomatosis remains unclear. In theory the essential steps in the development and regression of lesions of PIA seem logically to involve the initial

establishment of mucosalis in the lumen of the gut, followed by attachment to and penetration of susceptible host-cells, probably crypt epithelial cells. Intracellular parasitism is associated with a proliferative response of infected host enterocytes which fail to mature and this results in the typical adenomatous mucosa - devoid of goblet cells and villi, and consisting of enlarged, hyperplastic crypt glands. Recognition of the intracellular bacteria as "foreign" stimulates the production of host antibody, IgM and later IgA, which coat the bacteria. Host defensive mechanisms result in degeneration and lysosomal digestion of mucosalis, accompanied by extrusion of dead or dying bacteria and effete host cells. Removal of mucosalis from intracellular sites is followed by rapid return to normality - goblet cells reappear, there is attempted formation of villi and the lesions regress.

There is evidence to support the above hypothesis (Roberts, 1978 and others) but present knowledge of some areas is scant or absent (vide infra).

Epidemiology:

Mucosalis occurs in the oral cavity of a minority of pigs in the field (Lawson, Rowland and Roberts, 1975; Roberts, 1981) and will persist in the mouth of experimentally exposed piglets for up to 8 weeks, spreading to undosed littermates (Roberts, Lawson and

Rowland, 1980c). These studies suggest that such oral cavity infection may be of epidemiological importance.

The epidemiology of PHE suggests an infectious agent acting in a totally susceptible population (Love, Love and Edwards, 1977). What is unclear is how infection persists and spreads in an infected and presumably partially immune herd, and whether there is any connection between infection of the oral cavity and development of PIA. It may be that mucosalis from the oral cavity is responsible for establishment of infection in the gut by constantly seeding the alimentary tract with saliva containing the bacteria.

Pathogenesis:

Specific factors which promote infection of the gut lumen are unknown. Roberts (1978) produced some evidence, using benzetimide, that reduction of gut motility may be important.

Maternally - acquired antibody and the indigenous enteric flora may also inhibit establishment of mucosalis, but there is little experimental evidence concerning the role that these play.

Attempts to clarify these issues are described in this thesis (Chapters 4 and 5).

The mechanisms whereby mucosalis gains entry into host epithelial cells are not clear. This problem is

difficult to attempt in experimental animals since it has not proved possible to reproduce the disease consistently, and the enormous surface area of the gut mucosa relative to the size of mucosalis makes improbable the visualisation of chance encounters between mucosalis and host enterocytes. Recent work in cell culture systems has proved promising however (Rajasekhar, 1981), and is still progressing (Lawson and Okereke, unpublished results).

Host Response:

There is evidence that pigs mount both mucosal and humoral immune responses to mucosalis (Lawson et al., 1979; Lawson et al., 1982). It is not clear however in what way the production of IgM and IgA which coat the intracellular bacteria is related to eventual degradation and expulsion of the bacteria, and neither is it known whether serum antibodies are produced as a result of infection of the oral cavity, the gut lumen or host enterocytes. This area in particular warrants further investigation.

POSSIBLE REASONS FOR THE LACK OF SUCCESS IN THE TRANSMISSION OF PIA, AND ALTERNATIVE APPROACHES TO THE PROBLEM.

(i) Involvement of Other Agents.

Despite the association of Campylobacter sputorum ss

mucosalis with lesions of PIA, it has been difficult to implicate this organism as the causative agent, given the numerous attempts at transmission in conventional pigs using inocula containing *mucosalis*, and the limited success (Roberts, 1978). There may be another agent or agents involved in initiating lesions or in promoting intracellular parasitism by *mucosalis* in some way.

Adenomatous cells resemble undifferentiated crypt epithelial cells. It is difficult to imagine that infection of mature villous cells with *mucosalis* results in dedifferentiation of these cells to become immature cells. It seems more likely that it is crypt enterocytes which, once infected, fail to mature but continue to proliferate resulting in a mucosa of adenomatous glands. There is some evidence that infection of crypt epithelial cells precedes adenomatous change (Rowland *et al.*, 1980). Thus it may be possible to enhance infection of such cells by denuding the mucosa of mature villar epithelial cells and increasing the number of immature cells available for infection. Enteric viral pathogens such as TGE (Bohl, 1981) and rotavirus (Woode and Bohl, 1981) cause these changes in young piglets. If crypt enterocytes are susceptible *per se* to *mucosalis* infection the above viruses could in theory predispose to adenomatosis by increasing the number and

availability of crypt cells for infection by mucosalis, provided mucosalis had already been established as part of the indigenous flora of the gut lumen.

The possible role played by rotavirus in the pathogenesis of PIA is investigated in Chapters 5 and 6 of this thesis.

Immature crypt cells have poorly developed microvilli (Trier, 1963; Trier and Rubin, 1965) and it may be that this feature of the cell membrane allows attachment and penetration by mucosalis, which presumably must occur prior to intracellular multiplication. Agents which interact with the cell membrane of pig enterocytes, such as E.coli (Moon, 1974) and cryptosporidia (Kennedy, Kreitner and Strafuss, 1977), may promote attachment or penetration of mucosalis, possibly by reducing the effectiveness of the cell membrane as a barrier to extracellular mucosalis. Investigation of these possibilities is described in Chapters 6 and 7 of this thesis.

There are other instances where more than one agent is required to produce a specific lesion, the best known in the pig being swine dysentery. Treponema hyodysenteriae, the primary aetiologic agent of swine dysentery (Taylor and Alexander, 1971; Harris and Glock, 1981), will not induce the disease or colonise the gut in germ-free swine (Harris and Glock, 1981). The prevailing theory is that other

anaerobes, part of the normal enteric flora, are a prerequisite to colonisation of the lower intestine by I. hyodysenteriae and that synergism with other organisms is necessary for expression of pathogenicity by the spirochaete (Harris and Glock, 1981).

Another example in pigs where more than one agent is considered necessary to produce a recognised syndrome is pasteurellosis in which Pasteurella multocida is generally accepted as a secondary invader to Mycoplasma hyopneumonia in the lungs (Farrington, 1981).

Although attempts were made in this thesis to evaluate the possible role of other agents in the aetiology of PIA the work was limited to dual infections of mucosalis and rotavirus, mucosalis and enterotoxigenic E. coli, and mucosalis and cryptosporidia (Chapters 5, 6 and 7).

(ii) Virulence of Campylobacter sputorum ss mucosalis.

If mucosalis is the sole causative agent of PIA, the question remains as to why the disease is not consistently transmissible using a variety of inocula containing viable mucosalis (Roberts, 1978). It is possible that on culture or storage mucosalis loses attributes of virulence. An example of temporary loss of virulence attributes is found in the 987P bearing strains of enterotoxigenic E. coli which lose the ability to produce 987P pili during prolonged

storage or repeated subculturing. Pili such as 987P facilitate intestinal adhesion and colonisation by pathogenic E. coli and are therefore important virulence attributes (Nagy, Moon and Isaacson, 1977). Although such enteropathogenic strains regain the ability to produce 987P pili in vivo, this may not be true of possible virulence attributes lost by mucosalis on storage or culture. Repeated passage of viruses in cell cultures are known to result in attenuation of many viruses, and it is possible that a similar phenomenon occurs with mucosalis.

(iii) Involvement of Mucosal or Other Factors.

The only published apparently successful transmission of PIA/NE has been with an inoculum containing fresh, homogenised adenomatous mucosa (Roberts, Rowland and Lawson, 1977). Repetition of this experiment using the same mucosa after storage for some weeks at -80°C did not result in transmission of PIA, despite the fact that mucosalis could still be recovered in large numbers from this stored mucosa (Roberts, 1978).

It is possible that storage at -80°C, although not greatly affecting the viability of mucosalis, could have caused the destruction of other more delicate organisms or as yet undetermined mucosal "factors" essential for the pathogenesis of PIA.

If other infectious agents or undetermined "factors" are involved in the aetiology of PIA they may not always be present in developed lesions. Perhaps other

agents or factors, as yet unknown, act on host enterocytes and promote change in these cells such that for a limited period of time the host cells are receptive to mucosalis infection. If mucosalis infection establishes, or is already established in the lumen of the gut when host cells become receptive, then intracellular parasitism and PIA results. The initiating agent or factor may have absented itself or been absented by host defence mechanisms before lesions of PIA developed. Thus the successful transmission reported by Roberts, Rowland and Lawson (1977) could have been due to the presence of a vital initiating factor which did not survive storage at -80°C, and other experiments using different inocula failed because of the absence of the essential initiating factor in the inoculum.

In Roberts' experiments (Roberts, 1978) and in this thesis efforts were made to limit the possible effects of the points discussed in (ii) and (iii) (vide supra) by

(a) Using recently isolated strains of mucosalis and subculturing as little as possible before inoculation.
(b) Wherever possible using freshly prepared mucosal homogenates as inocula in the relevant experiments.
(c) Where relevant using both cultures of mucosalis and adenomatous mucosal homogenates as inocula.

(iv) Host Susceptibility.

(a) Age at Exposure:

Since the PIA complex occurs most commonly in the

post-weaned category of pigs many transmission attempts have used weaned pigs as recipients. However Roberts (1978) has shown that neonatal piglets are more susceptible to infection with mucosalis than post-weaned pigs and his successful transmission experiment was in piglets exposed within the first few days of life. In these piglets gross lesions of PIA/NE appeared after weaning and some 52 days after dosing, indicating that important factors in the pathogenesis of the disease operate before weaning and that there is a relatively long incubation period.

In the majority of the work described in this thesis piglets were exposed to infection at a similar early age, although in a few experiments and for other reasons (e.g. decline in maternal immunity of older piglets) older pigs were used.

(b) Genetic Predisposition:

A familial occurrence of PIA has been observed and was discussed in the literature review (see Aetiology of the Complex, and Adsersen, 1932; Emsbo, 1951; Hoorens, 1962; Jackson, 1980). It is possible that some lack of success in transmission could be expected if litters of resistant stock were used as experimental animals. The resistance of some piglets to enteropathogenic K88 E. coli is partly due to genetic factors and is well-documented (Sellwood et al., 1975; Sellwood, 1979). A similar mechanism may operate

with mucosalis and the pig enterocyte. It is likely that attachment of mucosalis to the surface of the cell must occur before penetration and intracellular multiplication can occur. Large White boars are more susceptible to PHE than other breeds (Jackson, 1980). However most transmission experiments in the past have used pigs of mixed breeding from herds where PIA cases were still occurring or had been known to occur in the past (Roberts, 1978), and hence one would reasonably expect that at least some pigs experimentally exposed should have been susceptible.

Similarly in the work reported here litters of mixed breeding and from a variety of herds, all of whom had experienced PIA in the past, were used in an attempt to exclude the possibility of exposing resistant stock. In one experiment (Chapter 3) one of the secondary aims was to investigate the possible existence of genetically susceptible pigs by cross-suckling two litters and exposing one group containing piglets from each litter. If PIA had occurred in the exposed group it would have been possible to assess whether pigs of one particular strain were more susceptible than pigs of another strain. (The primary aim of cross-suckling was to provide unexposed littermate controls for the exposed group).

(c) Host Defence Mechanisms

Innate defences: Possible innate defence mechanisms of the pig gut which could inhibit the establishment of

mucosalis infection in the lumen (I to III below) or intracellularly (IV below) have been discussed at length by Roberts (1978) and to avoid repetition will be mentioned only briefly here. Roberts considered that the following may be of importance in preventing the establishment of such infection:

I. gastric acidity

II. normal bowel motility

III. indigenous enteric flora

IV. the absence of specific receptors on host enterocytes.

Roberts attempted to prevent the destruction of orally administered mucosalis in the stomach by neutralising gastric acidity with chalk administered orally before the infective inoculum. The effect of gastro-enteric motility was reduced in many of his experiments by oral doses of benzetimide, an anticholinergic drug.

The possible role of indigenous gut flora in preventing the establishment of a luminal infection of mucosalis was considered to be manifold:

(i) through the influence of the resident flora on gastro-intestinal peristalsis

(ii) through the production of volatile fatty acids

(iii) through competition between mucosalis and the resident flora for available nutrients.

Roberts conducted several experiments where he treated piglets with antibiotics prior to exposure to adenomatous mucosa and mucosalis; in one instance the specific reason was to inhibit or remove the indigenous flora.

Dietary manipulation was also considered as a possible way to enhance the establishment of mucosalis since starvation may increase susceptibility to enteric infections possibly through a reduction in gastric secretion (Grady and Keusch, 1971; cited by Roberts, 1978) and a decrease in the antibacterial mechanisms which operate in the small intestine (Freter, 1974; Knop and Rowley, 1975; both cited by Roberts, 1978), or through the effect of food and water deprivation (Tannock and Savage, 1974; cited by Roberts, 1978) and alterations in the diet (Mickelson and Klipstein, 1975; cited by Roberts, 1978) on the gastro-intestinal microbial flora. In some of his experiments Roberts exposed new-born starved piglets to mucosalis but this was essentially to study whether exposure before or after colostrum ingestion affected the establishment of mucosalis rather than to study the effect of deprivation of food.

In this thesis when an attempt was made (Chapter 3) to repeat Roberts' successful transmission, chalk and benzetimide were given beforehand to reduce gastric acidity and gastro-intestinal motility. The effect of the indigenous gut flora on the establishment of

mucosalis infection was studied in a different way however by the use of gnotobiotic piglets (Chapter 5).

Specific Immunity to Mucosalis:

Preliminary studies have been reported by Lawson et al., (1982). A limited number of sow colostral samples were examined and found to have high titres of specific agglutinating antibody to mucosalis. This is presumably rapidly transferred to the blood stream of piglets sucking such sows and indeed serum samples from sucking piglets also had high titres of specific agglutinating antibody to mucosalis (Lawson et al., 1982). A number of sow milk samples have been examined and also found to contain specific agglutinating antibody to mucosalis (McCartney, Lawson and Rowland, unpublished results). Hence in addition to specific circulating antibody and perhaps of greater importance in determining whether mucosalis establishes in the gut, sucking piglets may ingest antibodies in sow milk which will agglutinate mucosalis.

Serum agglutinating antibodies to mucosalis are rare in pigs between 30 and 77 days of age (Lawson et al., 1982) and this probably reflects the diminishing passively-acquired immunity. Pigs in the field develop circulating agglutinating antibody by about 77 days of age and in general titres increase with age until at slaughter a high proportion of pigs have specific agglutinating antibodies to mucosalis. That

these antibodies are specific for mucosalis is suggested by the observation that none of a group of pigs prevented from exposure to mucosalis developed antibody by slaughter at 70 - 91 days of age while exposed littermates did develop antibody (Lawson et al., 1982).

Pigs affected with PIA also have high serum titres of specific agglutinating antibody to mucosalis. It is unclear to what extent the presence of specific serum antibodies reflects a previous episode of intracellular parasitism by mucosalis, or whether such antibody could arise as a result of oral cavity infection or extracellular infection in the gut lumen.

The failure to transmit PIA may be related to maternally-acquired immunity or to the development of active immunity by the growing pig. The lack of consistently successful transmission and the variation in response between litters (Roberts, 1978) may reflect differences in the immune status of different litters. Such differences in immune status may also partly explain the sporadic incidence of PIA in the field. Accordingly in this thesis attempts were made to transmit PIA in animals devoid of antibodies i.e. in colostrum-deprived (Chapter 4) and gnotobiotic (Chapter 5) piglets. It seemed reasonable also to attempt transmission in older post-weaned pigs of about 30 days of age since these rarely have circulating agglutinating antibody to mucosalis (Lawson et al.,

1982).

(d) Dose Response

In transmissible ileal hyperplasia of hamsters, a proliferative enteropathy similar in many respects to PIA and reviewed in the next part of this chapter, it has been shown that the disease is consistently transmissible using homogenised affected mucosa as the inoculum, but that if the inoculum is sufficiently dilute the percentage transmission is reduced (Frisk, 1976; La Regina, 1979).

Roberts (1978) postulated that in PIA the failure of transmission using weaned pigs as recipients of the inoculum was due to the difficulty in giving a sufficiently large dose. Greater success in transmission was achieved using much younger pigs where the dose of adenomatous mucosa was perforce much greater relative to body weight and surface area of the gut.

However it is unlikely that pigs in the field which develop PIA have been exposed to such large doses of infective material as experimental animals and so the failure to transmit PIA experimentally is probably not simply related to the size of the infective dose.

HUMAN AND OTHER ANIMAL ENTEROPATHIES AND THEIR RELATIONSHIP TO THE INTESTINAL ADENOMATOSIS COMPLEX OF PIGS.

(i) Transmissible Ileal Hyperplasia (Hamster Enteritis).

Transmissible ileal hyperplasia is a disease complex affecting hamsters in the post-weaning period. The complex has been recently reviewed (Frisk, 1976; Frisk and Wagner, 1977b, La Regina, 1979) and has a proliferative component similar to that seen in PIA. Like the intestinal adenomatosis complex, transmissible ileal hyperplasia has been referred to by a number of terms e.g. proliferative ileitis, regional enteritis, terminal ileitis, enzootic intestinal adenocarcinoma, atypical ileal hyperplasia and "wet-tail" of weanling hamsters (Frisk, 1976). The disease complex will be referred to as transmissible ileal hyperplasia (TIH) here. Unlike the intestinal adenomatosis complex, TIH is easily transmitted by oral inoculation of weanling hamsters with suspensions of ground ilea from affected hamsters (Jacoby, Osbaldiston and Jonas, 1975; Amend et al., 1976; Frisk, 1976; La Regina, 1979).

(a) Historical and Geographical Aspects:

This disease has been reported exclusively from America (Friedman, 1965; Jonas, Tomita and Wyand, 1965; Boothe and Cheville, 1967; Jackson and Wagner, 1970;

Wagner, Owens and Troutt, 1973), probably because the hamster is widely used as a laboratory animal there and large colonies are maintained commercially.

Friedman (1965) described the first major natural outbreak of TIH and since then there have been numerous reports in the American literature of the occurrence of the complex in hamster colonies (Jonas, 1965; Jonas, Tomita and Wyand, 1965; Boothe and Cheville, 1967; Jackson and Wagner, 1970; Goldman, Andrew and Lang, 1972).

(b) Occurrence:

Like PIA, transmissible ileal hyperplasia is seen most commonly in the post-weaned age-group – hamsters aged from 3-8 weeks (Frisk and Wagner, 1977b; La Regina, 1979). It is reputed to be the most common spontaneous disease of hamsters with a variable morbidity (20-60%) and a high mortality (90%) (Frisk, 1976; Frisk and Wagner, 1977b).

(c) Clinical Signs:

Early reports of colony outbreaks and experimental transmission of TIH described acute, subacute and chronic stages (Jacoby, Osbaldiston and Jonas, 1975; Frisk, 1976; Frisk and Wagner, 1977b), although a later report did not refer to an acute phase (Jacoby, 1978) and more recent work has suggested that the acute clinical phase is not essential for the development of the later proliferative lesions (La Regina, 1979; La Regina, Hales and Wagner, 1980).

The acute clinical phase, now considered as a separate disease entity from the proliferative syndrome (La Regina, 1979; La Regina, Hales and Wagner, 1980), was characterised by profuse, sometimes bloody diarrhoea and rapid dehydration. This phase occurred 7-10 days post-inoculation (p.i.) in experimental transmissions of the complex (Jacoby, Osbaldiston and Jonas, 1975; Frisk, 1976). Subacute signs were manifest 21-30 days p.i. and included diarrhoea, stunted growth and palpable abdominal masses (Jacoby, Osbaldiston and Jonas, 1975). Frisk (1976) reported deaths occurred from day 1 p.i. to day 43 p.i. and in his experimental transmissions 97% mortality occurred in inoculated hamsters by day 35 p.i. In the chronic phase surviving hamsters had improved growth rates from earlier in the disease and palpable abdominal masses. Deaths were few (Jacoby, Osbaldiston and Jonas, 1975).

(d) Pathology of Transmissible Ileal Hyperplasia.

Gross lesions:

In hamsters dying in the acute phase of TIH gross lesions were those of a severe enteritis of the terminal small intestine. The ileum was thin-walled, hyperaemic and dilated, and contained foetid yellow fluid which was sometimes blood-stained. Ileal and colonic intussusceptions frequently accompanied this acute phase (Jacoby, Osbaldiston and Jonas, 1975; Frisk, 1976; La Regina, 1979). In the subacute

phase there was grossly visible mild diffuse enlargement of the ileum which commenced by day 15 p.i. and continued to thicken until day 25 p.i. (Frisk, 1976; Jacoby, 1978). La Regina (1979) reported that in her experimental transmissions the proliferative phase was well-advanced by 21 days p.i. and suggested that slight differences in the time-scale of the development of the lesion were inevitable due to the crude nature of the inoculum. By 30 days p.i. the ileal walls were thickened up to 3-4 times normal size and in addition subserosal granulomas and abscesses were visible grossly. Affected portions of gut were turgid and friable and the lumen was occasionally occluded by necrotic mucosa and debris (Frisk, 1976; Jacoby, 1978; La Regina, 1979).

Lesions of chronic ileitis were observed in hamsters surviving the early acute phase. In these there was thickening and fibrosis of the ileum and stricture of the ileo-caecal junction. Scar tissue contraction at this site often resulted in partial or complete obstruction (Jacoby, Osbaldiston and Jonas, 1975; Frisk, 1976). Adhesions between the ileum and adjacent structures in the chronic phase were considered by Frisk and Wagner (1977b) to be a result of rupture of subserosal abscesses and subsequent focal peritonitis.

Histological Changes: Microscopic lesions in the acute phase varied in severity from mild catarrhal enteritis to severe diffuse haemorrhagic necrosis of the mucosa (Jacoby, Osbaldiston and Jonas, 1975). Frisk (1976) described the most consistent lesion of the acute phase as blunting and fusion of villi in the ileum and infiltration of the lamina propria with polymorphonuclear leucocytes. Hyperplasia of the mucosa was not a feature in the acute phase (Frisk, 1976; Jacoby, Osbaldiston and Jonas, 1975; La Regina, 1979).

Proliferation of epithelial cells of the mucosa commenced in the crypts by 10 days p.i. (Frisk, 1976; Jacoby, 1978) and was focal in distribution (Frisk, 1976), an interesting finding in the light of the postulated early pathogenesis of PIA (Rowland et al., 1980) where early formation of adenomatous glands was observed at the base of the mucosa. In TIH focal hyperplasia of the crypts spread progressively from day 10 p.i. - days 20-23 p.i. by which time the mucosa was greatly thickened and largely composed of vigorously proliferating, poorly-differentiated epithelial cells from the crypts to the by now greatly elongated villi (Frisk, 1976). As in PIA the mucosal glands were enlarged, misshapen and tortuous, and lined by immature enterocytes among which goblet cells were rare or absent. Many cells

were seen in mitosis and the cells often overlapped, giving a pseudostratified appearance to the epithelium (Boothe and Cheville, 1967; Frisk, 1976; La Regina, 1979). A comparison of the histology of PIA and TIH showed that at this stage the lesions were closely similar (Rowland, personal communication, 1982).

From day 20 p.i. there was a vigorous inflammatory response associated with the hyperplastic mucosa (Frisk, 1976; Jacoby, 1978; La Regina, 1979) which progressed from infiltration of the lamina propria with polymorphs and mononuclear cells to massive pyogranulomatous inflammation of the lamina propria and submucosa by days 30-35 p.i. (Frisk, 1976; La Regina, 1979). Coagulative necrosis of the entire depth of the thickened mucosa and penetration of the submucosa and muscle layers by hyperplastic, pus-filled crypts was not uncommonly described in the later stages of the proliferative phase (Boothe and Cheville, 1967; Jacoby, Osbaldiston and Jonas, 1975; Amend et al., 1976; Frisk, 1976; Frisk and Wagner, 1977b). The chronic stage of TIH was characterised microscopically by a relatively normal absorptive epithelium, subserosal granulomatous tissue formation, and often markedly hypertrophied muscle layers, sometimes with associated fibroplasia (Frisk, 1976).

(e) TIH and the Intestinal Adenomatosis Complex.

From the above descriptions it is clear that TIH

has components, particularly in the subacute and later phases, which bear a striking resemblance to the proliferative enteropathies of pigs. The acute phase of TIH has no parallel in PIA, but recent experimental studies suggest that the acute phase of TIH is a separate disease entity and an inessential precursor to the proliferative phase (La Regina, 1979; La Regina, Hales and Wagner, 1980).

The proliferative phase of TIH and its sequelae are reminiscent of uncomplicated PIA and the possible sequelae NE and RI.

(f) Aetiology of Transmissible Ileal Hyperplasia.

The aetiology of TIH is still in question. Despite the relative ease of transmission Koch's postulates have not been fulfilled (Frisk, 1976).

Numerous bacterial agents have been suggested as of possible aetiological importance and these were listed by Frisk (1976) and Frisk and Wagner (1977b). Other possible aetiologic agents which these authors discussed included viruses, rickettsia, protozoan or helminth parasites, and nutritional deficiencies. Frisk (1976) discounted most of these but concluded that the cause was most probably infectious and possibly a synergism existed between locally-invasive enteropathogenic E. coli (EPEC), involved in the acute phase of the disease, and campylobacter-like organisms, responsible for the proliferative phase. In ultrastructural studies of experimentally-inoculated hamsters Frisk (1976)

observed locally-invasive EPEC in villous epithelial cells shortly after dosing with ground proliferated ilea. Campylobacter-like organisms appeared in crypt cells from 5 days p.i. and were observed occasionally along with EPEC in villar cells. Later in the disease campylobacter-like organisms were observed in constant association with proliferating cells and EPEC were absent.

Frisk (1976) isolated strains of EPEC from hamsters with TIH but could only produce the acute enteric stage with these. He also isolated campylobacter-like organisms but could not produce the proliferative phase with these, whether given in pure culture or whether inoculated after previous EPEC. However immunofluorescent studies indicated that the campylobacters isolated by Frisk were not those observed in proliferating epithelial cells. Frisk was unable to produce the proliferative phase by inoculating ground ilea from hamsters in the acute phase (5 days p.i.) but was able to produce proliferative changes by inoculating ground ilea from hamsters in the early proliferative phase (9 days p.i.).

Jacoby, Osbaldiston and Jonas (1975) also implicated bacteria in the aetiology of TIH and were able to reproduce proliferative lesions by inoculating mixed bacterial flora from ileal lesions, or with filtrates of ileal homogenates. Morbidity was zero when the homogenate was passed through a 0.22 μm filter and reduced if filtered through 0.45 μm

mesh. These findings suggested a bacterial aetiology and tended to exclude viral agents.

Until recently EPEC were commonly considered to be involved in the aetiology of TIH (Wagner, Owens and Troutt, 1973; Jacoby, Osbaldiston and Jonas, 1975; Amend et al., 1976; Frisk, 1976) probably because these bacteria were frequently isolated from proliferated ilea and also caused a local-invasive acute colibacillosis in weanling hamsters which was, however, only infrequently followed by proliferative changes (Jacoby, Osbaldiston and Jonas, 1975).

Interestingly Wagner, Owens and Troutt (1973) published electron-micrographs of intracellular bacteria in the proliferative phase of TIH. These had the ultrastructural morphology of vibrios and were similar to the campylobacters observed intracellularly in PIA (Rowland and Lawson, 1974). However Wagner, Owens and Troutt (1973) concluded that E. coli were of aetiological importance since these were cultured in high numbers from the lesions. Despite strongly implicating campylobacters as of aetiological importance in TIH (Frisk, 1976), Frisk does not mention them in a later review (Frisk and Wagner, 1977b).

La Regina (1979) and La Regina, Hales and Wagner (1980) have recently presented compelling evidence that the acute phase of TIH is a distinct clinical and pathological entity caused by locally-invasive EPEC. This acute phase is inessential for the subsequent development

of epithelial cell proliferation and the proliferative phase is characterised by the constant association of an intracellular bacterium which strongly resembles a campylobacter.

La Regina (1979) inoculated hamsters with proliferated ilea and then treated groups of her experimental animals with various antibiotics. She treated two groups with neomycin which was effective against EPEC strains isolated from the original proliferated ilea. These groups did not develop acute enteritis but did develop proliferative changes in the ileum associated with the presence of an intracellular campylobacter-like organism. Another inoculated group was treated with dimetridazole, to which the EPEC strains were resistant. In this group there was some reduction in the numbers of hamsters developing proliferative lesions, hence La Regina concluded that anaerobic bacteria and not E. coli were implicated in the aetiology. She also isolated campylobacter-like bacteria from proliferative lesions but did not reproduce proliferative changes in hamsters inoculated with pure cultures of these.

Jacoby, Onderdonk and Jonas (1978) were able to isolate in cell culture a Gram-negative organism from proliferated ilea. The organism was found by immunofluorescent studies to possess the same antigens as organisms found intracellularly in proliferated epithelial cells of hamsters with TIH. This organism

remained viable for 2 weeks in cell culture, an interesting observation considering the recent work of Rajasekhar (1981) who has been able to achieve similar results with Campylobacter sputorum ss mucosalis in cell culture. However it has not proved possible to reproduce TIH using the cell-cultured organism (Jacoby and Johnson, 1981), which led these authors to suggest that transmission of TIH requires more than one agent.

Jacoby and Johnson (1981) observed that the severity of a TIH outbreak could be exacerbated by environmental and husbandry factors such as overcrowding, excess noise and experiment-related treatments. Notably hamsters fed certain closed formula commercial diets did not develop TIH after experimental exposure, while 90-100% of hamsters fed other diets (also closed formula) developed typical lesions of TIH (Jacoby and Johnson, 1981).

(ii) Regional Enteritis in Sheep

In 1962 Wensvoort described a condition in ill-thriven lambs of the Texel breed which he referred to as 'stretchers' due to the characteristic tendency in affected animals for the fore and hind limbs to be extended. At post-mortem examination the ileum was thickened by chronic inflammation. The mucosa appeared nodular and thickened in the photographs this author published.

Hoorens et al. (1977) described a case of regional enteritis in a 4 month old lamb, also of the Texel breed

and noted that the clinical picture of wasting and poor growth was similar to that described by Wensvoort (1962) for lambs with 'stretchers'. At post-mortem the jejunun and ileum were grossly thickened and areas of the mucosal surface showed a 'cobblestone appearance'. Histologically there was a chronic inflammatory reaction in the lamina propria and serosa and in some areas a distinct hyperplasia of the glands of Lieberkhun. On electron-microscopic examination campylobacter-like bacteria (0.2 - 0.3 μm X1 - 1.5 μm) were observed in the apical cytoplasm of the hyperplastic cells.

The above two reports described lesions which resemble regional ileitis of pigs in that only islands of proliferative mucosa remained surrounded by chronic granulomatous inflammation.

Cross, Smith and Parker (1973) described a terminal ileitis in 7 lambs ranging in age from 4 to 6 months. The clinical signs were poor live weight gains. At post-mortem the terminal ileum was thickened by rugose corrugations of the mucosa which histologically had an adenomatous appearance. No electron microscopy was undertaken.

In 1980 Vandenberghe and Hoorens isolated two strains of campylobacters from 2 lambs with clinical signs similar to those described by Wensvoort (1962) and adenomatous mucosal proliferation of the terminal jejunun and ileum. These strains resembled mucosalis

in many respects; they were catalase-negative micro-aerophilic campylobacters with similar biochemical characteristics to mucosalis apart from failure to reduce nitrite. These authors concluded that regional enteritis in lambs could be a manifestation of a similar disease to PIA.

(iii) Mouse Colon Hyperplasia

This is a transmissible disease of mice caused by a variant of Citrobacter freundii (Barthold et al., 1976). The disease has similarities to both TIH and PIA although the causative organisms do not appear intracellularly. In natural outbreaks in mouse colonies the disease is associated with diarrhoea, runting and high mortality in sucking and weanling mice (Barthold et al., 1978). The fundamental change is proliferation of epithelial cells, usually of the colon, although caecal and ileal hyperplasia have been observed (Barthold et al., 1978). Experimental inoculation of Citrobacter freundii results in development of the lesion in all ages of mice (Barthold et al., 1978), unlike experimentally-produced TIH in which weanlings are more susceptible than adults (Frisk, 1976).

Barthold et al., (1976) inoculated Citrobacter freundii into germ-free mice and induced colonic mucosal hyperplasia, recovering the organism in pure culture from affected animals. Grossly the colons were rigid

and the number of cells lining the crypts of hyperplastic colons was 2 - 3 times greater than in controls. The mitotic rate of the epithelium was increased and goblet cells were decreased in number or absent. Hyperplasia without inflammation occurred in the earlier lesions and inflammatory cell infiltrates were irregularly present in later stages. The location of Citrobacter freundii was not reported at that time but Johnson and Barthold (1979) conducted an ultrastructural study of transmissible murine colonic hyperplasia, correlating light microscopic changes with scanning and transmission electron-microscopic findings, and observed that bacteria morphologically identical to Citrobacter freundii became attached to the surface of the mucosa between 4 and 10 days after inoculation. Hyperplasia of the mucosa was most severe at 10 days post-inoculation and thereafter underwent regression until near normality returned at 45 days post-inoculation. These authors concluded that the severe mucosal proliferation with minimal inflammatory change resulted from attachment of bacteria to the surface mucosal epithelium and that this response was a defence mechanism in which cells infected with C. freundii were replaced with newly-migrated, uninfected epithelium.

(iv) Crohn's Disease in Man (Regional Enteritis).

Regional ileitis in the pig has been likened to

Crohn's disease due to the gross similarity of thickening of the wall of the terminal ileum (Emsbo, 1951). Opinions have varied over the years as to the validity of this comparison (Roberts, 1978). In man, Crohn's disease may present macroscopically in 3 patterns; aphthous ulceration of the mucosa may predominate, strictures can develop or the mucous membrane may have a "cobblestone" appearance (Morson and Dawson, 1972). Any part of the alimentary canal may be involved. Histologically there is a transmural inflammation with thickening of the submucosa by oedema, granulation tissue formation and an inflammatory infiltrate of predominantly lymphocytes and plasma cells.

Despite the gross resemblance between Crohn's disease and regional ileitis, the histological and ultrastructural findings in the human enteropathy are quite distinct from those in the pig. Epithelial hyperplasia, the fundamental change in the porcine enteropathies (Rowland and Lawson, 1975a), has not been described in Crohn's disease, although as Roberts (1978) pointed out any proliferative lesion in Crohn's disease may have regressed by the time the mucosa was sampled. Bacteria in the apical cytoplasm of enterocytes, described in the porcine proliferative enteropathies (Rowland and Lawson, 1975a), have not been observed in ultrastructural studies of Crohn's disease (Ranlov, Nielsen and Wanstrup, 1972; Cook and

Turnbull, 1975).

Many different aetiologies have been postulated for Crohn's disease and these are discussed more fully by Frisk (1976) and Roberts (1978).

In a recent serological study Matthews *et al.*, (1980) observed that sera from patients with Crohn's disease did not agglutinate *Campylobacter sputorum* ss *mucosalis* although they considered that such a negative agglutination test did not exclude the possibility that similar but antigenically-distinct organisms may play a part.

CAMPYLOBACTERS IN MAN AND ANIMALS

(i) The Genus Campylobacter

(a) Origins: The original genus Vibrio was made up of two groups of bacteria with widely different characters (Cowan and Steel, 1965; *vide infra*). The genus Campylobacter was formed from vibrio-like, curved microaerophilic bacteria originally described under the names *Vibrio fetus* (Smith and Taylor, 1919), *Vibrio jejuni* (Jones, Orcutt and Little, 1931), *Vibrio sputorum* (Prévot, 1940), *Vibrio coli* (Doyle, 1948) and

Vibrio bubulus (Florent, 1953). The reasons for designation of a new genus for these organisms were that they showed significant genotypic and phenotypic differences from the genus Vibrio (Sebald and Véron, 1963; Véron and Chatelain, 1973). Whereas members of the genus Vibrio grow aerobically, ferment glucose and contain DNA with a base composition of guanine and cytosine (G+C) between 40 and 53 mol %, campylobacters require reduced oxygen tension for growth, do not ferment carbohydrates and have DNA with a base composition of G+C between 29-36 mol % (Véron and Chatelain, 1973).

Véron and Chatelain (1973) further defined the genus Campylobacter as comprising Gram-negative, slender and curved bacteria which are motile by means of a single, polar flagellum and which are microaerophilic with a strictly respiratory metabolism. These authors advocated placing the genus Campylobacter in the family Spirillaceae due to the morphological and physiological similarities between the genera Campylobacter and Spirillum. In particular there were striking anatomical similarities observed ultrastructurally:

a) an outer wavy membrane or "integument", easily separated from the inner membrane with a large vacant space between both membranes.

b) a "complex cytoplasmic membrane" consisting of

a triple-layered membrane associated with short bar-like elaborations making up centripetally oriented compartments.

c) individual flagellar basal granules.

(Véron and Chatelain, 1973).

(b) Nomenclature: Unfortunately prior to 1980 two different taxonomic schemes existed for the Campylobacter genus, that of Véron and Chatelain (1973) and that of Smibert (1974). Since the names given by Véron and Chatelain (1973) have been adopted by the International Journal of Systematic Bacteriology (the official organ of the International Committee on Systematic Bacteriology of the International Association of Microbiological Societies) and are listed in the Approved Lists of Bacterial Names (Skerman, McGowan and Sneath, 1980), the nomenclature used below will largely follow that of Véron and Chatelain (1973). Previous names and Smibert's (1974) nomenclature will follow in brackets where confusion is likely to occur.

Broadly the genus Campylobacter can be divided into two main groups, the catalase-positive organisms associated with animal and human disease and a single catalase-negative species whose recognised subspecies prior to 1974 were commensals of the human oral cavity or the bovine genital tract (Lawson et al., 1981). A third subspecies of the catalase-negative species was found associated with the intestinal

adenomatosis complex of pigs and was described and characterised by Lawson and Rowland (1974) and Lawson, Rowland and Wooding (1975).

The names used in this thesis will be those underlined below: AI - IV; BI - III.

A. Catalase-Positive Campylobacters

I Campylobacter fetus subspecies fetus
(Vibrio fetus ss intestinalis Florent 1959, Campylobacter fetus ss intestinalis Smibert 1974).

II Campylobacter fetus subspecies venerealis
(Vibrio fetus ss venerealis Florent 1959, Campylobacter fetus ss fetus Smibert 1974).

III Campylobacter coli
(possibly Vibrio coli Doyle 1948, Campylobacter fetus ss jejuni Smibert 1974).

IV Campylobacter jejuni
(Vibrio jejuni Jones, Orcutt and Little 1931, Campylobacter fetus ss jejuni Smibert 1974).

B. Catalase-Negative Campylobacters

I Campylobacter sputorum subspecies sputorum
(Vibrio sputorum Prévot 1940, Vibrio sputorum ss sputorum Loesche, Gibbons and Socransky 1965, Campylobacter sputorum ss sputorum Smibert 1974).

II Campylobacter sputorum subspecies bubulus
(Vibrio bubulus Florent 1953, Vibrio sputorum ss bubulus

Loesche, Gibbons and Socransky 1965, Campylobacter sputorum ss bubulus Smibert 1974).

III Campylobacter sputorum subspecies mucosalis

This organism is not listed in the Approved Lists of Bacterial Names (Skerman, McGowan and Sneath, 1980), a surprising omission since a valid description of the subspecies was published in 1975 (Lawson, Rowland and Wooding, 1975) and acknowledged as such by the editor of the International Journal of Systematic Bacteriology in 1975 (Lawson, personal communication, 1981). Unequivocal validation of this subspecies was finally achieved in 1981 (Lawson et al., 1981).

(c) Characters which Differentiate the Campylobacters in the Laboratory: The biochemical characteristics which allow differentiation are summarised in Tables 1.1. and 1.2.

A. Catalase-Positive Campylobacters: These are subdivided into two main groups:

(i) Campylobacter fetus: the 2 subspecies, ss fetus and ss venerealis, form a group which grow at 25°C but not generally at 43°C. Further differentiation between these 2 subspecies is by biochemical tests (Table 1.1) and of individual strains by serology (Véron and Chatelain, 1973; Smibert, 1974; Firehammer, 1979).

(ii) "Thermophilic Campylobacters": These are Campylobacter coli and Campylobacter jejuni. Strains of these 2 species do not grow at 25°C but will grow at

TABLE 1.1.

CHARACTERS WHICH DIFFERENTIATE THE CATALASE-POSITIVE CAMPYLOBACTERS.

Species (Véron and Chatelain, 1973)	BIOCHEMICAL TESTS						
	1	2	3	4	5	6	7
	Catalase	Growth 25°C	at 43°C	Growth in 1% glycine	Production of H_2S	Hydrolysis of Hippurate	Growth in Nalidixic Acid
Campylobacter fetus ss *fetus*	+	+	v	+	+[a]	-	+
Campylobacter fetus ss *venerealis*	+	+	v	-	-[a]	-	+
Campylobacter coli } "Thermophilic							
Campylobacter jejuni } Campylobacters"	+	-	+	+	+[b]	+	-
"NARTC" [c]	+	-	+	+	+[b]	+	+

+ = positive test or growth
- = negative test or no growth
v = variable
5a = Production of H_2S in 0.05% cysteine media
5b = Production of H_2S in iron-containing media
c = NARTC = Nalidixic-acid-resistant-thermophilic-campylobacters (Skirrow, 1980)
1-5a (Véron and Chatelain, 1973)
5b-7 (Skirrow and Benjamin, 1980a).

TABLE 1.2

CHARACTERS WHICH DIFFERENTIATE THE CATALASE-NEGATIVE CAMPYLOBACTERS.

Species (Smibert, 1978) Results (Lawson, Rowland and Wooding, 1975)	BIOCHEMICAL TESTS							
	1	2	3	4			5	
	Catalase	Oxidase	Growth in 1% glycine	Growth in NaCl 1.5%	2.0%	3.0%	Growth in Na deoxycholate 0.5%	0.2%
Campylobacter sputorum subspecies sputorum	-	D	+	+	-	-	v	-
Campylobacter sputorum subspecies bubulus	-	+	+	+	+	+	+	-
Campylobacter sputorum subspecies mucosalis	-	D	-	+	v	-	+	-

+ = positive test or growth

- = negative test or no growth

D = delayed positive

V = variable or inconclusive results

43°C (Skirrow, 1980). Differentiation between species is more difficult but can be achieved by biochemical tests (Table 1.1) and possibly by serology (Kosunen, Danielsson and Kjellander, 1980; Firehammer and Davies, 1981). Skirrow (1980) and Skirrow and Benjamin (1980a) have described a distinct group of nalidixic-acid-resistant-thermophilic campylobacters which may constitute a third species. Hence despite the efforts of the Judicial Commission of the International Committee on Systematic Bacteriology (Skerman, McGowan and Sneath, 1980) there are still taxonomic uncertainties, at least among the thermophilic campylobacters.

B. Catalase-Negative Campylobacters (Table 1.2).

Differentiation between catalase-negative strains is relatively simple by biochemical and serological means (Lawson, Rowland and Wooding, 1975; Smibert 1978; Lawson et al., 1981).

The DNA base composition of Campylobacter sputorum ss mucosalis has recently been established as 33.9 mol % G+C, placing this organism firmly in the genus Campylobacter and adding a third subspecies to Campylobacter sputorum (Lawson et al., 1981). There is also a heterogenous group of catalase-negative campylobacters similar to ss mucosalis and associated with the mouths, faeces and alimentary tracts of pigs

Lawson et al., 1981). These strains (NAC's) are serologically and biochemically distinct from ss mucosalis and of uncertain taxonomy (Lawson et al., 1981; Table 1.3).

(d) Distribution and Pathogenicity:

I. Campylobacter fetus ss fetus: Strains of this organism occur principally in the gut of sheep, cattle, pigs, birds and humans, and are usually commensal. All strains may cause sporadic outbreaks of abortion, mainly in cattle and sheep, due to their ability to multiply in the placenta after passage from the gut to the viscera. Some strains are venereally transmitted and can survive in the genital tracts of cattle (Véron and Chatelain, 1973). According to Firehammer (1979) campylobacteriosis of sheep due to C. fetus ss fetus occurs at a low level throughout the world including the U.K., whilst human disease due to this subspecies has been reported from the U.S.A. and the Federal Republic of Germany. Such human disease is not intestinal and generally takes place in compromised individuals.

II. Campylobacter fetus ss venerealis: This organism is strictly adapted to the bovine genital tract and is unable to survive in the gut. It causes enzootic infertility in cattle and abortion in cows (Véron and Chatelain, 1973). Bovine campylobacteriosis due to ss venerealis occurs occasionally in the U.K. and is endemic in many areas of the world (Firehammer,

TABLE 1.3

CHARACTERS WHICH DIFFERENTIATE CAMPYLOBACTER SPUTORUM SS MUCOSALIS FROM OTHER CATALASE-NEGATIVE CAMPYLOBACTERS ISOLATED FROM PIGS.

Results (Lawson et al., 1981)	BIOCHEMICAL TESTS					Agglutination with antisera against Serotype A mucosalis
	Nitrite Reduction	H_2S Production	Growth in 1% Glycine	Growth in NACl 1.5%	Growth in NACl 2.5%	
Campylobacter sputorum ss mucosalis (Serotype A strains)	+	+	-	+	-	+
Non-agglutinating, catalase-negative porcine campylobacters (NAC'S))Strain 1189/74	-	+	-	+	-	-
)Strain 179/75	-	-	-	-	-	-
)Strain 20/74	+	+	+	+	+	-

\+ = positive test or growth.

\- = negative test or no growth.

1979).

III. Campylobacter coli and Campylobacter jejuni: The distribution and pathogenicity of these two species will be considered together as differentiation between the strains has not always been achieved and many strains have intermediate characteristics (Skirrow and Benjamin, 1980b). Collectively C. coli and C. jejuni are known as the "thermophilic" campylobacters (Skirrow, 1980) and can be found in the gut and faeces of normal calves, lambs, pigs, wild birds and poultry (Smibert, 1978). According to Skirrow and Benjamin (1980b) most strains derived from cattle and man resemble C. jejuni, most of the pig strains resemble C. coli, while the poultry strains occupy an intermediate position.

The thermophilic campylobacters have emerged recently as a common cause of acute enteritis in man (Butzler et al., 1973; Skirrow, 1977; Skirrow, 1980). The disease is a zoonosis and although the origin of sporadic infections is unknown, probably many are acquired via the food chain and especially from poultry. Infected pet animals suffering from campylobacter enteritis are occasional sources of human infection (Skirrow, 1980).

The role of thermophilic campylobacters as animal pathogens is less clear cut. Certainly some strains of C. jejuni cause abortion in sheep (Smibert, 1974;

Firehammer, 1979) and there have been reports of the occurrence of thermophilic campylobacters in enteritis of dogs (Blaser et al., 1978), cats (Gruffydd-Jones, Marston and White, 1980), primates (Tribe, McKenzie and Fleming, 1979), cattle (Al-Mashat and Taylor, 1980) and pigs (Taylor and Olubunmi, 1981). However Prescott and Bruin-Mosch (1981) found no significant difference in the carriage of strains between healthy and diarrhoeic animals. It is possible that further work on the role of thermophilic campylobacters in enteric disease of animals will clarify present doubts.

Distribution of thermophilic campylobacters is probably world-wide. They have been reported from several continents; Europe (Skirrow and Benjamin, 1980b), North America (Prescott and Bruin-Mosch, 1981) and Australia (Williams and Deacon, 1980). They are considered by the World Health Organisation Scientific Working Group (1980) as possibly of greater importance as a cause of diarrhoeal disease in developing countries than in industrialised countries.

IV Campylobacter sputorum:

Campylobacter sputorum ss sputorum is non-pathogenic and occurs as part of the normal flora of the oral cavity of man, where it forms about 5% of the cultivable organisms (Loesche, Gibbons and Socransky, 1965; Smibert, 1978).

Campylobacter sputorum ss bubulus is also considered non-pathogenic and is found in the vaginal mucosa of cattle and sheep and in the prepuce of bulls (Smibert, 1978). Experimental infection of the bovine uterus resulted in mild or no histopathological changes although the organism could be recovered up to 24 days post-infection (Dozsa, 1965).

Campylobacter sputorum ss mucosalis: The distribution of this organism, probably world-wide (Roberts, 1978), and its possible pathogenicity have been discussed in detail in the preceding sections of this chapter. Its constant association with the intestinal adenomatosis complex of the pig and its intimate relationship with the host cell are unusual. Although mucosal invasion can occur in enteric disease associated with thermophilic campylobacters (Duffy, Benson and Rubin, 1980) the resulting lesions are of a destructive enteritis, quite unlike the proliferation associated with intracellular mucosalis infection in the pig.

Similar relationships between campylobacter-like organisms and host cells have been recognised in sheep (Vandenberghe and Hoorens, 1980) and hamsters (La Regina, 1979), suggesting that such an association, although unusual, is perhaps not due to an idiosyncracy of the pig but more likely a unique feature of certain strains of campylobacters.

CHAPTER 2

PLAN OF WORK AND GENERAL MATERIALS AND METHODS.

PLAN OF WORK.

The primary aim of the work reported in this thesis was to reproduce intestinal adenomatosis in experimental piglets, thus enabling a study of the role of Campylobacter sputorum ss mucosalis (mucosalis) in the pathogenesis of the disease.

Field cases of porcine intestinal adenomatosis (PIA) were studied, initially to provide experience for the author in the techniques used, and later to obtain infective mucosa and strains of mucosalis with which to orally dose experimental piglets.

Naturally-farrowed, cross-suckled piglets were exposed to adenomatous mucosa and cultures of mucosalis, using a similar experimental design to a previous successful transmission (Roberts, 1978). This method did not result in reproduction of lesions of PIA in exposed piglets, and as a result alternative approaches to the problem were tried.

Naturally-farrowed, colostrum-deprived piglets were exposed to adenomatous mucosa, but did not survive for long enough to assess whether intestinal adenomatosis would have occurred. Caesarean-derived, gnotobiotic piglets were exposed to cultures of mucosalis alone and in combination with rotavirus, to assess the pathogenicity of mucosalis in the absence of maternal antibodies and a conventional enteric flora, and to investigate whether rotavirus predisposes

the enteric mucosa to intracellular multiplication of *mucosalis* leading to the development of PIA.

Pilot studies were carried out in weaned pigs, exposed to either dual *mucosalis* and rotavirus infections or dual *mucosalis* and enterotoxigenic *Escherichia coli* (ETEC) infections. The purpose was to study whether rotavirus or ETEC could predispose to the establishment of *mucosalis* in the enteric mucosa of weaned pigs and hence initiate lesions of PIA.

Finally a series of experiments was carried out, on piglets under various systems of management, to investigate whether cryptosporidia, protozoan parasites of the surface of mucosal epithelial cells, could promote intracellular parasitism of pig enterocytes by *mucosalis*.

GENERAL MATERIALS AND METHODS.

The following materials and methods were used throughout the work. Details of particular techniques used in individual experiments will be found in the relevant chapters.

(a) CULTURE MEDIA. The following media were used: (All Oxoid media were prepared according to

the manufacturer's instructions).

Blood Agar (BA), Hartley's digest agar (Cruickshank, 1965) containing 5% oxalated horse blood.

Columbia Blood Agar (CBA), Columbia agar base (Oxoid, CM 331) incorporating 7% oxalated horse blood.

Desoxycholate Citrate Agar (DCA), Oxoid, CM 35).

Diphasic Slopes (CBA/TPB) were prepared either by overlaying a 10ml CBA slope in a McCartney bottle with 5ml tryptose phosphate broth (TPB), or by overlaying a 30ml CBA slope in a 100ml medical flat bottle with 30ml TPB.

Glucose Agar Slopes (GA), Blood agar base No. 2 (Oxoid, CM 271) incorporating 0.1% glucose.

MacConkey Agar ($M^{C}C$), (Oxoid, CM 7 b, without salt).

Minca - IsoVitalex Agar (Minca - Is) was prepared according to the method of Guinée, Veldkamp and Jansen (1977).

Novobiocin-Brilliant Green - Trimethoprim Agar (NBGT). This selective medium, used for the isolation of Campylobacter species from pig tissues, was developed by Dr. Gordon Lawson, Department of Veterinary Pathology, University of Edinburgh, to replace the previous selective medium (NBG, Lawson and Rowland, 1974) in an effort to improve the recovery of mucosalis from contaminated tissues. NBGT differs from NBG in the base employed, in the inclusion of trimethoprim and in the use of lysed horse

blood. The change in base was introduced as on occasions the previous base had failed to support the growth of test strains of mucosalis.

NBGT formula:

Columbia agar base (Oxoid, CM 331) incorporating:

1% yeast extract (Oxoid, L21).

* Novobiocin, 5 µg/ml.

** Trimethoprim lactate, 5 µg/ml.

*** Brilliant Green, 1 in 80,000.

5% oxalated, lysed horse blood.

The Columbia agar base and the yeast powder were added to distilled water, boiled to dissolve and then autoclaved at 1.05 kg/cm^2 for 15 minutes, 121°C. The solution was then cooled in a water-bath and at 56°C the other ingredients were added.

Nutrient Gelatin, (Oxoid, CM 135a).

Reinforced Clostridial Medium (RCM), (Oxoid, CM 149).

Sabouraud Broth, (Oxoid, CM 147).

Sabouraud Dextrose Agar (SDA), (Oxoid, CM 41).

* Upjohn Ltd., Crawley, Sussex.

** Burroughs-Wellcome Ltd., London.

*** G.T. Gurr Ltd., London.

Selenite Broth (SB), (Oxoid, CM 3 a).

Serum Agar, Blood agar base No. 2 (Oxoid, CM 271) containing 10% inactivated horse serum.

Sheep Blood Agar (SBA), Hartley's digest agar (Cruickshank, 1965) incorporating 5% defibrinated sheep blood.

Thioglycollate Broth (Brewer) (TB), (Oxoid, CM 23).

Tryptone Soya Broth (TSB), (Oxoid, CM 129).

Tryptose Phosphate Broth (TPB), (Oxoid, CM 283).

(b) TESTING SELECTIVE (NBGT) MEDIUM.

To be acceptable batches of selective medium must not depress the viable counts of mucosalis by more than 1/10 in comparison with CBA (Lawson and Rowland, 1974; Roberts, 1978). The inhibitory effect of brilliant green solution was tested by inoculating nutrient agar, to which had been added various amounts of brilliant green, with known numbers of mucosalis. The lowest dilution of brilliant green solution which did not bring about a reduction in the counts of mucosalis was chosen for inclusion in the selective medium.

Occasional batches of NBGT plates were found to be more inhibitory than expected, as had been observed with NGB plates (Roberts, 1978). Each batch of NGBT plates was therefore checked using two strains of

mucosalis, 253/72* and 106/75, and the modified method of Miles and Misra (1938) described by Roberts (1978).

(c) BACTERIAL STRAINS.

All strains of mucosalis used were serotype A (Lawson et al., 1979).

253/72 (1248/72 - 2C2 [Roberts, 1978]).

The neotype strain (National Collection Type Culture 11000) was isolated in 1972 from the terminal ileum of a post-weaned pig with lesions of adenomatosis. The pig came from an Animal Breeding Research Organisation (ABRO) farm (see Sources of Experimental Pigs). This strain was maintained on BA or CBA plates and used for assessment of the selective medium and for preparation of serotype A antigen as a control in the tube agglutination test.

106/75 - S1 - 10 - 3 (106/75).

This strain was more sensitive to inhibition by NBG (Roberts, 1978) and was used to test batches of NBGT plates in parallel with the neotype strain. 106/75 was isolated in 1975 from a case of adenomatosis in a post-weaned pig from an ABRO farm.

1075/78 A-F.

This culture was derived from an adenomatous pig slaughtered at Gorgie abattoir in 1978. The affected small intestine was cultured (see later for technique)

* Roberts (1978) refers to this strain as 1248/72-2C2.

and 6 mucosalis colonies were subcultured from the CBA plate of the 4th dilution onto BA plates. Approximately equal amounts of each subculture were suspended in glycerinated serum broth, bulked, and stored at -80°C in small aliquots. Thus piglets dosed with culture 1075/78 A-F were exposed to organisms descended from 6 colonies, hence reducing the chance, when selecting a single colony, of developing a strain lacking in possible essential virulence factors.

209/80 L1-4.

This strain was derived in 1980 from a post-weaned runt pig from an ABRO farm. Lesions of intestinal adenomatosis were present in the terminal ileum, caecum and spiral colon. A colony from the spiral colon, isolated on CBA at the 4th dilution, was selected. This strain was used to infect pigs in later experiments as it was the most recent field isolate available and was considered less likely to have undergone changes such as loss of virulence due to prolonged storage.

(d) ANTISERA.

(i) Rabbits.

Rabbits used for the production of antisera were New Zealand Whites obtained from the Edinburgh University Centre for Laboratory Animals and housed and fed under standard management procedures.

(ii) Mucosalis Antisera.

Hyperimmune antisera against whole cells of Campylobacter sputorum ss mucosalis were obtained from Dr. Lawson, or prepared by the author using the method of Lawson and Rowland (1974). The sera ('OH') were prepared by the intravenous inoculation of rabbits with the surface growth, suspended in normal saline, of 48 hour BA plate cultures. Rabbits were injected four times in all, at 3 - 5 day intervals, and bled 7 days after the last injection. Antisera used in slide agglutination tests were preserved in 30% glycerol and stored at 4°C. Antisera used in tube agglutination tests and in fluorescent staining were stored without additives at -20°C. The mucosalis antisera used were as follows:-

(1) 253/72 - 2C2 - 'OH'.

(2) 1075/78 A - 'OH'.

(3) 209/80 LI - 4 - 'OH'.

(4) 982/76 - 'OH', Serotype B Campylobacter sputorum ss mucosalis (Roberts, Lawson and Rowland, 1977).

(iii) Escherichia coli Antisera.

Polyvalent Pig 'OK' antisera.

Small aliquots were obtained from Dr. Lawson and consisted of individual 'OK' antisera prepared by the method of Edwards and Ewing (1972), mixed in equal proportions, preserved with 30% glycerol and stored

at 4°C. The individual strains used in the preparation were as follows:-

0149 : K91 (B) K88 ac (L)

0147 : K89 (B) K88 ac (L)

0141 : K85 ab (B) K88 ab (L)

0141 : K85 ac (B)

0139 : K82 (B)

08 : K87 (B) K88 ab (L)

987P$^+$'OK' antiserum

This was prepared by the author using the method of Edwards and Ewing (1972). A 24 hour SBA culture of 987 P$^+$ *E. coli* [O9 : K103 (A) 987(P)] was seeded onto 0.1% glucose nutrient agar slopes and incubated overnight. The growth from these was emulsified in normal saline and the opacity adjusted to approximately Brown's tube No. 3. Rabbits were inoculated intravenously, five times in all, at 3 day intervals, initially with small amounts (0.1ml, 0.2ml) of bacteria suspended in 0.5% formol saline and subsequently with live bacteria (0.4ml, 0.8ml, 1.6ml). Rabbits were bled out 4 - 6 days after the last inoculation and the sera preserved at -20°C. Small aliquots were thawed, preserved with 30% glycerol, stored at 4°C and used in slide agglutination tests.

Absorbed 987P$^+$ antiserum

A small amount was obtained from Dr. R. Sellwood, Agricultural Research Council Institute for Research

on Animal Diseases, Compton. It was prepared by absorbing 987P$^+$ 'OK' antiserum (Edwards and Ewing, 1972) with non-piliated, capsulated (P^-K^+) and non-piliated, non-capsulated (P^-K^-) variants of the strain as described for K and O absorption (Edwards and Ewing, 1972).

Slide agglutination tests were performed using a 1 in 10 dilution of this antiserum in phosphate-buffered-saline (PBS, pH 7.2, 0.01M), stored when not in use at 4°C.

Absorbed K99$^+$ antiserum

A small volume was obtained from Mr. David Sherwood, Moredun Research Institute, Edinburgh. It was prepared using similar methods to Edwards and Ewing (1972) except that more dense live suspensions of E. coli* (K99$^+$) grown on Minca-1s plates were used. Rabbits were bled 1 - 2 weeks after the final injection and the sera absorbed with a homologous strain grown on BA at 18°C and boiled to denature any remaining pili. The serum was used at 1 in 10 dilution in PBS (pH 7.2, 0.01M) in slide agglutination tests. The serum was stored at 4°C.

(iv) Polyvalent Salmonella 'O' Antiserum (A-G)

Wellcome Reagents Ltd., Beckenham, Kent.

Small aliquots were stored at 4°C.

* K(A) type capsular antigen.

(v) Non-hyperimmune Rabbit Serum

Inactivated rabbit serum, Wellcome Reagents Ltd., Beckenham, stored at 4°C.

(e) FLUORESCENT REAGENTS

FITC - GAR:

Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit-globulin serum (FITC-GAR) was obtained from Dr. Lawson. It was prepared by the hyper-immunisation of goats with a crude preparation of rabbit gamma-globulin. The goat serum was precipitated by "Rivanol" and saturated ammonium sulphate (Mostratos and Beswick, 1969) and the purified globulin conjugated with FITC (Nairn, 1964). The conjugated serum was subsequently absorbed with fresh, washed, porcine red blood cells and acetone-dried porcine liver powder.

FITC - SAR:

FITC conjugated sheep anti-rabbit-globulin serum (FITC-SAR) (Wellcome Reagents Ltd., Beckenham) was also used, unabsorbed, according to the manufacturer's instructions.

FITC - RASw:

FITC conjugated rabbit anti-swine-globulin serum (FITC-RASw) (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used unabsorbed, according to the manufacturer's instructions.

RASw - IgA:

Rabbit anti-swine IgA antiserum (RASw-IgA) (Nordic Immunological Laboratories, Tilburg, The Netherlands) was used according to the manufacturer's instructions.

Conjugates were stored in small aliquots at -20°C except the Nordic reagents which were stored at -80°C.

(f) PATHOLOGICAL PROCEDURES

(i) Necropsy

Sampling was carried out under terminal anaesthesia or after euthanasia.

Terminal Anaesthesia (Figure 2.1)

Piglets were anaesthetised by cardiac puncture and injection of 'Sagatal' (Pentobarbitone Sodium BP (Vet) 6mg/ml, May and Baker Ltd., Dagenham, Essex). Immediately after sampling the piglets were killed by injection of 'Expiral' (Pentobarbitone Sodium B Vet C 200mg/ml, Abbott Laboratories Ltd., Queenborough, Kent).

Euthanasia

Weaner pigs were shot with a captive bolt or stunned electrically before being bled out from the neck. Younger piglets were anaesthetised by intracardiac injection of 'Expiral' and then bled out from the neck. The gnotobiotic piglets of Experiment 1, Chapter 5 were shot with a captive bolt.

Sampling Protocol

Where euthanasia was employed, a blood sample was collected while the carcase was bleeding out. The pig was then placed in dorsal recumbency and the abdominal cavity opened along the mid-line. The alimentary tract from the oesophagus, at its point of emergence through the diaphragm, to the rectum was removed, along with the liver and spleen.

The mesentery of the alimentary tract was opened and examined along with associated lymph nodes. At predetermined sites, samples were taken for bacteriological, histological, immunofluorescent, enzymological and electron-microscopic examination. (Deviations from this protocol are indicated in the relevant chapters).

Tissue was first taken for bacteriology, the portion immediately distal to this was removed, opened out on a clean glass slide, mucosal surface uppermost, and areas for transmission electron microscopy (TEM) and immunofluorescence selected. A second intact segment distal to this was washed out and then immersed in 10% buffered formol saline. A third portion was opened, mucosal side uppermost, on to a flat card and also immersed in 10% buffered formol saline. Where samples for enzymology were taken a fourth segment of gut was gently rinsed in PBS

(pH 7.2, 0.01 M) to remove any contents, and immediately stored at -80°C.

Portions of liver, lung, spleen, kidney, mesenteric lymph nodes and any tissue with gross abnormality were immersed in 10% buffered formol saline. Finally the remaining alimentary tract was opened and the mucosa examined.

In a few animals samples for histology and immunofluorescence were taken from the tongue, tonsils, gums and hard palate. These are detailed further in the appropriate chapter.

Where samples were taken under terminal anaesthesia a blood sample was drawn from the heart and the animal placed in dorsal recumbency. A mid-line incision was made from the umbilicus to the level of the caudal teats and the gut sampled *in situ* using artery forceps as haemostats and markers. Subsequently the pig was killed and the procedure for animals destroyed by euthanasia followed.

The sites from which samples were taken are shown in Figure 2.2 and listed below. Variations in the procedure are recorded in the appropriate chapters.

Stomach: The fundus area was sampled.

Site 1 : Immediately distal to the pylorus - corresponds to the duodenum or the upper small intestine (USI).

Site 2 : Midway between sites 1 and 3 - corresponds to the jejunum.

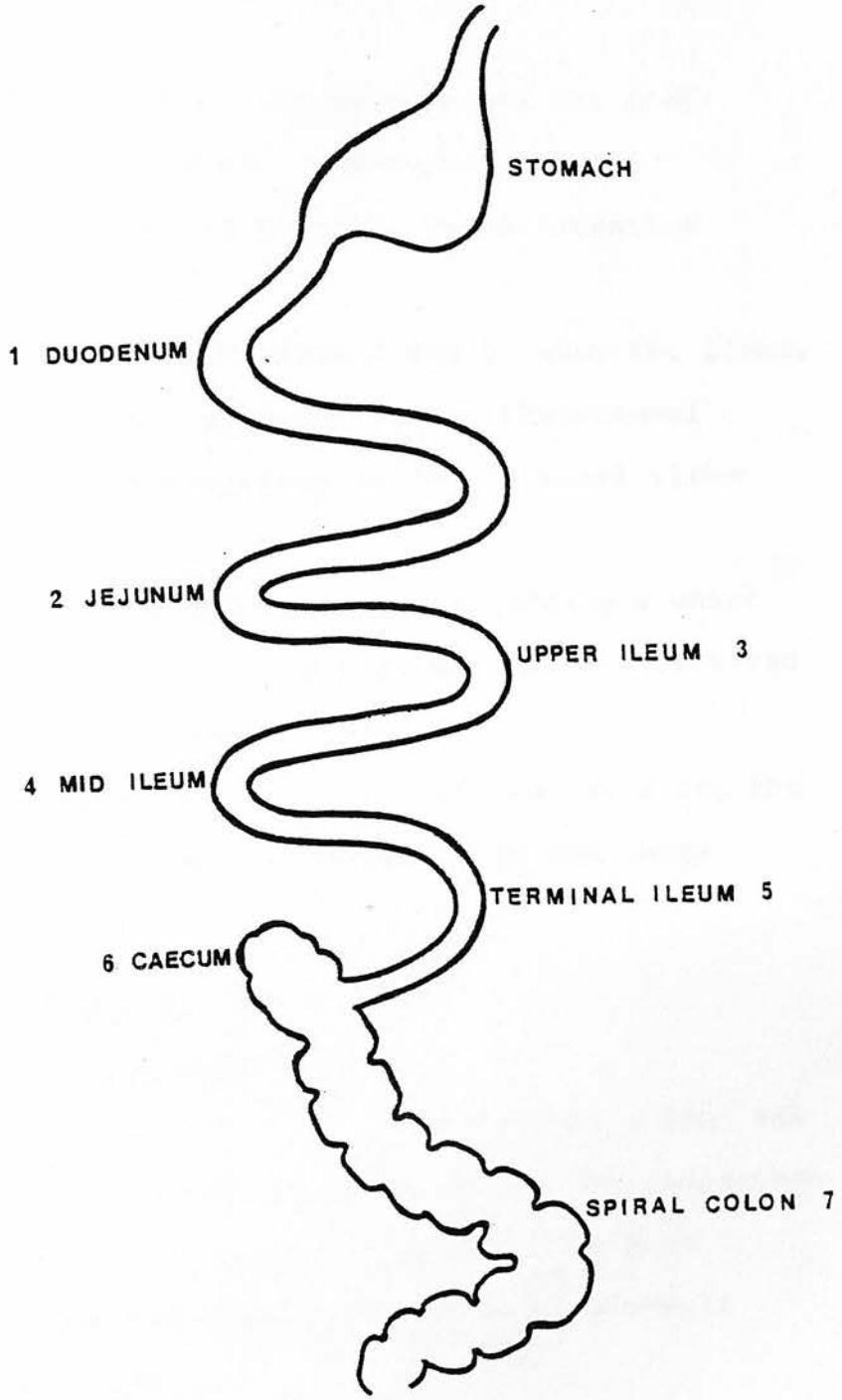


Figure 2.2: Alimentary sites sampled at necropsy.

Site 3 : Midway between the pylorus and the ileo-caecal junction - corresponds to the upper ileum or the mid- small intestine (MSI).

Site 4 : Midway between sites 3 and 5, also the ileum.

Site 5 : Immediately proximal to the ileo-caecal valve - corresponds to the terminal ileum (TSI).

Site 6 : The caecum and sometimes extending a short distance along the proximal colon - referred to as the caecum (Caec).

Site 7 : Approximately one third of the way along the spiral colon - referred to as the large bowel (LB).

(ii) Light Microscopy (LM)

Rapid Cryostat Sections:

A small area of fresh mucosa (10mm x 5mm) was quenched onto a cryostat chuck using dry ice/isopentone slurry. Sections 8µm thick were cut on a Slee cryostat and stained with alum haematoxylin (Mayer, 1903) and eosin (H&E).

Impression Smears of Mucosa for Intracellular Campylobacters.

Impression smears of fresh mucosa were made on clean, dry glass slides, air-dried and stained as follows:

Dilute carbol fuchsin 5 minutes

Wash in tap water

Acetic acid 0.5% 25 seconds

Wash in tap water

Fast green (0.4% in distilled water) 10 - 15 seconds

Wash in tap water

Tissues Fixed in Formol Saline:

These were processed routinely, embedded in paraffin wax, and sections cut at 5µm. All sections were routinely stained with H & E. Demonstration of campylobacter-like organisms in selected sections employed Young's modification of the Warthin-Starry staining technique (Young, 1969), and Levaditi's method for spirochaetes (Levaditi and Manouelian, 1906). Giemsa stains were used in selected sections for the demonstration of luminal bacteria and cryptosporidia.

Kerr's and Toluidine Blue Stains:

These were used on sections cut from blocks of tissue processed for TEM (vide infra), in order to select areas for ultrastructural examination and to supplement other observations made by light microscopy.

Sections to be stained by Kerr's method were cut at 1.5µm and the araldite removed in saturated alcoholic sodium hydroxide. These were then stained with a modification of the Warthin-Starry technique (Kerr, 1938). Sections to be stained with toluidine blue were cut at 0.5µm and stained with 0.5% toluidine blue in 1% borax at 60°C.

(iii) Transmission Electron Microscopy (TEM)

Blocks of tissue, approximately $2mm^3$ were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.3, for 24 hours at 4°C. The blocks were then rinsed in buffer and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.3, for 1-2 hours. Finally the blocks were rinsed in buffer, dehydrated through graded ethanols and embedded in araldite (Polaron Ltd.). Ultrathin sections were cut at 600-1200Å using glass knives on a Cambridge-Huxley Ultramicrotome Mark II. The sections were then mounted on copper grids and stained as follows:-

Saturated uranyl acetate in 50% alcohol	15 minutes
10% ethanol	20 seconds
Distilled water	20 seconds
Lead citrate (Reynolds, 1963)	5 minutes
Distilled water	20 seconds

Occasionally bacterial cultures were examined ultrastructurally. These were grown in appropriate liquid media (CBA/TPB for Campylobacter species, TSB for *E. coli*) and centrifuged at 1,900 relative centrifugal force (Xg) for 15 minutes. The resultant bacterial pellet was fixed, processed and stained as described for tissue blocks. Occasionally negative staining of bacteria was employed. For this a bacterial pellet was obtained as described above or by scraping growth from a BA or CBA plate. The pellet

was suspended in phosphate buffer at pH 7.0. An equal volume of 2% phosphotungstic acid (adjusted to pH 7.0 with N sodium hydroxide) was added and mixed well with the bacterial suspension. One drop of the mixture was placed on carbon-coated copper grids and allowed to dry.

All grids were examined as soon as possible using a Phillips EM400 transmission electron microscope.

(iv) Scanning Electron Microscopy (SEM).

Tissues were fixed in 10% buffered formol saline. Blocks were trimmed to a surface area of approximately $1cm^2$, dehydrated through graded acetone, and dried in a Polaron E 3000 critical point drying apparatus using carbon dioxide as the drying liquid. As soon as possible after drying the blocks were coated with gold in a Polaron sputtering system and examined on a Cambridge 5150 scanning electron microscope.

(v) Fluorescent Microscopy.

Blocks of tissue approximately $5mm^3$ were quenched in dry ice/isopentane slurry and stored either in liquid nitrogen or in air at -80°C. Sections were cut at 8µm on a cryostat microtome (Slee, London) operating at -25°C. One section from each block was stained H & E and parallel sections were used for immunofluorescent microscopy.

Air-dried sections were overlaid with appropriate antiserum and incubated for 30 minutes in a moist chamber at 37°C. Parallel sections were overlaid with PBS (pH 7.2, 0.01M) or inactivated rabbit serum and treated similarly. The sections were then washed gently in PBS (pH 7.2, 0.01M) for 20 minutes with 3 changes of PBS. After blotting off excess PBS the sections were stained with FITC - conjugated goat or sheep anti-rabbit-γ globulin and reincubated as before. Finally they were washed as before, rinsed in distilled water and air-dried.

Sections were examined on a 'Wild M20' Microscope (Wild, Heerbrugg, Switzerland) using transmitted blue light from an H.B.200 Mercury Vapour lamp with a 2mm B.G. 38 and a 2mm B.G. 12 filter.

Control tissues from adenomatous material and normal pig gut were stained and examined with the sections under test. Where suitable adenomatous material was unavailable or where it was considered more appropriate (see gnotobiotic experiments, Chapter 5) control material was prepared from live mucosalis organisms as follows:-

> Overnight or 48 hour CBA/TPB cultures were checked for purity by a Gram-stained smear and examination under phase-contrast microscopy. The bacteria were centrifuged at 1.900 Xg for 20 minutes, the pellet resuspended in a small amount of nutrient gelatin at 37°C or in a

small amount of 'Tissue-Tek II OCT Compound' (Miles Laboratories, London NW10 6JC), frozen on a metal chuck in dry ice/isopentane slurry and sectioned on the cryostat microtome at 8 μm. Control material prepared in this way is referred to as "Gelatin Control Material" (Chapter 5).

(g) BACTERIOLOGICAL AND SEROLOGICAL PROCEDURES.

(i) Isolation and Quantification of Mucosalis from the Mucosa of Naturally Infected or Experimental Pigs.

A loop of gut was ligated, placed in a sterile tray, opened with sterile instruments and the mucosa washed three times with PBS (pH 7.2, 0.01M). Approximately 1g mucosa was then removed by scraping, weighed and homogenised in a small volume of RCM using an MSE homogeniser (30 seconds at maximum speed). The homogenate was made a 1 in 20 dilution by adding RCM and a further 4 serial 1 in 20 dilutions were made in RCM. CBA and NBGT plates were inoculated in parallel with 0.1ml of each dilution and the inoculum spread over the whole surface with a sterile glass spreader. The plates were incubated microaerophilically at 37°C for at least 5 days, the gaseous atmosphere being replenished after 24 hours. At 48 hours the plates were examined, mucosalis - like

colonies counted and representatives of these sub-cultured on to BA or CBA plates. All plates were incubated for at least 5 days before discarding except plates showing extensive contamination which were discarded sooner. Mucosalis - like colonies were recounted after 4 days incubation and from consideration of the total counts of mucosalis - like colonies and the proportion of representative colonies which proved to be mucosalis, estimates were made of the numbers of mucosalis present in the mucosa.

(ii) Isolation of E. coli.

Where coliform isolation was attempted the surface of a ligated loop of gut was seared and the contents aseptically pipetted. A few drops were plated out conventionally on SBA and $M^{C}C$ plates and incubated aerobically.

(iii) Isolation and Identification of Other Bacteria.

Where applicable, other tissues or organs were sampled aseptically and inoculated conventionally on to relevant media. Colonies were identified according to the methods of Cowan and Steel (1974), unless otherwise indicated in the text.

(iv) Identification of Campylobacter sputorum ss mucosalis and Differentiation from other Porcine Campylobacters.

Colonies which on primary plates exhibited

typical colonial morphology of porcine campylobacters (Lawson and Rowland, 1974; Lawson, Rowland and Wooding, 1975; Roberts, 1978) were subcultured on to BA and CBA plates and the growth after 24-48 hours examined by a Gram-stained smear. Isolates which could be classified as vibrios on colonial and morphological bases were further examined by a slide catalase test and a slide agglutination test (vide infra). Those which were morphologically vibrioid, catalase-negative, and which agglutinated in hyperimmune mucosalis antiserum were considered to be mucosalis. Of these, selected isolates were purified by replating twice from single colonies and then examined in slope catalase and tube agglutination tests (vide infra). Strains which were catalase-negative but did not agglutinate in hyperimmune mucosalis antisera (versus serotypes A and B), were loosely termed "non-agglutinating campylobacters" (NAC's). Isolates which were vibriod on colonial and morphological bases but catalase-positive in the slide catalase test were designated as catalase-positive campylobacters.

(v) Biochemical Tests

Slide Catalase Test

Using a platinum loop, surface bacterial growth from a BA or CBA plate was emulsified with a drop of 3% hydrogen peroxide solution on a clean glass slide. If effervescence was not observed within 2

minutes a negative result was recorded.

Slope Catalase Test

This test was carried out on 48 hour cultures grown on serum agar slopes (Cowan and Steel, 1974).

Other Biochemical Tests

These were carried out according to the methods of Cowan and Steel (1974), to assist in the identification of bacteria other than mucosalis.

(vi) Agglutination Tests.

Slide Agglutination Test

Bacterial surface growth was emulsified in 0.85% sterile saline on a clean glass slide. Using a platinum loop a drop of antiserum was added to the emulsion. The slide was rocked gently and examined against a dark background, using a hand lens if necessary. If no agglutination occurred within two minutes a negative result was recorded.

Tube Agglutination Test

The organisms used in the test were grown for 48 hours on 30ml CBA slopes, checked for purity by a Gram-stained smear, the growth washed off with 0.3% formol PBS (pH 7.2, 0.01M) and then filtered through several layers of gauze. The antigen suspension was washed three times by centrifugation (1,900 X g, 15 minutes), discarding the supernatant after each wash and finally resuspending the deposit in a small volume of 0.3% formol PBS. This suspension was diluted with PBS (pH 7.2, 0.01M) to a standard

density (equivalent to Brown's Opacity Tube No. 2), using a nephelometer.

Doubling dilutions of hyperimmune antiserum were made in PBS (pH 7.2, 0.01M) and unit volumes of antigen were added to unit volumes of the serum dilutions. The tubes were then placed in a 56°C waterbath for 30 seconds to allow thorough mixing, and thereafter incubated at 37°C overnight. Controls included a known antigen with homologous antiserum and test antigen without serum, tested at the same time in an identical manner. The result was recorded as the amount of deposit visible to the naked eye, the end point taken as the highest dilution of serum to give 50% agglutination. This test was used to confirm the identity of mucosalis strains, to estimate the amount of mucosalis-agglutinating antibody in pig and rabbit sera, in sow colostral whey, and in sow milk.

Tube agglutination tests to estimate the titre of E. coli OK antibody in rabbit serum were performed according to the method of Edwards and Ewing (1972).

(vii) Incubation Procedures.

All plates were incubated at 37°C.

Aerobic Incubation

Aerobic incubation was undertaken for the isolation of aerobes where applicable.

Anaerobic Atmosphere

Plates and bottles were placed in a

Mackintosh and Fildes Anaerobic Jar containing a catalyst and the jar evacuated to -700mm of mercury. Hydrogen was then allowed to fill the jar; after a delay more hydrogen was added to confirm the activity of the catalyst and 10% of this atmosphere then removed and replaced by carbon dioxide.

Microaerophilic Atmosphere

This was employed for the isolation of Campylobacter sputorum ss mucosalis and other porcine campylobacters. Plates and bottles were placed in an anaerobic jar without a catalyst. The jar was evacuated to -650mm of mercury, allowed to stand for 10 minutes and the negative pressure checked before adding hydrogen to atmospheric pressure and then replacing 10% of the atmosphere with carbon dioxide.

(viii) Storage of Cultures

The medium used was Hartley's digest medium (Cruickshank, 1965) incorporating 5% horse serum with 17% tyndallised glycerol. Cultures were grown for 24-48 hours on suitable solid media and a loopful of the bacterial growth suspended in approximately 0.5ml of the storage medium. Within 15 minutes of preparation the suspension of organisms in the glycerinated serum broth was placed in the -80°C store.

To recover the organisms from the frozen state cultures were thawed at room temperature and a few

drops immediately inoculated on to a BA or CBA plate.

(ix) Separation of Whey from Colostrum or Milk for use in the Tube Agglutination Test.

The sample was centrifuged at 1,900 X g for 10 minutes and the milk pipetted carefully from beneath the cream. The separated milk was then heated at 37°C and sufficient calcium chloride added to give at least 5mg Ca^{++}/20ml. For every 20ml of the sample at least 0.5ml rennin was added and then the milk was incubated at 37°C for at least 30 minutes. The formed clot was carefully sliced and the whey obtained by centrifugation at 1,900 X g for 10 minutes (Sharpe, 1965).

(h) PROCUREMENT AND PREPARATION OF INFECTIVE MATERIAL

Field cases of PIA were obtained from 4 sources:-

(i) Gorgie Abattoir.

(ii) Easter Howgate Piggery.

(iii) ABRO Skedsbush Farm.

(iv) ABRO Mountmarle Farm.

The origin of cases slaughtered at Gorgie was largely unknown. Details of the ABRO farms are given in the next section of this chapter. Easter Howgate Piggery, belonging to the East of Scotland College of Agriculture, submits stock to the Veterinary Field Station for routine post-mortem examination. The herd is free

of enzootic pneumonia but has a spirochaetal colitis syndrome similar to that described by Taylor, Simmons and Laird (1980), is not free of helminths and from time to time suffers a colibacillotic episode. Chronic mange and lice have also been observed.

Preparation of Infective Mucosa (Chapters 3 and 4 only).

Immediately after euthanasia of the pig the abdominal viscera were removed for examination. Material was taken from areas grossly affected with adenomatosis and processed as previously described for histology, immunofluorescent and electron microscopy. For rapid confirmation that the lesions were adenomatous, cryostat sections were stained H&E and examined, and impression smears of the mucosa were stained and examined for intracellular campylobacters as previously described. The remainder of the affected intestine was used to provide material for oral dosing of experimental piglets.

The selected portion of gut was opened in a sterile tray using sterile instruments and the surface of the mucosa washed vigorously with sterile PBS (pH 7.2, 0.01M) in order to remove gut contents and any adherent necrotic debris. The washed mucosa was scraped off using a sterile scalpel, weighed and homogenised at maximum speed in an MSE homogeniser together with a small volume of sterile TPB. More

TPB was added to give a 1:1 ratio (w/vol) of mucosa: TPB. A 2ml aliquot of this was added to 18ml sterile RCM and used as the initial 1 in 20 dilution for estimating the numbers of mucosalis per gram of original mucosa (see Bacteriological and Serological Procedures). The remainder of the homogenate was used for oral inoculation of experimental piglets as soon as possible after its preparation.

Preparation of Pure Cultures of Mucosalis for Oral Exposure of Experimental Piglets.

A fresh aliquot of the selected strain of mucosalis was removed from the -80°C store, thawed at room temperature and a few drops inoculated onto BA or CBA plates which were then incubated micro-aerophilically. After 24 or 48 hours growth was checked for purity by Gram-stained smears and the identity confirmed by a slide agglutination test versus serotype A antiserum. Growth from plates, assessed as morphologically pure mucosalis, was then inoculated into large diphasic CBA/TPB slopes. These were plugged with sterile cotton wool plugs, incubated microaerophilically for 24 or 48 hours and then small aliquots of the TPB overlay were examined by phase contrast microscopy and Gram-stained smears. The liquid moieties of cultures judged by the above criteria to consist only of vigorously motile mucosalis were then bulked and used to orally dose piglets. The numbers of mucosalis in the inoculum

were assessed by decanting aseptically 1ml of the bulked suspension, diluting this serially in 9ml amounts of PBS (pH 7.2, 0.1M), and carrying out the method described by Miles and Misra (1938).

(i) SOURCES OF EXPERIMENTAL PIGS.

Sows whose litters were used in experiments attempting transmission of PIA were obtained from 3 sources.

(i) Animal Breeding Research Organisation (ABRO) Skedsbush Farm.

(ii) ABRO Mountmarle Farm.

(iii) Easter Bush Piggery.

Post-mortem examinations are routinely carried out on stock from these premises and so the general health status of the herds is monitored.

Skedsbush and Mountmarle Farms, owned by the Animal Breeding Research Organisation, have enzootic pneumonia and helminths, although apply satisfactory worming procedures, and have occasional outbreaks of colibacillosis. Chronic mange and lice are also problems.

Easter Bush Piggery was established from Caesarean-derived stock and is free of enzootic pneumonia, chronic mange, lice, atrophic rhinitis and swine dysentery. Colibacillosis is a problem

from time to time.

These 3 herds have all suffered cases of PIA in the past. Further details of the experimental animals will be recorded in the appropriate chapters.

(j) TECHNIQUES FOR INFECTION.

Chalk Suspension.

Where this was used to neutralise gastric acidity prior to infecting, 1g of chalk powder was suspended in 5ml sterile distilled water. This was poured into the back of the piglet's mouth and the throat massaged to encourage deglutition.

Benzetimide Solution.

Benzetimide was kindly donated by Janssen Pharmaceuticals and used in some experiments to slow passage of ingesta through the gut (Marsboom, Temmerman and Symoens, 1973; Roberts, 1978). The drug was administered orally at a dose rate of 0.25mg/kg, dissolved in a suitable volume (approximately 2ml) of sterile distilled water.

Oral dosing.

Inocula (e.g. mucosal homogenates, faecal suspensions, benzetimide, bacterial cultures) were given using sterile syringes with an attached length of plastic tube. The tube was inserted to lie on the

midline of the dorsum of the tongue and the inoculum injected slowly into the oral pharynx. The throat was massaged to promote deglutition.

(k) IN VITRO SAMPLING TECHNIQUES.

Oral Swabs.

Initially (Chapter 3), oral swabs were taken by thoroughly massaging the gingival margin with a cotton wool swab. The swab was then placed in 5ml of 0.85% sterile saline and mixed for 5 minutes on a Mattburn mixer. The sample was then filtered through a 1.2μm Millipore filter and the last few drops inoculated conventionally onto CBA and NBGT plates.

Subsequently swabs were taken without particular attention to the gingival margin but ensuring that the swab was well-moistened with saliva. These swabs were inoculated directly onto CBA and NBGT plates.

Rectal Swabs.

Cotton tipped swabs were gently inserted into the rectum and a small amount of faeces collected. It was estimated that swabs collected approximately 0.2g faeces. Swabs for isolation of mucosalis were placed in 2ml PBS (pH 7.2, 0.1M) and mixed for 10 minutes on a Mattburn mixer giving approximately a 1 in 10 dilution. Three further 1 in 10 dilutions

were made and 0.1ml from each spread over CBA and NBGT plates. The identification and quantification of mucosalis was thereafter as described for mucosa.

Blood.

Blood samples were collected conventionally with vacutainers. In sows an ear vein was sampled, in piglets the anterior vena cava.

(1) STATISTICAL ANALYSES OF DATA.

Statistical analyses of data, where undertaken, were performed using methods described by Bailey (1969), and Sokal and Rohlf (1981).

CHAPTER 3

EXPOSURE OF CROSS-SUCKLED PIGLETS TO CAMPYLOBACTER SPUTORUM SUBSPECIES MUCOSALIS IN CULTURE AND FRESH HOMOGENATES OF ADENOMATOUS MUCOSA.

INTRODUCTION

Since the recognition of the condition of porcine intestinal adenomatosis there have been many attempts at transmission, most of them unsuccessful. These experiments are described more fully in Chapter 1.

Roberts (1978) described one experiment in which some piglets of one litter were dosed within 24 hours of birth with cultures of mucosalis, homogenised adenomatous mucosa, chalk and benzetimide. The use of chalk to neutralise gastric acidity and benzetimide to reduce intestinal motility were aimed at encouraging the establishment of mucosalis in the gut. Eight of the 9 dosed piglets developed gross or microscopic evidence of PIA or NE between 50-65 days post-exposure with corresponding increasing numbers of mucosalis isolated from the mucosa of affected pigs. Mucosalis was also isolated but in lower numbers from the gut of the 9th piglet, killed prior to the development of lesions in littermates. As controls Roberts killed 2 piglets prior to exposure of littermates and did not isolate mucosalis from these, or observe adenomatous change.

The obvious criticism of this experiment is that Roberts was unlikely to find developed lesions of PIA in a very young piglet, since field cases of PIA occur much later, and so it is possible that the development of PIA in littermates was unrelated to the exposures

in the neonatal period. In the field PIA can occur "spontaneously" in several pigs from the same litter. It could be argued that these experimental pigs became infected naturally at a later date or were "genetically prone" to PIA and would have developed lesions without being experimentally exposed.

Nevertheless the development of adenomatous lesions in piglets exposed as described above and the absence of other published reports of successful transmissions indicated that the method used by Roberts warranted further investigation. In order to overcome the difficulty of providing adequate controls, two sows were allowed to farrow naturally and half of each litter were suckled on the other sow. The piglets sucking one sow were exposed in a similar fashion to those of Roberts described above, while the piglets sucking the second sow were kept as unexposed controls.

It was hoped that the development of adenomatous lesions could be studied in the exposed piglets, using the unexposed littermates as controls of the same age range and under similar management. Since piglets from two litters would be exposed it would also be possible, in the event of adenomatous lesions developing, to compare the incidence of the disease between pigs of different lineage. It is well known that certain strains of pigs are resistant to enterotoxigenic Escherichia coli bearing the K88 antigen (See Chapter 6)

due to the absence of certain receptors on enterocytes, and there is also evidence that Large White boars are more susceptible to PHE than other breeds (Jackson, 1980).

MATERIALS AND METHODS

(i) Source and Management of Sows.

Two pregnant sows, both at 111 days of gestation, were purchased from ABRO Mountmarle Farm (See Chapter 2), and were housed in clean, separate, freshly-fumigated isolation rooms.

The sows were confined in farrowing crates, bedded on straw and fed a proprietary diet ("Sowlac" cubes, Seafield Mill, Roslin) plus water ad libitum.

On the 115th day of gestation one sow (Sow A) farrowed 13 piglets of which 9 were live, 2 were stillborn, and 2 were crushed soon after birth. A few hours later the other sow (Sow B) farrowed 12 piglets, of which 6 were live, 4 were stillborn and 2 were crushed soon after birth.

Both sows remained healthy thereafter.

(ii) Identification and Management of Piglets.

Surviving piglets were ear-tagged with different lettered ear-tags each letter (A and B) relating to the original dam (Sow A or Sow B). The piglets sucked

their original dam for 6 - 9 hours and then half of each litter was removed and placed with the other sow (Table 3.1).

Surviving piglets were offered a proprietary creep feed ("Finisher Creep", Seafield Mill, Roslin) from 7 days of age and were weaned from the sow at 25 days of age; thereafter the diet was creep feed and water ad libitum.

(iii) Experimental Exposures and When Killed or Died (Table 3.1).

On the day of birth, immediately after the litters were cross-suckled, the piglets sucking Sow A were each dosed orally with 1g chalk in 5ml sterile distilled water, 2ml of 0.025% benzetimide solution and 5ml of a homogenate containing equal volumes of TPB and fresh adenomatous mucosa, all prepared as described in Chapter 2. The adenomatous mucosa was from an 84 day old runt pig (M364/78), submitted for necropsy by ABRO Mountmarle Farm. This pig had typical lesions of intestinal adenomatosis in the lower ileum, becoming necrotic for the terminal 10cm. Quantification of mucosalis was carried out as described in Chapter 2. Each piglet received 2.32×10^8 mucosalis (serotype A) (8.37 log 10) in the oral inoculum.

At 26, 27, 28 and 29 days of age the piglets which were exposed thus while sucking Sow A, were orally dosed with 1g chalk in 5ml sterile distilled

TABLE 3.1.

IDENTIFICATION OF PIGLETS, EXPERIMENTAL EXPOSURES, AND WHEN KILLED OR DIED.

Piglet Number	ORAL INOCULA AT DAYS OF AGE					Age (days) at Death
	1	26	27	28	29	
*A36 (BA 111/78)	C,B,PIA	Na	Na	Na	Na	2 (D)
*A43 (BA 126/78)	C,B,PIA	Na	Na	Na	Na	13 (D)
*A37 (BA 217/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	63 (K)
*A46 (BA 218/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	64 (K)
*A38 (BA 225/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	70 (K)
*B91 (BA 246/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	79 (K)
*B93 (BA 253/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	82 (K)
*B94 (BA 254/78)	C,B,PIA	C,B,M	C,B,M	C,B,M	C,B,M	83 (K)
** B92 (BA 114/78)	ND	Na	Na	Na	Na	3 (D)
** A34 (BA 211/78)	ND	ND	ND	ND	ND	62 (K)
** A40 (BA 230/78)	ND	ND	ND	ND	ND	71 (K)
** A39 (BA 245/78)	ND	ND	ND	ND	ND	78 (K)
** A35 (BA 247/78)	ND	ND	ND	ND	ND	79 (K)
** B90 (BA 252/78)	ND	ND	ND	ND	ND	81 (K)
** B95 (BA 255/78)	ND	ND	ND	ND	ND	83 (K)

C = chalk
B = benzetimide
PIA = adenomatous mucosa
M = culture of mucosalis

Na = not applicable
ND = not dosed
D = died
K = killed

* = piglets which sucked Sow A (exposed piglets).
** = piglets which sucked Sow B (controls).

water, 1ml/kg body weight of 0.025% benzetimide solution, and 10ml of a 24 hour diphasic culture of mucosalis, strain number 106/75 S1 10-3, all prepared and quantified as described in Chapter 2. Each piglet received the following numbers of mucosalis:

26 days of age - 3.25×10^9 (9.51 log 10)

27 days of age - 5.00×10^{10} (10.70 log 10)

28 days of age - 5.00×10^{10} (10.70 log 10)

29 days of age - 5.00×10^9 (9.70 log 10)

Piglets were killed or died as indicated in Table 3.1. Selection of piglets for necropsy was accomplished using a cumulative summation technique described by Roberts (1978); a method which identifies the poorest-growing pig over a selected time period.

(iv) Other Procedures.

(a) Surviving piglets were weighed weekly from 2 - 9 weeks of age.

(b) Oral swabs were taken from surviving piglets every week from 2-9 weeks of age. These were cultured for mucosalis by the filtration technique described in Chapter 2.

(v) Necropsy Procedure and Sites Sampled.

The general procedure described in Chapter 2 was followed. Samples from the US1, MS1, TS1, Caec and LB were processed for histology, electron microscopy and immunofluorescence.

The US1, MS1, TS1, Caec and LB of piglets A34, A37, A46, A38 and A40, and the TS1 of piglets A36 and A43, were cultured for mucosalis as described in Chapter 2.

The gut contents of piglets A36 and B92 were cultured for enterotoxigenic E. coli. Samples (0.01 ml loopfuls) of spleen and liver were taken aseptically from piglet A43, plated conventionally on to BA and McC plates and incubated aerobically at 37°C.

Piglets A39, A35, B91, B90, B93, B94 and B95 were not cultured for mucosalis at necropsy.

RESULTS

(i) Clinical Findings/Daily Observations.

The main clinical findings are summarised in Table 3.2. Apart from piglet B92 which was found dead at 3 days of age, the control piglets remained healthy and vigorous throughout. Piglet A36 from the exposed group was found crushed to death at 2 days of age. Piglet A43 became progressively weaker from 2-12 days of age, was observed to scour at 8 days of age and was found dead at 13 days of age. By 9 days of age the exposed piglets were noticeably less vigorous than the controls and suffered intermittent

TABLE 3.2.

SUMMARY OF CLINICAL FINDINGS.

Days of Age	Exposed Piglets	Control Piglets
2	A36 dead, crushed by sow A43 weak	B92 weak
3	A43 weak and lethargic	B92 found dead
4-8	A43 thin and weak A43 diarrhoeic at 8 days of age	Surviving piglets healthy
9	Surviving piglets less vigorous than controls. B91, B93 and A46 diarrhoeic. A43 not sucking well. Considerable variation in body size.	All piglets healthy and fairly uniform in body size.
10	A43 diarrhoeic and lethargic	Surviving piglets healthy
13	A43 found dead	Surviving piglets healthy
16	A46 weak, bullied by littermates, becoming stunted in appearance.	Surviving piglets healthy
17	Surviving piglets diarrhoeic	Surviving piglets healthy
25	Sow A removed	Sow B removed
28-32	Surviving piglets diarrhoeic	Surviving piglets healthy
40-43	Surviving piglets diarrhoeic	Surviving piglets consuming approximately 50% more creep than exposed piglets

periods of diarrhoea (Table 3.2). Comparison of weekly body weights of the two groups showed that the exposed piglets were always lighter than the controls and there was considerable variation in body weight among exposed piglets, unlike the controls (Figure 3.1).

(ii) Bacteriological Findings.

(a) Oral Swabs.

Mucosalis was isolated from piglet A46 when it was 28 days old. No campylobacters were isolated from any other piglets at any other oral sampling except that catalase-positive campylobacters were isolated from piglet A37 when it was 14 days old.

(b) Sites Cultured at Necropsy.

There were no isolations of mucosalis from any of the sites cultured at necropsy. Catalase-positive campylobacters were isolated from the TS1 of piglet A43, the US1 and Caec of piglet A46, the TS1 and Caec of piglet A40, the Caec of piglet A34 and the TS1 of piglet A37 (Table 3.3).

No enterotoxigenic E. coli were isolated from the gut contents of piglets A36 and B92, found dead early in the experiment. Pure cultures of β-haemolytic streptococci were isolated from the spleen and liver of piglet A43 which died at 13 days of age. The streptococci were examined by the precipitin test but could not be classified as any of Lancefield's

Figure 3.1: Mean bodyweight (± 1 standard deviation) of Exposed Group and Control Group.

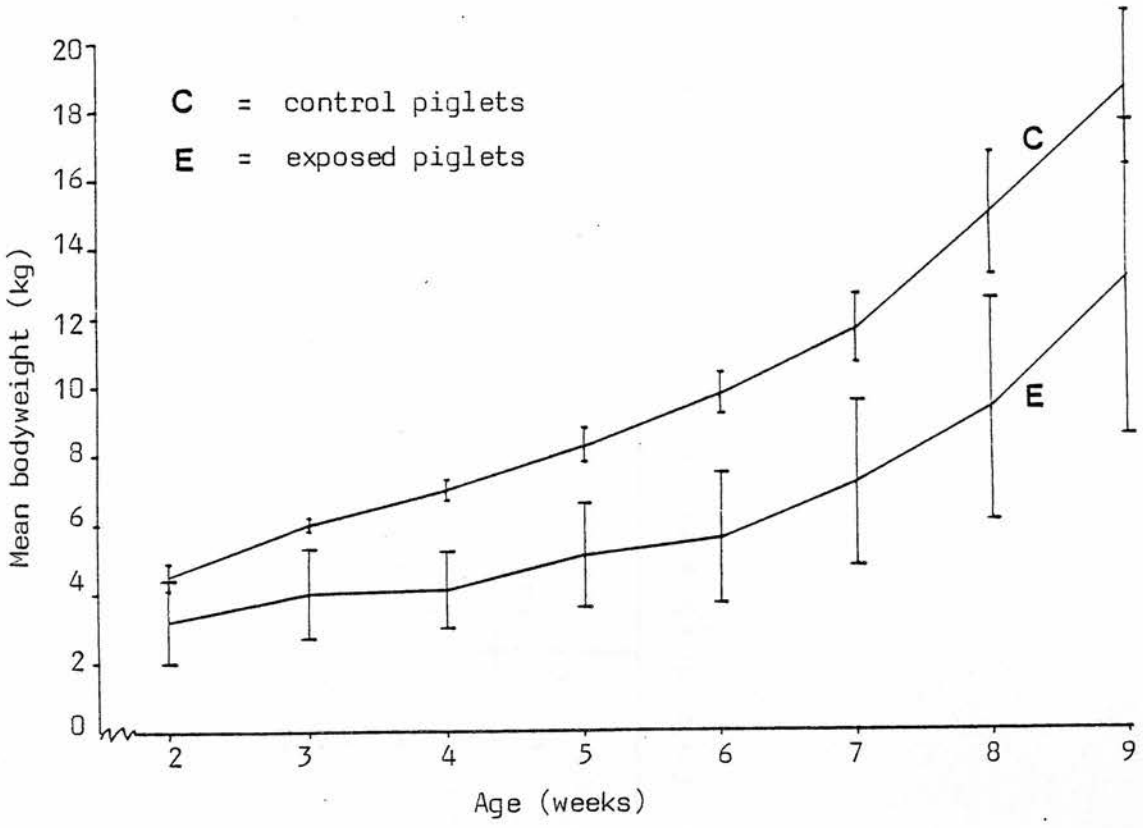


TABLE 3.3.

ISOLATIONS OF CATALASE-POSITIVE CAMPYLOBACTERS FROM THE GUT AT NECROPSY OF CROSS-SUCKLED PIGLETS.

PIGLETS EXPOSED TO PIA MUCOSA AND MUCOSALIS				CONTROL PIGLETS			
Piglet Number	Site	log 10/g of mucosa	Plates on which isolated*	Piglet Number	Site	log 10/g of mucosa	Plates on which isolated*
A43 (BA126/78)	TSl	6.60	$(NBGT)^{2}$	A34 (BA211/78)	Caec	5.38	$(CBA)^{3}$
A37 (BA217/78)	TSl	4.90	$(CBA)^{3}$	A40 (BA230/78)	TSl	2.78	$(NBGT)^{1}$
A46 (BA218/78)	USl	3.08	$(NBGT)^{1}$		Caec	2.90	$(NBGT)^{1}$
	Caec	5.20	$(NBGT)^{3,2}$				

* Figures denote dilutions [X 20^{x}] from which successful isolations were made.

groups C, D, L, N or as Streptococcus suis.

(iii) Pathological Findings.

(a) Gross findings at Necropsy.

Gross abnormalities were found in piglets B92, A43 and A46. Piglet B92 was found to have a hard cast of meconium in the rectum and descending colon. There was distension and congestion of the gut anterior to the meconium cast. Piglet A43 had cyanosis of the tips of the ears and tail, hypostatic congestion of the right side of the body, and an enlarged spleen. Piglet A46 was in very poor bodily condition and was pot-bellied. The right kidney was cystic. There were no gross abnormalities in the other pigs although exposed pigs were less well grown at necropsy than control pigs of the same age. There was no macroscopic evidence of adenomatous change in any pig.

(b) Histopathology (H & E, Gram's Giemsa and Young's Stains).

There were no histopathological abnormalities in any piglet other than piglets A43 and A46. In piglet A43 all tissues and organs had evidence of a streptococcal septicaemia. There were acute inflammatory cell aggregates in the interstices of all major organs including the gut, and Gram's stains showed chains of gram-positive cocci present in the capillaries and interstices of all major organs. The cystic right kidney of piglet A46 was confirmed as such on

histological examination.

There was no evidence in any piglet of adenomatous glands. Young's stains of gut tissues revealed no close association of campylobacter-like bacteria with enterocytes. On occasions a few campylobacter-like organisms (CLO's) were found in the lumina of crypt glands of the TS1 e.g. in piglets A46 and A38. The lumina of crypts of the Caec and LB of all piglets were frequently found to contain a mixed bacterial flora, amongst which CLO's were observed.

Kerr's Stains: The MS1 and TS1 of control piglets A34 and A40, and of exposed piglets A37, A46 and A38 were examined using Kerr's staining technique (See Chapter 2). At all of these sites in all of these piglets there were aggregations of argyrophilic bodies visible in the apical cytoplasm of villar enterocytes (Figure 3.2). These bodies were not visible on the Young's stained sections. Although within the size range of campylobacters the bodies were very pleomorphic and often did not conform to typical campylobacter morphology.

(c) Immunofluorescence.

The sites which on Kerr's staining showed the presence of argyrophilic bodies (vide supra) were examined for mucosalis by the immunofluorescent

technique described in Chapter 2.

There was no fluorescence which could be attributed to mucosalis in any section examined.

(d) Electron Microscopy.

The MS1 of exposed piglet A46 and the TS1 of exposed piglet A38 were examined ultrastructurally. Particular attention was paid to the apical cytoplasm of villar cells where the argyrophilic bodies had been observed in Kerr's stained sections. No structures recognisable as campylobacters were observed at any site. The argyrophilic bodies were found to be extremely pleiomorphic structures resembling myelinosomes (Figures 3.3 - 3.7). Myelinosomes are occasionally found in a wide variety of normal cells and may show infinite diversity of form. They are found in great numbers in certain inborn errors of metabolism, known commonly as lysosomal storage diseases (Ghadially, 1982).

Interestingly, Roberts (1978) observed similar structures in enterocytes of some of his experimental piglets. Their significance here is unknown.

There was no evidence of intracellular parasitism by CLO's, and no bacteria were observed in any close association with enterocytes.

DISCUSSION

There was no gross or microscopic evidence of adenomatosis in any of the experimental piglets. Thus it was not possible to confirm Roberts' (1978) evidence that exposure of neonatal piglets to adenomatous mucosa is likely to result in the later development of PIA. Neither was it possible to compare the incidence of the disease in piglets of different lineage.

The exposed piglets grew poorly in comparison with littermate controls and suffered intermittent diarrhoeic episodes. These findings are consistent with but not specific for the clinical picture observed in cases of PIA and NE. Roberts (1978) considered that exposure of neonatal animals to abnormal pig mucosa was likely to result in persistent, low-grade, non-specific bacterial enteritis in these animals. This is particularly true when the adenomatous mucosa used in the inoculum is partially necrotic which was the case in the experiment described in this chapter.

The experimental procedures used were not identical to those of Roberts (1978). In his successful experiment he dosed piglets in the neonatal period with cultures of mucosalis as well as adenomatous mucosa. In the experiment described here cultures of mucosalis were not given until the piglets were weaned. This difference may have contributed to the failure of

transmission although in Roberts' successful transmission some strains of mucosalis isolated from the experimentally-produced lesions were examined and their surface antigens were found to more closely resemble the strain from the adenomatous inoculum than the cultured strain to which the piglets were exposed (Roberts, 1978). Lawson and Rowland (1977, unpublished results) have carried out experiments closely similar in design to the successful transmission described by Roberts (1978) but have also failed to reproduce intestinal adenomatosis.

There was in this experiment no evidence that mucosalis established in the gut of any piglet. The failure to isolate mucosalis from mucosal sites at necropsy may have been due partly to the author's inexperience. However isolations of catalase-positive campylobacters were made (Table 3.3). Some members of this heterogenous group of campylobacters grow more slowly than mucosalis and it is unusual for mucosalis colonies to appear on plates after the growth of these organisms (personal observations). Hence the failure to isolate mucosalis, which are of similar colonial morphology to other campylobacters, was probably a genuine observation.

The sows used in this experiment were from the same source as the sow used in Roberts' successful transmission, a herd in which a low level of PIA has

been recognised over a number of years. It is unlikely therefore, but possible, that the animals were resistant due to some genetic factor. A more likely explanation of resistance under these circumstances is the possible protective role played by passively-acquired maternal antibodies. The sows used would be likely to have experienced mucosalis since they originated from an infected herd and may in fact have produced colostral and milk antibodies directed against mucosalis.

A low level of infection in the herd could result in considerable variation in the immune status of different sows from the same herd. Thus maternally-derived serum antibodies in piglets could be low due to a low level in the dam's colostrum, or due to other factors such as failure of piglets to suck adequately in the first few hours after birth. If maternally-acquired immunity is an important factor in determining whether exposed piglets develop PIA then variations in maternally-acquired immunity could account for the success or failure of experimental transmissions. The mechanisms whereby maternal antibody could affect the establishment of mucosalis infection are discussed more fully in Chapter 4, where the possible inhibitory effects of colostrum and sow milk are circumvented by the use of artificially-reared colostrum-deprived piglets.

In conclusion it can be said that despite Roberts' apparent success in the transmission of PIA, the methods he used have not led to a consistently reliable technique for reproduction of the disease, and thus therefore of elucidation of the aetiological process. This observation has resulted in the attempts in this thesis to define more fully the behaviour of mucosalis in experimental piglets under varying systems of management and to investigate the role which agents other than mucosalis could play in the pathogenesis of the disease.

CHAPTER 4.

EXPOSURE OF COLOSTRUM-DEPRIVED PIGLETS TO ADENOMATOUS MUCOSA.

INTRODUCTION

Colostrum-deprived piglets are defined in this work as piglets not allowed to suck the dam (i.e. colostrum and sow-milk deprived), but not maintained free of microbial flora.

Effects of Colostrum-Deprivation

(i) Immunity to Infectious Agents

Mammals rely on maternally-derived passive immunity in the first weeks of life in order to resist disease as a result of infection by microbial agents. This immunity is largely provided by protein antibodies (immunoglobulins) which are transferred to the young either across the placenta or by ingestion of colostrum, and in some species by both routes (Brambell, 1958; Kekwith, 1959; Brambell, 1970). Piglets are born virtually devoid of immunoglobulins owing to the epithelio-chorionic placentation of the sow, which, in health, effectively prevents the passage of maternal antibodies to the foetus (Brambell, Hemming and Henderson, 1951; Sterzl, Rejnek and Travnicek, 1966).

Colostral proteins are absorbed non-selectively by the pinocytotic activity of the neonatal pig enterocytes and the bulk of maternal antibodies found in the sera of neonatal suckled piglets has been absorbed in this manner in the initial 36 hours of sucking (Lecce 1966; Ullrey, Long and Miller, 1966). There are 3 recognised classes of immunoglobulins in sow colostrum

and milk : IgA, IgM and IgG, each with important defensive roles against micro-organisms (Porter, 1969; Bourne, 1973; Porter and Allen, 1972). IgG becomes predominant in the circulation of the sucking piglet (Bourne, 1973) while defence of the enteric mucosa is primarily attributed to the continued ingestion of antibodies in the milk, predominantly of the IgA class (Porter, 1969). IgM, although not quantitatively predominant in either colostrum (and hence the serum of sucking neonatal piglets) or milk is considered to have important functions in defence against Gram-negative bacteria in particular (Porter, 1969; Porter and Hill, 1970). A fuller discussion of the quantitative significance and qualitative properties of porcine immunoglobulins can be found in articles by Knop et al., (1971), Porter and Allen (1972) and Chidlow and Porter (1978).

In addition to the specific defensive functions of antibodies in colostrum and milk, lacteal secretions contain less specific systems such as lactoferrin, lysozyme and the lactoperoxidase system which provide the neonatal gut with protection against bacteria.

(ii) Nutritional and Physiological Roles of Colostrum and Milk.

Newborn piglets are not only seriously deficient in circulating immunoglobulins but also lack other serum proteins, notably albumin (Lecce and Matrone, 1960; Metzger, Milen and Bordieu, 1978) which has

physiological functions as a carrier protein of essential fatty and amino acids and later in the suckling process is itself digested to provide essential amino acids for de novo protein synthesis. Albumin and the other colostral proteins are protected from digestion in early neonatal life by a trypsin inhibitor in colostrum (Aumaitre and Seve, 1978).

Energy is provided by lactose which is one of the few disaccharides baby pigs are capable of digesting owing to a deficiency of certain enzymes in the first week of life (Aherne et al., 1969). By 4 days of age however, provision of energy is largely from the fat content of milk (Aumaitre and Seve, 1978). Lysozyme and other enzymatic proteins in milk govern the metabolism of glycoproteins in the young pig and once the effect of trypsin inhibition has passed hydrolysis of albumin, casein and the proteose-peptone fraction of milk provide amino acids for synthesis of proteins (Aumaitre and Seve, 1978).

Lecce and Matrone (1960) in a study on the relation between diet, serum proteins and performance in baby pigs concluded that colostrum-fed piglets were more vigorous and resistant to disease than piglets deprived of colostrum. Although these authors acknowledged that the increased viability of suckled piglets was partly attributed to colostral antibodies they postulated that the delay in the synthesis of serum proteins by colostrum-deprived piglets was also a factor.

To summarise, colostrum and milk have important nutritional and physiological roles in addition to the provision of systemic and local enteric immunity. Colostrum-deprived piglets are not only more susceptible to microbial pathogens capable of causing disease in suckled animals (vide infra) but are also extremely vulnerable to environmental organisms not normally pathogenic to conventional suckled piglets (Alexander and Roe, 1962).

The difficulty of previous workers in transmitting PIA has been discussed in Chapter 1. Roberts (1978) produced lesions of PIA by exposing a litter of sucking piglets in the neonatal period, but failed to repeat these results with other litters. Clearly the variation in maternally-derived immunity between litters may have contributed to the lack of success in his later experiments. The use of colostrum-deprived piglets in attempted transmission of PIA removes therefore an important factor, namely the acquisition of antibodies of maternal origin, whose influence on the pathogenicity of mucosalis for the young animal is largely unknown, but which may have a major influence in resistance to the disease.

LITERATURE REVIEW.

Factors Relevant to the Derivation and Rearing of Colostrum-Deprived Piglets.

Most researchers in this field have developed their own techniques, based on published information, previous experience and available facilities. Little comparative work has been reported although Whitehair and Waxler (1963) and Young (1964) indicate the major advantages and disadvantages of various systems of management. Some pertinent considerations in the derivation and management of colostrum-deprived swine are now discussed in the light of published accounts.

(i) Health Status of the Dam.

Placentation in the pig is complete by 30 days of gestation (Young, Underdahl and Hinz, 1955) and the placental membranes are an effective barrier to many, but not all, infectious agents. Prior to 30 days many pathogens may gain access to the uterine environment particularly if blood-borne as in a viraemia or bacteraemia (Young, Underdahl and Hinz, 1955; Christie, 1963; Goodwin, 1965). For this reason these authors recommended that dams showing evidence of ill-health (such as a febrile episode) during pregnancy should not be selected for the derivation of colostrum-deprived litters.

Other agents which are likely to be important are

those in which the sow may show no clinical signs of illness yet produce infected piglets at term. Possibly the best example of infections of this type is porcine cytomegalovirus (Edington et al., 1977). Whilst many other organisms may pass the placental barrier, most of these episodes are accompanied by obvious ill-health in the sow and a high proportion of still-born or less viable piglets.

(ii) Microbial Status of the Piglets.

In utero infections of piglets are potential hazards but generally can be avoided (vide supra). The microbial flora to which the piglets are exposed at birth however, is considered an important factor influencing the success of subsequent rearing. For example Young and Underdahl (1951) disinfected the perineal area of the sows, and then allowed them to farrow naturally, catching the piglets in sterile bags. Later they adopted a hysterectomy technique of derivation (Young, Underdahl and Hinz, 1955) as it was found that, despite the aseptic precautions of the former method, the piglets became contaminated by organisms from the faeces or flatus of the straining sow. These contaminating organisms were considered responsible for diarrhoea and death of piglets within a few days of birth (Done, 1955; Young, 1964).

Whitehair and Waxler (1963) attributed disease in their colostrum-free piglets to the "build-up" of

infection in the isolation units. The piglets exhibited diarrhoea, swollen joints, high temperature and poor growth rates, and responded well to antibiotic therapy. Coliforms and pleuropneumonia-like organisms (PPLO) were isolated from affected animals.

Escherichia coli were the most frequently implicated organisms in accounts of disease problems due to infectious agents (Girard and Mitchell, 1962; Lecce and Reep, 1962; Calverley, 1963; Goodwin, 1965; Campbell, Brough and Fell, 1971; Cooper, 1975).

Better survival rates were achieved when piglets were derived and maintained free of microbial contamination until 3 or 4 days of age (Betts, Lamont and Littlewort, 1960; Girard and Mitchell, 1962). However several authors have encouraged the establishment of a "non-pathogenic" flora by feeding bacterial cultures (Calverley, 1963), or a non-sterile diet (Hill and Larson, 1955; Young, Underdahl and Hinz, 1955; Abelseth, 1962; Henry, 1965). It was thought that a non-pathogenic enteric flora would be beneficial by preventing colonisation by potential pathogens.

Combs (1960) and Girard and Mitchell (1962) favoured a period of strict isolation followed by a period of environmental exposure in rearing specific pathogen free (SPF) pigs for minimal disease (MD) herds. Henry (1965) also advocated the maintenance of a balance between minimal contamination and gradual exposure to organisms.

(iii) Method of Derivation of Piglets.

(a) Natural Farrowing:

Early workers allowed sows to farrow naturally in isolation and caught the piglets in sterile containers using aseptic precautions (McRoberts and Hogan, 1944; Bustad, Ham and Cunha, 1948; Young, Underdahl and Carpenter, 1949; Catron et al., 1953; Shaw, 1953; Bauriedel et al., 1954; Done, 1955). A large percentage of piglets derived in this way developed diarrhoea and died within a few days. This was thought to be due to build up of organisms, originally acquired from the sow, in the rearing accommodation (Young, 1964). Other disadvantages were the unpredictable farrowing times of sows - often at nights or weekends where labour was unavailable. Collection of piglets during natural farrowing was time consuming and skill was required to limit contamination (Young, 1964). The advantages were that no elaborate equipment was required and for a small number of piglets good survival rates could be achieved particularly if the isolation rooms had no previous contact with swine (Young, Underdahl and Carpenter, 1949; Bauriedel et al., 1954; Whitehair and Waxler, 1963).

(b) Surgical Derivation:

In order to prevent contamination of piglets by organisms in the dam's environment hysterectomy (Young, Underdahl and Hinz, 1955; and others) and hysterotomy

(Hoerlain, Adams and Meade, 1956; and others) were employed. Most workers stressed the importance of obtaining accurate service dates and performed surgery between 110-114 days of gestation (Young, Underdahl and Hinz, 1955; Betts, Lamont and Littlewort, 1960; Christie, 1963; Henry, 1965; Campbell, Brough and Fell, 1971; Coalson, Maxwell and Hillier, 1971). Whitehair and Waxler (1963) prolonged pregnancy by feeding oral progesterone and found that this resulted in heavier, more vigorous piglets. Young (1959b) reported that few piglets survived if obtained prior to 108 days of gestation and at 109 days litters were difficult to revive.

Where hysterectomies have been performed, carbon dioxide (CO_2) has been the anaesthetic most commonly used (Young, Underdahl and Hinz, 1955; Christie, 1963; Schneider and Sarett, 1966 and others). Betts (1960), Goodwin (1965) and Henry (1965) stunned their sows electrically prior to CO_2 anaesthesia. The use of CO_2 was considered advantageous due to the stimulatory effect on respiration of the piglet (Young, Underdahl and Hinz, 1955). However sows ceased to breathe after 1 or 2 minutes, so there was a limited time in which to obtain viable piglets (Hoerlain, Adams and Meade, 1956; Underdahl and Young, 1959).

A variety of anaesthetic techniques have been used for derivation by hysterotomy. Hoerlain, Adams and Meade (1956) used CO_2 to avoid the depressant effect

of barbiturates on the piglets. Most other workers have used local anaesthesia with or without a tranquilliser (Bauriedel et al., 1954; Whitehair and Thompson, 1956; Abelseth, 1962; Girard and Mitchell, 1962). Robertson et al., (1971) used trichlorethylene general anaesthesia.

These surgical procedures meant that the piglets were protected from contact with potentially harmful agents in the dams' environment and procurement could usually be arranged at more suitable times than natural farrowings. The main disadvantages were that the techniques were expensive in terms of labour and equipment and the piglets were less viable if derived too early in gestation or subjected to an anoxic or anaesthetic mishap during surgery.

(iv) Isolation Accommodation.

A variety of types of isolation units have been described and their success in terms of percentage piglet survival largely reflects their efficiency in excluding environmental or other organisms. In early days many workers used pre-sterilised cages without a filtered air supply (Bustad, Ham and Cunha, 1948; Sheffy et al., 1951; Thompson et al., 1952; Catron et al., 1953; Bauriedel et al., 1954; Bellis, 1957; Pond et al., 1961; Lecce and Reep, 1962). Many reported mortalities approaching 50% (Pond et al., 1961; Lecce and Reep, 1962). Goodwin (1965) achieved a

survival rate of 86% using presterilised cages with an unfiltered air supply but later recorded 'catastrophic losses' when production was increased. Young and Underdahl (1951) considered that poor isolation was responsible for high mortality in their early work.

Survival rates of 70.5% were achieved with the adoption of individual isolation units incorporating a filtered air supply (Young and Underdahl, 1953). These units were modified Horsfall-Bauer isolators (Horsfall and Bauer, 1940), pre-sterilised by formalin-fumigation and supplied with filtered air. The pressure within the units was less than atmospheric in order to prevent escape of infectious agents from the unit. The problem with colostrum-deprived piglets however was to prevent contamination of the isolators by microbial agents from without and soon positive pressure ventilation systems were designed (Haelterman, 1956) and adopted in various forms by most workers (Henry, 1965; Roe and Alexander, 1961; Schneider and Sarett, 1966; Robertson et al., 1971).

Further refinements in procurement and isolation techniques for colostrum-deprived piglets have resulted in the establishment of gnotobiotic piglets which are 'germ-free', or have a limited 'known flora'. This type of animal is reviewed in Chapter 5.

(v) Diets for Colostrum-Deprived Piglets.

Not surprisingly the most adequate diets

nutritionally are those whose composition closely approximates to sows' milk (Alexander, 1960; Whitehair and Waxler, 1963; Alexander, 1969; Lecce and Coalson, 1976). According to Perrin (1954) the composition of sows' milk throughout most of lactation is as follows:-

Total solids	21%
Fat	5%
Solids not fat	11%
Protein	5%
Lactose	5%
Ash	1%

Piglets adapt readily to cows' milk based diets, the disadvantage being a poorer growth rate due to the low energy content compared with sows' milk (Whitehair and Waxler, 1963). Some early diets were nutritionally inadequate and probably contributed to mortality (McRoberts and Hogan, 1944; Bellis, 1957).

Diets for colostrum-deprived piglets have developed along two main themes, 'synthetic' milks whose composition reflects sows' milk, and diets based on cows' milk but with added supplements to approximate to the composition of sows' milk. Thus Bustad, Ham and Cunha (1948), Catron et al., (1953), Betts (1961), Girard and Mitchell (1962), Goodwin (1965), Henry (1965) and Schneider and Sarett (1966) employed synthetic sow milk formulae while Bauriedel et al., (1954), Young, Underdahl and Hinz (1955) and Coalson,

Maxwell and Hillier (1971) adopted cows' milk based diets.

Many adopted diets similar to Young, Underdahl and Hinz (1955) which contained pasteurised cows' milk fortified with whole egg and a mineral mixture e.g. Alexander (1960); Combs (1960); Abelseth (1962); Campbell, Brough and Fell (1971).

Amtower and Calhoun (1964) compared three diets:-

(a) a commercial sow milk replacer, " SPF lac ".

(b) a similar diet to Young, Underdahl and Hinz (1955), but not sterilised, and

(c) diet b) sterilised with Beta-propiolactone.

They found no significant difference in growth rates or percentage piglet survival at 21 days of age.

More recently canned evaporated cows' milk diets have been used, either diluted with sterile tap water or with sterile vitamin and mineral mixtures e.g. Cooper (1975); Makin and Tzipori (1980; see Chapter 5).

(vi) Frequency and Volume Fed of Diet.

Sow-suckled piglets feed approximately every hour, and in the first days of life ingest 25-30mls at each feed (Morgan and Lewis, 1962; Yang, Howard and Macfarlane, 1980). Colostrum-deprived piglets grow and survive better as the frequency and volume fed approach that of suckled piglets. Berry et al., 1962) compared performances following

3 and 7 daily feeds. They found that piglets fed 7 times daily grew faster and had less diarrhoea than piglets fed 3 times daily, although none grew as well as sow-suckled piglets.

Lecce and Coalson (1976) used an automatic feeding device and achieved better growth and a higher survival rate (96%) than naturally - reared littermates (80%). It is interesting that Lecce and Coalson were able to achieve such high percentage piglet survival by careful dietary control without elaborate isolation or derivation. They caught piglets at birth and kept them in open cages employing 'reasonable' sanitary precautions. Their results support the theory that 'nutritional' enteritis results from flooding the upper small intestine with undigested milk thus predisposing to overgrowth of certain bacteria. Animals devoid of enteric flora, in contrast, can be fed large volumes infrequently without inducing diarrhoea (see Chapter 5).

(vii) Temperature and Humidity.

The thermoregulatory powers of piglets are poor especially in the first 24 hours after birth (Newland, McMillan and Reineke, 1952) and piglets are extremely susceptible to chilling (Mount, 1959). The preferred temperatures for young pigs are as follows:-

	°F	°C
Day 1	89.6 - 93.2	32 - 34
Days 2-41	85	29

Mount (1963).

Most workers have appreciated this and recommend initial temperatures above 32°C e.g. Young, Underdahl and Hinz (1955), Abelseth (1962), Amtower and Calhoun (1964), Schneider and Sarett (1966) and others, with a gradual reduction in temperature after a few days to 29°C.

The ratio of surface area: body weight is large in piglets and at high environmental temperatures relative humidity should be maintained between 60 - 70% to minimise loss of moisture (Imlah, personal communication, 1978).

(viii) Antibiotic Administration.

The administration of antibiotics did not appear to present any clear cut advantages in the control of diarrhoea or in improving performance (Bustad, Ham and Cunha, 1948; Sheffy et al., 1951; Thompson et al., 1952; Young, Underdahl and Hinz, 1955; Combs, 1960; Mandel, Lane and Syrinek, 1960; Abelseth, 1962; Berry et al., 1962; McCallum, Elliott and Owen, 1977), although a minority of authors favoured their use (Hill and Larson, 1955; Bellis, 1957; Pond et al., 1961; Robertson et al., 1971).

(ix) Feeding of Colostrum - Substitutes.

Improved survival rates have been achieved by feeding or injecting a variety of 'colostrum-substitutes' such as porcine serum or plasma, bovine or porcine gamma globulins, or bovine colostrum (Bustad, Ham and Cunha, 1948; Barrick, Matrone and Osborne, 1954,

Done, 1955, Whitehair and Thompson, 1956; Owen et al., 1961; Pond et al., 1961; Gomez-Garcia and Matrone 1967; Senft and Klobasa, 1971 a, b, c; Scoot, Owen and Agar, 1972; Elliott, Owen and McCallum, 1975; McCallum, Elliott and Owen, 1977).

Colostrum-substitutes are an advantage when the piglets are destined for minimal disease herds where the important requirement of stock is freedom from pathogens rather than freedom from antibodies. In disease transmission studies however there is a possibility that colostrum substitutes may interfere with the establishment or expression of the agent under study. A good example is the protection conferred to rotavirus - challenged piglets by feeding serum containing antirotavirus antibodies (Corthier, Cohen and Scherrer, 1980). The possibility of a predisposing agent in the pathogenesis of PIA has been discussed in Chapter 1. If such an agent were, for example, rotavirus, the feeding of colostrum-substitutes, most of which contain rotaviral antibodies would be likely to inhibit transmission.

USES OF COLOSTRUM - DEPRIVED PIGLETS.

Colostrum-deprived and surgically-derived piglets have been used in many ways, particularly for creating

'minimal disease' or 'pathogen-free' herds of superior health status, in investigations into the immune response, and for nutritional studies on growth and the genetic basis of growth. Of more direct relevance to the work described in this thesis is the particular use of these animals in the investigation of infectious diseases.

Disease Transmission Experiments.

Because neonatal piglets rely on antibodies derived from colostrum and sow milk to a great extent for protection from infectious agents their susceptibility is often inversely related to the previous disease 'experience' of the dam (Young and Underdahl, 1951).

For disease-transmission studies unsuckled piglets were preferred by some as they were uniformly susceptible to infection and no other agents were present to complicate the host-parasite relationship (Young, Underdahl and Hinz, 1955). These authors cite the use of such piglets in studies on transmissible gastroenteritis (TGE), swine fever, virus pig pneumonia and human diseases.

Alexander (1960) used colostrum-deprived piglets in experimental transmissions of an encephalomyelitis, probably of viral aetiology. Betts (1960) and Betts and Jennings (1960) described the production of polioencephalomyelitis in colostrum-deprived piglets with a virus isolated from the tonsils of 'normal' piglets and then shown to cause clinical disease in

the field. Similarly Sibalin and Lannek (1960) and Izawa, Howarth and Bankowski (1962) worked with porcine enteroviruses and demonstrated their pathogenicity in colostrum-deprived piglets where no disease could be reproduced in sucking piglets or demonstrated in the field.

Lecce (1960) successfully reproduced a Glässer's disease syndrome in colostrum-deprived piglets using Haemophilus suis and a pleuropneumonia-like organism. Sow-raised piglets showed no clinical signs after a similar experimental exposure.

In recent years colostrum-deprived gnotobiotic piglets have been used extensively in the field of disease transmission and their use is further discussed in Chapter 5.

POSSIBLE APPLICATIONS OF COLOSTRUM-DEPRIVED PIGLETS IN THE TRANSMISSION OF PIA.

As discussed previously colostrum-deprived piglets have been successfully used to transmit disease to which sow-suckled piglets were not susceptible.

Susceptibility to enteric pathogens often depends on the presence or absence of local enteric immune mechanisms e.g. where antibody from milk exerts a protective effect. Haelterman (1965) has demonstrated

protection from TGE virus in sucking piglets due to the presence of milk antibodies. Snodgrass and Wells (1976) have shown that lambs challenged with rotavirus are protected only if antibody to rotavirus is present in the gut and not if antibody to rotavirus is restricted to the circulation.

It seems logical that antibody to mucosalis, if present in the gut, could interfere with the establishment of mucosalis and the development of PIA. Roberts (1978) has shown that although sucking neonatal piglets can be colonised by mucosalis more readily than post-weaned pigs, transmission of PIA in piglets exposed in the neonatal period is not regularly achieved. It is possible that the immune status of the dams, particularly dams secreting antibody to mucosalis in their milk, may have been responsible for the inconsistent results.

Mucosalis can be isolated from lesions of PIA in pigs and occasionally from the oral cavity and the alimentary tract of pigs in the field, not all of which are affected by PIA (Roberts, 1978; Roberts, Lawson and Rowland, 1980c; Roberts, 1981). In addition Lawson, Roberts and Rowland (1980) have examined the sera of arbitrarily selected slaughterhouse pigs and found agglutinating antibody to mucosalis in a high percentage of the sera examined. It is likely that such circulating antibody has resulted

from previous exposure to *mucosalis*.

In parturient sows and gilts, prior exposure to *mucosalis* may be reflected in the secretion of specific antibodies to *mucosalis* in colostrum and milk. To support this hypothesis agglutinating antibody to *mucosalis* has been found in both colostrum and milk of a small number of sows examined (Lawson *et al*., 1982; McCartney, Lawson and Rowland, unpublished).

Such antibodies could influence either establishment of *mucosalis* in the gut lumen or uptake of *mucosalis* by piglet enterocytes. Colostrum-deprived piglets, lacking these antibodies, would seem a more susceptible animal to use in attempted transmission of PIA.

Previous apparent experimental transmission of PIA has only been achieved using adenomatous mucosa in the inoculum. It is possible that adenomatous mucosa contains unidentified 'factors' essential for the establishment of *mucosalis* in epithelial cells. *Mucosalis* organisms within adenomatous mucosa may be 'cell-adapted', and lose essential virulence attributes when cultured, or the infective mucosa may contain other agents such as occult virus which are essential for the reproduction of PIA. The possibility of a viral predisposing agent to PIA has been discussed in Chapter 1, and several workers have attempted to demonstrate viruses in adenomatous tissue

(see Chapter 1). The guaranteed susceptibility of colostrum-deprived piglets increases the likelihood of demonstrating such "occult" virus, if it is present in adenomatous tissue, and examining its possible role in the pathogenesis of PIA. Exposure of colostrum-deprived piglets to such abnormal porcine tissue however, is likely to involve exposure to other potentially pathogenic organisms possibly unrelated to the development of PIA but fatal to these susceptible animals e.g. pathogenic E. coli, rotavirus. Despite the risk of high mortality after such exposure it was considered that valuable information could be obtained regarding the early relationship of mucosalis with pig enterocytes in a completely susceptible host.

In transmissible ileal hyperplasia (TIH) of hamsters transmission is achieved more readily by using minced mucosa from affected hamsters as the inoculum, rather than bacterial flora cultured from the lesions (Jacoby, Osbaldiston and Jonas, 1975; Amend et al., 1976). PIA has many similarities to TIH and it seemed reasonable therefore to expect better results with homogenised mucosa than cultured bacteria.

Other possible disadvantages were the high cost of colostrum-deprived piglets in terms of labour and equipment. Clearly however colostrum-deprived animals were more appropriate where exposure to crude material was intended than gnotobiotic piglets whose major advantages (i.e. no competitive flora) would be destroyed by the very nature of the inoculum.

MATERIALS AND METHODS

(i) Plan of Experiment

A litter of piglets was caught at birth, housed individually in cages, and some of the litter dosed orally at 4 days of age with fresh adenomatous mucosa. The piglets were killed serially, or died; at post-mortem selected areas from the small and large intestine were cultured for mucosalis, and parallel areas were examined by light, transmission-electron, and immunofluorescent microscopy.

(ii) Animals, Accommodation and General Procurement Procedures

At 103 days of gestation a Large White gilt from Easter Bush Farm (see Chapter 2) was introduced into a disinfected tubular steel farrowing crate in a clean, formalin-fumigated isolation room*. She was fed approximately 3kg daily of commercial pellets suitable for sows in milk ('Sowlac cubes', Seafield Mill, Roslin) plus water ad libitum. The animal was observed at least twice daily, ate well and remained clinically normal throughout.

Parturition was induced by the intramuscular injection of 200µg cloprostenol** on day 112 of gestation.

*Ministry of Agriculture (1965) Advisory Leaflet No. 514, HMSO, London.

** 'Estrumate', ICI, Prostaglandin $F_2\alpha$ analogue.

Labour had started by the following day and at the first signs of straining the vulva and perineum of the gilt were thoroughly washed in dilute cetrimide*. Each piglet was caught at birth by personnel wearing sterile gloves and a disinfected rubber calving gown**. The piglets were ear-tagged, washed briefly in dilute cetrimide and then transferred to a separate, clean, formalin-fumigated room where they were housed individually in cages (vide infra).

Ten healthy piglets were born with no still-births and normal placentae.

The piglets were housed individually in stands of aluminium rabbit cages which had been autoclaved immediately prior to placement in the room. The room was concrete walled and floored, with an asbestos roof, and had been scrubbed with lysol and formalin-fumigated before introduction of the cages.

During the experiment the air temperature was maintained thermostatically between 30 - 35°C. Heat was provided by electric fan-heaters. Relative humidity was kept between 60 - 70% by wetting the floor and trays beneath the cages at every feed. Both temperature and relative humidity were continually monitored by a recorder (Casella, London).

(iii) Feeding

Piglets were fed four times daily a diet of tinned

* 'Savlon', ICI, 1.5% w/v Chlorhexidine gluconate & 15% w/v cetrimide.

** Scrubbed with carbolic disinfectant.

evaporated cows' milk* diluted with mineral mixture**. The relative proportion of each were 1:1, milk: mineral mixture in the first week of life and 4:3, milk:mineral mix thereafter.

Initially a small amount of the diet was poured into a flat-bottomed pottery dish and the piglet encouraged to drink by dipping its nose in the mixture. Most piglets rapidly learned to drink from the dishes and the amount offered at each feed was increased gradually to 150ml by the end of the first week of life and 175ml thereafter.

In the event of diarrhoea (see Results) milk was temporarily withdrawn and a sterile solution of 10% glucose in 0.85% NaCl offered instead.

(iv) Procedures to Prevent Introduction or Spread of Infection

All heat-resistant equipment was autoclaved before being placed in the room. Non-autoclavable items such as tinned milk were carried into the room in a bucket of dilute cetrimide. Metal trolleys (used to hold feeding and sampling materials) were scrubbed with lysol before introduction into the room.

As much as possible of the necessary equipment was placed in the room before the birth of the piglets.

* ' Carnation', Carnation House, London.

** Formula given in Appendix 5.2.

The room with the equipment *in situ* was then refumigated and kept closed until the introduction of the piglets.

Personnel entering the room during the experiment wore sterile face-masks, donned a disinfected calving gown, wore sterile gloves and dipped their boots in lysol.

Control piglets, housed in the same room as the piglets exposed to adenomatous mucosa, were fed and sampled first, using materials from a separate trolley. The attendant swabbed the cage fronts with dilute cetrimide after feeding and sampling the controls, then changed masks and gloves before feeding and sampling the exposed piglets, using materials from a second trolley and following the same procedure described for the controls.

The piglets were provided with sterile dishes at each feed. Any undrunk milk from the previous feed was discarded into a bucket of dilute cetrimide along with the dirty dishes.

Dilute cetrimide was added to the trays beneath the cages to reduce spread of faecal organisms.

When feeding and sampling was completed the attendant removed all dirty dishes, samples and effete materials such as empty tins.

(v) Details of Exposure to Adenomatous Mucosa.

Five piglets (F1 - F5) were dosed orally at 4 days

of age with 4mls of a homogenate of adenomatous mucosa in TPB. The method of dosing and the preparation of the mucosa for oral dosing are described in Chapter 2.

The adenomatous mucosa was prepared from Pig 486/78, a runt pig from Easter Howgate Farm (Chapter 2). This pig had lesions of adenomatosis in the TSI and caecum. The caecum was chosen to provide mucosa for the inoculum as this area was free of adherent necrotic debris, unlike the TSI. A 2ml aliquot of the inoculum was cultured for mucosalis as described in Chapter 2 and from this an estimate made of the total numbers of mucosalis given to each piglet.

Each piglet (F1 - F5) received (total dose)

6.4×10^7 mucosalis (7.81 log 10)

Three littermates (C1 - C3) were maintained as unexposed controls. [Two piglets died prior to 4 days of age (see Results) and are not regarded as belonging to either the controls or the exposed group].

(vi) Sampling and Treatment of Samples

(a) Oral and Rectal Swabs

Oral and rectal swabs were taken from each piglet at least once after exposure of piglets F1-F5 to adenomatous mucosa. These were cultured for mucosalis as described in Chapter 2.

(b) Faeces Samples

Where possible approximately 1g of faeces from

each piglet was obtained by rectal swabbing and placed in a sterile bijou container. The faeces were examined for rotavirus antigen by an enzyme-linked immunosorbent assay test (ELISA) based on the method described by Ellens and de Leeuw (1977)*. Aliquots were stored at -80°C and examined 21 months later by direct electron microscopy**.

(vii) Necropsy Procedures

In general the necropsy sampling and processing procedures were as described in Chapter 2, after euthanasia by intracardiac injection of "Expiral".

(a) Sites Cultured for Mucosalis

Two piglets which died of intercurrent disease (vide infra) prior to exposure of littermates to adenomatous mucosa were not cultured for mucosalis.

The remaining piglets were cultured for mucosalis at the sites listed below following the procedure described in Chapter 2. Piglet F4 was not cultured for mucosalis. It had been intended to adopt a standard procedure but the rapid collapse of a number of piglets necessitated the processing of a large number of samples in a short period of time. Hence sampling had to be curtailed.

* Performed by Dr. D. Snodgrass - Moredun Research Institute.

** Performed by Dr. S. Tzipori - - Moredun Research Institute.

(BA 489/78)	F1	USl	(BA 493/78)	Cl	TSl
		MSl			
		TSl			
(BA 494/78)	F2	MSl	(BA 495/78)	C2	MSl
		TSl			TSl
(BA 496/78)	F3	MSl	(BA 502/78)	C3	USl
		TSl			MSl
(BA 497/78)	F4	ND*			LB
(BA 498/78)	F5	USl			
		MSl			
		TSl			
		LB			

(b) Other Procedures

Tissues from all the piglets were examined histologically. These were the USI, MSI, TSI, LB, mesenteric lymph nodes, liver, lung, spleen and kidney. A selection of sites from the gut were examined by immunofluorescent and electron microscopy and are indicated in the text.

* ND - no sites cultured for mucosalis.

(viii) Additional Bacteriology

The MSI contents of all piglets were cultured for ETEC as described in Chapter 2.

Where piglets were found dead the liver and spleen were cultured as follows:-

The surface of the organ was seared, a loopful from beneath the seared surface plated onto SBA and $M^{C}C$ plates which were incubated aerobically at 37°C.

RESULTS

Intercurrent Disease

Three out of the 10 piglets in the litter died of intercurrent disease. Two of these (BA 484/78 and BA 485/78) died before exposure of littermates to adenomatous mucosa and will not be considered as belonging to either the infected or the control group. BA 484/78 showed nervous signs prior to death and post-mortem examination suggested a bacterial septicaemia as a result of umbilical infection.

BA 485/78 did not readily drink from the flat dishes and was bottle-fed instead. Death was due to a drenching pneumonia.

Control piglet C1 was found dead at 10 days of age, with evidence of acute diarrhoea. Post-mortem examination and the isolation of pure cultures of *E. coli* from the MSI, liver and spleen suggested colisepticaemia as the cause of death.

(a) Clinical Observations: (C1 - C3; F1 - F5).

Control piglets C2 and C3 remained healthy throughout and were killed at 10 (C2) and 12 (C3) days of age. C1 described above had remained healthy until its sudden demise at 10 days of age. Piglets F1 - F5 were clinically normal until dosing with adenomatous mucosa at 4 days of age. From 2 days post-infection these piglets had diarrhoea which worsened progressively and was accompanied by rapid dehydration. Attempts to rehydrate the diarrhoeic piglets by withdrawal of milk and substituting 10% glucose-saline were unsuccessful. Piglet F1 was killed at the onset of scour 2 days post-infection (at 6 days of age). Piglet F2 was killed when moribund at 6 days post-infection (at 10 days of age). Piglets F3 and F4 died late on the 6th day post-infection (at 10 days of age). Piglet F5 was severely dehydrated by 7 days post-infection and was killed at this point (11 days of age) when it seemed unlikely to survive much longer.

The considerable dehydration of the piglets which scoured is indicated in Table 4.1 which lists the body-weights at death of exposed and control piglets.

TABLE 4.1.

Body-weight at Death (kg)

Piglets exposed to adenomatous mucosa				Controls			
Piglet Number		Age (days)	Weight (kg)	Piglet Number		Age (days)	Weight (kg)
(BA 489/78)	F1	6(k)[a]	ND[c]	(BA 493/78)	C1	10(d)	1.73
(BA 494/78)	F2	10(k)	0.80	(BA 495/78)	C2	10(k)	1.65
(BA 496/78)	F3	10(d)[b]	1.00	(BA 502/78)	C3	12(k)	1.56
(BA 497/78)	F4	10(d)	0.90				
(BA 498/78)	F5	11(k)	1.00				

a (k) - piglet killed

b (d) - piglet died

c ND - piglet not weighed at death.

(b) Findings at Necropsy

(i) Gross Findings

a) Controls (C2-C3)

Both piglets were in good bodily condition. Their stomachs contained clotted milk and the small intestines were irregularly full of fluid digesta. The large intestines contained creamy yellow contents becoming solid towards the rectum. The lacteals in the small intestinal mesentery were prominent and appeared as white 'veins' running in the mesentery due to the presence of milk fat from the diet. The washed surface of the mucosa of the small intestines had a pink velvety appearance due to the presence of villi.

b) Exposed Piglets (F1-F5)

F1 killed 2 days post-infection was moderately dehydrated. The stomach contained clotted milk but the small and large intestines contained watery fluid and foetid yellow clots. The lacteals in the mesentery were not prominent. The washed mucosa had a similar appearance to that of the controls.

Piglets F2-F5 had similar abnormalities at post-mortem which are summarised here. All were severely dehydrated and there was hair loss and erythema of the perineum and hocks due to the prolonged scour. The stomachs mostly contained a little clotted milk or glucose-saline but the small intestines were collapsed and empty with a dry, tacky serosal surface and a dull,

flat mucosal surface. The lacteals were not grossly visible. Watery fluid containing yellow clots of digesta was found in the large intestines.

(ii) Histopathology

Controls (C2-C3)

a) Small Intestine (H&E Stain)

The mucosa in the main conformed to descriptions given in standard texts (e.g. Dellman and Brown, 1976) for the pig species. The glandular mucosa consisted of finger-like villi, which surmounted simple crypt glands. The villi were longer in the jejunum than in the duodenum or the ileum and in the ileum they were shorter over the lymphoid tissues than in adjacent areas. Two cell types were recognised on the villi; columnar absorptive cells and goblet cells, whilst lining the crypts were undifferentiated crypt cells and goblet cells. The lamina propria was sparsely populated with lymphocytes and eosinophils. The lymphoid follicles of Peyer's patches were prominent in the lower ileum.

There were two main differences observed from the appearance of the small intestine of colostrum-fed piglets; first the villi of colostrum-deprived piglets were shorter than those of colostrum-fed piglets, and second there was a total absence of vacuoles in the villar enterocytes of colostrum-deprived piglets. These differences are demonstrated by comparing Figures 4.1 and 4.2.

b) Large Intestine (H&E stain)

The mucosa of the large intestine of piglets C2 and C3 was similar to descriptions in standard texts (Dellman and Brown, 1976). The mucosal surface was not folded and was devoid of villi. The crypts were short and straight and opened onto a smooth mucosal surface of simple columnar epithelium. Goblet cells were numerous in the large intestine. The lamina propria was scanty and contained mainly lymphocytes. Occasional small lymph follicles were observed at the base of the crypts.

c) Silver Stains (Young's and Kerr's) of Small and Large Intestines.

Examination of Young's stained sections showed that there was a mixed bacterial flora in the lumen of the small intestine. Bacteria were rarely observed in contact with host enterocytes and never observed below the crypt-villus junctions of the small intestine except for piglet C2 where a few of the crypt lumina in the TSI contained aggregations of bacillary or irregularly-curved bacteria.

In the large intestine there was often a dense bacterial population in the lumina of the crypts. Several different morphological forms were observed among which were irregularly-curved bacteria. In the large intestine bacteria were often closely associated with host cells, sometimes apparently adherent to the surface.

Exposed Piglets (F1-F5)

a) Small Intestine (H&E stain)

F1. The small intestine of the piglet was similar to the controls C2 and C3 except for the USI where in many areas examined the villi were blunted, fused or flattened.

F2-F5. The changes in these piglets were similar and are described together. The most striking feature was the severe blunting, fusion and extensive loss of villi particularly in the MSI and TSI. The surface epithelium in many areas was cuboidal or flattened and failed to cover the mucosa completely thus allowing the escape of inflammatory cells from the lamina propria into the gut lumen. The lamina propria contained aggregations of neutrophil polymorphs and pyknotic debris particularly beneath the surface of denuded villi. Compensatory crypt hyperplasia was observed in piglets F4 and F5. Many of the crypt lumina were swollen and contained necrotic cell debris. The typical appearance of the small intestinal mucosa of these piglets is shown in Figure 4.3.

b) Large Intestine (H&E stain)

F1. The large intestine was similar to the large intestines of controls C2 and C3.

F2-F5. The large intestines of these piglets showed evidence of a colitis of considerable severity. In many areas the mucosa was thin and the surface eroded.

The crypts were lined with flattened, degenerating epithelial cells and their lumina were packed with necrotic and inflammatory cell debris. Goblet cells were absent from many areas examined. The lamina propria was densely cellular and contained aggregations of lymphocytes, macrophages, neutrophil polymorphs and pyknotic debris (Figure 4.4). However in piglet F5 there remained a few areas where, although the lamina propria contained pyknotic debris and inflammatory cells, the crypt glands were composed of both goblet cells and apparently normal absorptive cells.

c) Silver Stains (Young's and Kerr's) of Small and Large Intestines.

F1. Young's stains showed that in the small intestine there was a mixed bacterial flora in the lumen of the gut. The organisms were not adherent to villar enterocytes and the flora rarely extended below the crypt-villus junctions. A few crypt lumina in the USI contained small numbers of irregularly-curved bacteria.

In the TSI small aggregations of bacteria of mixed morphology were observed in some crypt lumina.

In the large intestine many crypt lumina were packed with bacteria of various morphological types. Among these, irregularly-curved bacteria were prominent and in several instances were observed in intracellular sites (Figure 4.5). In other instances irregularly-curved bacteria appeared to be extracellular but

closely adherent to the surface of crypt cells (Figure 4.6).

F2. The USI contained a mixed bacterial flora and its distribution was restricted to the lumen of the gut as in Controls C2 and C3. A few crypt lumina in the MSI and TSI contained aggregations of bacteria among which were irregularly-curved forms. In one crypt only of the TSI, irregularly-curved bacteria were observed in the apical cytoplasm of crypt cells.

In the large intestine bacterial forms of varied morphology could be seen close to or adhering to the surface of the section. In severely damaged areas bacteria, including short irregularly-curved and longer spirally-curved organisms, were observed scattered through the lamina propria.

F3 and F4. Autolysis was considered to be too far advanced for useful interpretation of silver stains.

F5. Many of the crypt lumina in the small intestine contained irregularly-curved bacteria which often appeared closely adherent to epithelial cell surfaces. In a few crypts in the USI, MSI and TSI irregularly-curved forms were observed apparently within the apical cytoplasm of crypt cells.

In the large intestine curved organisms were commonly observed in the crypt lumina. Some of these bacteria appeared closely adherent to the crypt epithelial cells.

(iii) Bacteriological Results (Campylobacters)

a) Controls (C1-C3)

Mucosalis was not isolated from control piglets, neither from oral swabs taken at 9 days of age, nor from the gut at necropsy. No catalase-positive campylobacters were isolated.

b) Exposed Piglets (F1-F5)

F1. Mucosalis was not isolated from oral or rectal swabs taken prior to necropsy at 6 days of age (2 days post-exposure), and neither was mucosalis isolated from the gut at necropsy. Catalase-positive campylobacters were isolated from the TSI (Table 4.2).

F2 - F5. Mucosalis was isolated from oral swabs taken 5 days post-exposure. Mucosalis was not isolated from the gut at necropsy of piglets F2 and F3, but was isolated from piglet F5 (Table 4.2).

F4 was not cultured for mucosalis at necropsy. Mucosalis was isolated from an oral swab taken 5 days post-exposure

Catalase-positive campylobacters were isolated from the gut at necropsy of piglets F2, F3 and F5 (Table 4.2).

All isolations of mucosalis were made on NGBT plates. Recovery was not successful from CBA plates inoculated in parallel. Catalase-positive campylobacters could usually be recovered from CBA plates, although on some occasions overgrowth by other bacteria

TABLE 4.2.

ISOLATIONS OF CAMPYLOBACTERS FROM EXPOSED PIGLETS (F1 - F5)*

Piglet Number	Isolation of mucosalis prior to necropsy		Days post-exposure when necropsied	Isolations of mucosalis at necropsy		Isolations of catalase-positive campylobacters at necropsy	
	Oral Swabs	Rectal Swabs		log 10/g of mucosa	Sites, media** and highest dilution	log 10/g of mucosa	Sites, media** and highest dilution
F1(BA 489/78)	0	0	2	0	NA	4.90	TSl, CBA, $(20)^3$
F2(BA 494/78)	+	ND	6	0	NA	4.41	MSl, NBGT, $(20)^1$
F3(BA 496/78)	+	ND	6	0	NA	7.16	CBA, $(20)^4$ MSl, NBGT, $(20)^3$
						6.90	TSl, NBGT, $(20)^3$
F4(BA 497/78)	+	ND	6	NA	NA	NA	NA
F5(BA 498/78)	+	ND	7	3.60	USl, NBGT, $(20)^2$	7.50	USl, CBA, $(20)^5$
				3.60	MSl, NBGT, $(20)^2$	6.81	MSl, CBA, $(20)^4$
				3.60	TSl, NBGT, $(20)^2$		
				3.60	LB, NBGT, $(20)^2$	8.11	TSl, CBA, $(20)^5$

* Piglets C1, C2, C3 - No campylobacters isolated.
ND - not sampled. NA - not applicable. + - positive isolation. 0 - no isolation.
** This column gives the alimentary sites, the media, and the highest dilution of mucosa from which campylobacters were isolated.

possibly prevented their recovery. On these occasions it was still possible to isolate them from NBGT plates. The recovery of campylobacters from these piglets is summarised in Table 4.2.

(iv) Additional Bacteriology and Virology

Culture of the MSI contents on $M^{C}C$ and SBA invariably led to the growth of non-haemolytic coliforms and α-haemolytic streptococci. No ETEC were isolated from these piglets.

Examination of faeces samples for rotaviral antigen by the ELISA test indicated that piglets F2, F3, F4 and F5 were excreting rotavirus on the 5th and 6th days post-exposure. Further examination by direct electron microscopy 21 months later did not result in detection of rotavirus particles in the faeces.

Rotavirus was not detected in piglets F1, C1, C2 or C3 by either of the above methods.

Dr. Snodgrass who performed the ELISA test advised caution when interpreting these results as the ELISA test for detection of pig rotavirus was at the time in the early stages of development in his laboratory.

(v) Immunofluorescent Results

Detection of mucosalis antigen by immunofluorescence was attempted in piglets C2, C3, F1, F2 and F5. The results are summarised below:-

F1. TSI and LB examined. A few basal crypts in the TSI showed particulate apical fluoresence. This was somewhat duller than that observed in the positive

control. No specific fluorescence was observed in the LB.

F2. TSI and LB examined. No specific particulate fluorescence was observed in any of the blocks examined. In one area of the TSI there was diffuse apical fluorescence in crypt cells midway in intensity between the positive and negative controls.

F5. USI, TSI, MSI and LB examined. No specific particulate fluorescence was observed in any of the blocks examined but bright diffuse fluorescence was observed in the lumina of basal crypt gland in the TSI (Figure 4.7).

C2 and C3. TSI and LB examined. No specific particulate fluorescence was observed in any of the blocks sampled.

(vi) Electron Microscopy

Tissues from piglets F1, F2, F5, C2 and C3 were examined by transmission electron microscopy.

C2 and C3. USI, MSI, TSI and LB were examined. Ultrastructurally the areas of gut examined conformed in general to published descriptions (Rhodin, 1963; Sandborn, 1970; Cheville, 1976).

USI. Cells on the villi were tall and columnar, had basal nuclei a well-defined brush border of microvilli. Conspicuous by their absence were the large cytoplasmic vacuoles observed in young colostrum-fed piglets. However there was evidence of pinocytosis of a smaller

scale. Vesicles were observed forming beneath the bases of microvilli in the apical cytoplasm. The cell cytoplasm was finely granular and contained abundant rough endoplasmic reticulum. The mitochondria were compact with well-defined cristae. The nuclei were large and oval and had one or two prominent nucleoli. Goblet cells which contained characteristic cytoplasmic granules of mucus were occasionally interspersed with more mature absorptive cells. The lamina propria was sparsely populated with mononuclear cells. Occasional lymphocytes were observed migrating through the epithelium.

Cells lining the crypts were also tall and columnar with large basal nuclei. The microvilli, particularly on cells deep in the crypts, were less well-developed than on villar enterocytes. Further evidence of the immaturity of the crypt cells was demonstrated by the presence of apical secretory granules and abundant free ribosomes. Goblet cells were more numerous in the crypts than on the villi. The lamina propria of the crypts was scantily cellular. The crypt lumina were free of bacteria. Occasional argentaffin cells were observed among crypt epithelial cells.

Scattered fissures were observed throughout the mucosal areas examined. These were assumed to be artefacts of fixation or processing, and occurred in all sections examined, both control and exposed.

MSI. The sections examined were similar to those of the USI. Micropinocytosis was observed in the villar enterocytes but to a lesser degree than in the USI. Occasionally mucoid debris and granules similar to apical secretory granules were observed in the crypt lumina.

TSI. The villi were shorter here than in the anterior small intestine but the ultrastructural appearance was otherwise similar to the USI and MSI. No bacteria were seen in any areas of the sections examined.

LB. The crypt glands were lined by undifferentiated columnar epithelial cells interspersed with goblet cells and were within a relatively acellular lamina propria. The microvilli of the columnar cells were shorter and thicker than those on small intestinal villar enterocytes.

There were abundant free ribosomes throughout the cytoplasm and numerous apical secretory granules. The nuclei were oval or convoluted, large and basal in position. Scattered aggregations of rough and smooth endoplasmic reticulum were observed. The mitochondria were compact with well-defined cristae.

Goblet cells had basal more cuboidal nuclei and many large granules of mucus filled most of the cytoplasm. The surface membrane of one goblet cell was seen bulging into the crypt lumen and other crypt

lumina appeared distended with mucoid debris.

The lumina of many crypts contained numerous bacteria most of which were morphologically indistinguishable from campylobacters. These bacteria were never seen intracellularly and did not appear to be adherent to the surface of host cells (Figure 4.8).

Fl. Areas of the caecum and spiral colon were examined. The ultrastructural features were largely similar to those of the control piglets. There was no evidence of cellular degeneration.

As in the control piglets many crypt lumina were packed with bacteria indistinguishable from campylobacters. However there was evidence of much closer association with the host cells than in the controls (Figures 4.9 - 4.11).

Some of the campylobacter-like bacteria were apparently in contact with the surface of the host cells. In one crypt gland a campylobacter-like organism (CLO) was found deep in the cytoplasm near the basal lamina and not within a membrane-bound vacuole (Figures 4.12 - 4.14). In another area examined a CLO was observed free in the lamina propria (Figure 4.15). Two CLO's were observed within the apex of a goblet cell lying between membrane-bound granules of mucus (Figures 4.16 - 4.17). Considering the large numbers of extracellular CLO's, the numbers of CLO's observed intracellularly or within the lamina propria were

small and there was no evidence of division of these bacteria within host cells.

F2. Areas from the LB were examined and confirmed the light microscopic findings of a destructive colitis. The crypts were lined by cuboidal or flattened epithelial cells, lacking apical secretory granules. The microvilli were short, sparse and sometimes clumped together. The mitochondria were swollen with poorly-defined cristae. Many epithelial cells contained supranuclear apoptotic bodies. Goblet cells were absent.

The crypt lumina were swollen and packed with neutrophil polymorphs, extruded epithelial cells and debris. A few CLO's were observed in crypt lumina along with other morphological types of bacteria. No bacteria were observed in intracellular locations.

Lymphocytes were observed frequently migrating through the remaining crypt epithelium and in the lamina propria. The lamina propria contained increased numbers of macrophages, neutrophil polymorphs and plasma cells.

F5. Areas from the USI, MSI, TSI and LB were examined.

USI, MSI & TSI. The ultrastructural features of these areas were similar. The epithelial cells throughout were reduced in height to low columnar or cuboidal and showed evidence of mild degenerative changes such as irregularities and effacement of the

microvillous brush border, swelling and disruption of mitochondria and the presence of supranuclear apoptotic bodies in some sells. Villar enterocytes were less mature than in control piglets - many had apical secretory granules and numerous free ribosomes. The lamina propria was intensely cellular and contained neutrophil polymorphs, macrophages, plasma cells and lymphocytes. Lymphocytes were frequently observed migrating through the epithelium.

The crypt lumina were packed with mucin-like material and cell debris and aggregations of CLO's. Despite frequent close apposition of the CLO's to the surface of host cells (Figure 4.18) CLO's were not observed in intracellular sites except on one occasion where a group of CLO's were seen in an evacuated goblet cell (Figure 4.19).

LB. The crypt glands of the areas examined were composed of both undifferentiated cells and goblet cells. Most of the undifferentiated cells were swollen and the cytoplasm of these cells was very electron translucent. The cellular organelles were often bloated or disrupted and the microvilli were clumped or effaced. The lumina of the glands contained cellular debris and bacteria of mixed morphology among which CLO's were not as prominent as had appeared when parallel areas were examined under the light microscope. The lamina propria was densely cellular and contained neutrophils

and mononuclear cells.

On a few occasions isolated bacterial forms were observed free in the cytoplasm of swollen enterocytes or lying free in the lamina propria. Although some appeared morphologically indistinguishable from CLO's others were not recognisable as CLO's (Figures 4.20 - 4.21).

DISCUSSION

The results of this experiment indicate that exposure of colostrum-deprived piglets to a crude preparation of adenomatous mucosa is a procedure which is rapidly fatal to such susceptible hosts. The dosed piglets experienced severe enterocolitis which resulted in extreme dehydration followed by death. Grossly and histologically there was no evidence of adenomatous proliferation in any piglet, although none survived for long enough to assess whether adenomatosis could have occurred. The results of Roberts' (1978) apparently successful transmission and the epidemiological studies of Love, Love and Edwards (1977) suggest that the incubation period is at least several weeks.

Nevertheless some information was gained on the behaviour of mucosalis in the early period post-exposure of antibody-free pigs. Mucosalis did not appear to colonise the gut of exposed piglets readily, demonstrated by the failure to isolate mucosalis from the gut of 3 out of the 4 pigs cultured (F1, F2, F3). The last exposed piglet killed, F5, became colonised throughout the small and large bowel (Table 4.2) but the numbers of mucosalis isolated were several logs lower than from viable adenomatous tissue.

It is possible that the piglets killed earlier than F5 were also infected, but the numbers of mucosalis were not sufficiently great to be detected by the technique used. There was evidence from the light and electron microscope that CLO's were present in association with the mucosa of all the exposed piglets. Some of these organisms may have been mucosalis. If this was the case then there seems to be a lag period in the establishment of detectable numbers of mucosalis, even in susceptible piglets exposed to adenomatous mucosa.

Roberts (1978) argued that as long as most of the surface area of the inoculated plates was relatively free of contaminating bacteria, then mucosalis, if present, should have been recovered. However the factors governing successful isolation of mucosalis, particularly when present in low numbers in a

contaminated milieu, are probably complex. Lawson and McCartney (unpublished results) have observed that other bacteria, probably anaerobes, grow selectively on top of mucosalis colonies even when large areas of the plate are free of bacterial growth. This selective growth could be due to reduced oxygen tension provided by the microaerophilic mucosalis colonies which allows the growth of strict anaerobes. Hence it is likely that when the ratio of anaerobes: mucosalis is high (i.e. especially so when the numbers of mucosalis are low) isolation of mucosalis is not achieved due to selective overgrowth of anaerobes. For these reasons it is possible that low numbers of mucosalis colonising the gut of piglets F1, F2 and F3 were not isolated by the bacteriological techniques used.

The sites and mechanisms of colonisation of the pig by mucosalis are crucial to our understanding of the pathogenesis of intestinal adenomatosis. Mucosalis was recovered from the oral cavity of 4 out of 5 exposed piglets in this experiment and has been recovered from the oral cavities of piglets in the field, sampled sequentially over a period of weeks (Roberts, 1981), and from other experimentally-exposed animals (Roberts, Lawson and Rowland, 1980c). These results and in addition the close taxonomic relationship between mucosalis and Campylobacter sputorum ss sputorum, an oral commensal of man, suggest that the "normal" ecological niche of mucosalis is the porcine

oral cavity, and that perhaps infection of the alimentary tract is a rare occurrence, leading even more unusually to intracellular infection of enterocytes and the development of adenomatous change. Such a postulate is supported by the failure of Lawson and Rowland (1974) to isolate mucosalis from the gut of healthy pigs.

If mucosalis did colonise the alimentary tract of all the exposed piglets the question remains as to the exact site of the infection. The evidence of the light and electron microscope suggests that only rarely did CLO's penetrate epithelial cells whilst the vast majority remained extracellular, in crypt lumina. However the interpretation of these observations was complicated by the isolation of large numbers of catalase-positive campylobacters, morphologically very similar to mucosalis. It is possible that the few intracellular CLO's observed were mucosalis, present in numbers too low for successful culture or detection by immunofluorescence, and the extracellular CLO's observed were the more numerous (and hence easier to isolate) catalase-positive campylobacters.

The specific fluorescence observed in the crypt lumina of the TSI of piglet F5 suggests however that most of the mucosalis present were extracellular. There was no evidence that extensive intracellular colonisation by mucosalis occurred under the circumstances of this experiment.

Roberts (1978) suggested that colostrum, now known to contain specific antibody to mucosalis (Lawson et al., 1982), stimulates uptake of mucosalis by pig enterocytes. In addition Lawson et al., (1979) have shown that intracellular mucosalis in adenomatous tissue are coated with host antibody. In the neonatal pig exposed to mucosalis it is possible that luminal bacteria could become coated with colostral antibody. Such antibody, if non-lethal to mucosalis, could possibly protect mucosalis after non-selective uptake of the bacteria by neonatal enterocytes, for example by preventing recognition of "foreign" antigens by the host cell, or by protecting mucosalis from the action of lysosomal enzymes of the host cell. However when Roberts exposed suckling piglets in the neonatal period there was little evidence that ingestion of colostrum at the time of exposure resulted in extensive intracellular parasitism by mucosalis and in most of his experiments his results were similar to those described here i.e. a low level of intestinal colonisation by mucosalis with little evidence of intracellular multiplication. In this respect there seems little difference in the behaviour of mucosalis between exposed sow-suckled and exposed colostrum-deprived piglets.

On the other hand perhaps host antibody prevents the attachment of mucosalis to host cells. Since the

inoculum used was adenomatous mucosa, many of the *mucosalis* to which these piglets were exposed were presumably already coated with IgA (Lawson *et al.*, 1979). Rajasekhar (1981) has shown *in vitro* that specific antibodies to *mucosalis* prevent their attachment to certain cell lines. A similar mechanism may operate *in vivo*.

The severe enteric upset experienced by only the dosed piglets suggested the presence of an enteric pathogen probably derived from the inoculum. Rotavirus was tentatively diagnosed by Dr. Snodgrass of Moredun Institute. The failure to detect rotavirus in the frozen faeces 21 months later was not unexpected as rotavirus particles survive poorly under these conditions (Tzipori, personal communication, 1980). The histological changes observed were compatible with rotavirus infection and no enteropathogenic *E. coli* were isolated. Rotavirus was not observed in host enterocytes or the gut lumen of the areas studied by the electron microscope, but these areas were not those likely to result in observation of rotavirus. Rotavirus infects the villar enterocytes of the small intestine (McNulty, 1978) and only in piglet F5 was the small intestine examined, by which time most of the villar enterocytes had been shed.

Catalase-positive campylobacters were isolated in high numbers from the diarrhoeic exposed piglets but not from the controls. It is possible that these

bacteria originated from the inoculum and there is recent evidence that some members of this heterogenous group of bacteria may exert pathogenicity in piglets (Taylor and Olubunmi, 1981). Their role in the enteric disturbance of this group of piglets is unknown. Despite the failure to isolate campylobacters from control piglets, bacteria morphologically indistinguishable from campylobacters were observed in the LB of 2 controls. This suggests that failure to culture such fastidious organisms does not necessarily indicate their absence from the sample.

There was no clear evidence of the presence of occult virus in the adenomatous inoculum. Rotavirus may have been present, but its role in the pathogenesis of PIA is dubious (see Chapter 5). If occult virus plays a role in the adenomatosis complex it may be as an initiator and then no longer be present by the time the lesions have developed. Under the circumstances of this experiment it was not possible to establish the involvement of occult virus in the pathogenesis of PIA.

The loss of 3 piglets (C1, BA484/78, BA485/78) from intercurrent disease illustrates the hazards of rearing colostrum-deprived piglets in a non-sterile environment. Several other experiments, not reported here, were abandoned due to high mortality.

The results of this experiment indicate that lesions of adenomatosis in pigs from conventional herds are often multiply contaminated by potential pathogens, bacterial and viral. Unless adenomatosis is presented to us as a disease in otherwise disease-free herds the lesions are likely to be thus contaminated. Hence any attempts to expose further litters of colostrum-deprived or gnotobiotic piglets to such contaminated tissue in a search for mucosal "factors" or initiating agents are likely to be frustrated by high mortality in exposed animals. Exposure of older colostrum-deprived pigs may be more fruitful, especially since the disease occurs in the post-weaned age group in the field, but experience with other colostrum-deprived litters (vide supra) indicated that the facilities available were not adequate for the rearing of sufficient numbers of older colostrum-deprived pigs. There would still be the danger of mortality after exposing such older susceptible animals to contaminated mucosa and if lesions of adenomatosis did develop they would be likely to be complex, possibly involving other syndromes not related to PIA.

The problems encountered in this experiment, high mortality and bacterial contamination, were partially avoided by the use of gnotobiotic piglets (Chapter 5) to study the behaviour of mucosalis in antibody-free piglets.

CHAPTER 5

EXPOSURE OF GNOTOBIOTIC PIGLETS TO:

1) MUCOSALIS ONLY, 2) MUCOSALIS AND ROTAVIRUS.

TERMINOLOGY AND DEFINITIONS

The variability existing within and between groups of experimental animals maintained under traditional conditions imposes a limit on the nature and amount of information that can be obtained from experiments carried out with such animals. While *in vitro* test systems such as those utilising tissue culture may provide a convenient alternative under some circumstances, experimental animals remain essential for many research purposes. As a result there is a need for the production of standard experimental animals which has been fulfilled to some extent by the development of specific pathogen free (SPF) and gnotobiotic animals.

In 1964, the International Committee on Laboratory Animals published a list of recommended terms and definitions, some of which are relevant to the work described in this Chapter and are mentioned below:

(i) '*Gnotobiotic animal*' or '*gnotobiote*' denotes an animal in which any associated unicellular or multicellular organisms have been fully defined.

(ii) A '*germ-free*' animal is one which is free of all other organisms and is therefore a particular type of gnotobiote although a gnotobiote is not necessarily germ-free. It is worth noting here that animals regarded as 'germ-free' may not be so designated in the future if improved monitoring techniques reveal the presence of previously unsuspected organisms.

(iii) A gnotobiotic animal which has become associated with a single, two or several known species of organisms, whether intentionally or accidentally introduced, is often referred to as being mono-, duo-, or poly-contaminated respectively. However in this thesis "contamination" will be used to mean organisms accidentally introduced.

(iv) 'Specific Pathogen Free' (SPF) Animals are free of specifically stated organisms which are usually but not invariably pathogenic. Apart from these specified organisms the animals are associated with a variety of other species which have not been defined.

(v) 'Conventional' Animals have a microbiological status which is largely unknown but which is assumed to include a variety of pathogenic and non-pathogenic organisms.

It should be noted that these categories define the microbiological status of the animal and not the method of derivation or maintenance for which terms such as Caesarean-derived, hysterectomy-produced, colostrum-deprived, artificially-reared and barrier-maintained may be used and are largely self-explanatory.

The 'gnotobiotic piglets' described in this work were derived by aseptic Caesarean section into a sterile environment. They were maintained using germ-free techniques according to the general principles of Coates (1968). They were colostrum-deprived and reared on

an artificial diet. Their microbiological status was defined as fully as possible by the techniques available to the laboratory where the work was performed. The term 'isolator' is used to designate the apparatus in which the gnotobiotic piglets were maintained (vide infra).

A SHORT HISTORY OF GNOTOBIOTES.

Interest in rearing animals free of associated micro-organisms was expressed as early as 1885 (Pasteur, 1885; cited by Waxler, Schmidt and Whitehair, 1966). Pasteur considered that animals would not survive without their associated microbial flora, a theory which was later disproved.

Numerous attempts were made in the late 19th and early 20th centuries to rear germ-free chickens and small mammals and improvements in the techniques used have led to the present availability of germ-free laboratory animals for many research purposes (Luckey, 1963; Coates, 1968).

Germ-free techniques for the derivation and maintenance of the larger mammals, including pigs, were based in principle on the methods used for small mammals and were greatly facilitated by the introduction of transparent flexible polyvinyl chloride (PVC) film for the construction of isolator systems (Trexler and

Reynolds, 1957). Isolators constructed of PVC film proved to have many advantages. They were inexpensive, easy to construct, versatile in design, and readily sterilised, usually by peracetic acid (Meyer, Bohl and Kohler, 1964; Makin and Tzipori, 1980).

The problem of transferring piglet foeti from the uterine environment (considered 'sterile' if reasonable precautions were observed in selecting the dam, see Chapter 4) to a sterile environment for rearing was overcome by a number of methods, all based on preventing or eliminating possible contamination by the extra-uterine environment:

a) Hysterectomy

The sow was suitably restrained and anaesthetised and an aseptic laparotomy performed. A ligature was passed around the uterus anterior to the cervix. The uterus was then excised and passed through a liquid germicidal trap into a sterile isolator. The piglets were quickly removed from the uterus and passed through a connecting PVC sleeve into a separate sterile isolator for rearing (Meyer, Bohl and Kohler, 1964; Waxler, Schmidt and Whitehair, 1966; Miniats and Jol, 1978).

b) Closed Hysterotomy

The sow was suitably restrained and anaesthetised. A sterile box-shaped PVC isolator was stuck on with sterile adhesive to the surgically prepared flank of the sow. An aseptic Caesarean section was performed using

electrocautery for the initial incision through the floor of the 'surgical' isolator and the skin of the sow. The piglets were excised from the gravid uterus directly into the sterile environment of the 'surgical' isolator. They were then passed through a connecting PVC sleeve into a separate sterile isolator for rearing (Meyer, Bohl and Kohler, 1964; Miniats and Jol, 1978; Makin and Tzipori, 1980).

c) Open Hysterotomy

The sow was restrained and suitably anaesthetised. An aseptic Caesarean section was performed. The piglets were removed from the uterus and passed through a liquid germicidal trap into a sterile PVC isolator for rearing (Miniats and Jol, 1978).

Miniats and Jol (1978) compared the three methods described above and found that the open hysterotomy was more efficient and equally successful in providing microbiologically sterile piglets. Most other authors compared only the first two methods and preferred closed hysterotomy to hysterectomy (Makin and Tzipori, 1980).

Once the animals were procured into a sterile environment accidental contamination was generally avoided by:

i) maintaining a positive-pressure, filtered-air ventilation system for each isolator (see Chapter 4 for principles).

ii) introducing only materials which had been sterilised (e.g. food, water and equipment), and these

through a disinfectant lock attached to the wall of the isolator (Trexler and Betts, 1975).

APPLICATIONS OF GNOTOBIOTIC PIGS IN BIOMEDICAL RESEARCH

The applications of gnotobiotic laboratory animals in research are manifold and are discussed more fully elsewhere (e.g. Luckey, 1963; Pollard, 1967; Coates, 1968). The gnotobiotic pig, likewise, is useful in research relating to a wide range of problems, as well as in fields directly applicable to the pig species, and in some areas of great relevance to man (Meyer, Bohl and Kohler, 1964). The experimental value of the gnotobiotic pig, and other species of gnotobiotes, derives largely from their defined microflora and from the absence of protective antibodies acquired from colostrum. Some applications of gnotobiotic pigs are discussed below:

a) Sources of Tissues and Fluids

Tissues derived from gnotobiotic pigs have been used in the preparation of primary pig cell cultures or for the initiation of semicontinuous cell-lines free from detectable mycoplasmas and latent viruses (Christofinis et al., 1972). Sera from gnotobiotic pigs that have been deprived of colostrum are free from most inhibitory substances and are particularly useful

as constituents of cell culture media used for the growth of viruses. Tissues from such pigs are of value in histological investigations where tissues are required which have not been altered by exposure to micro-organisms or other antigenic stimuli (Trexler and Betts, 1975).

b) Control of Infectious Diseases

Herds of pigs free of many infectious diseases have been established by breeding from stock originally derived by Caesarean and reared under gnotobiotic conditions in early life (Trexler and Betts, 1975). In spite of the high failure rate in the early stages of these projects, due to mistakes that with hindsight appear obvious, there is clear evidence that the establishment from gnotobiotic animals of a few elite herds kept under conditions of strict hygiene is a practical way of controlling respiratory, enteric and perhaps other diseases of pigs (Heard and Jollans, 1970). These elite herds have been used to provide breeding stock for more conventional farms (Trexler and Betts, 1975).

c) Immunological Studies

Gnotobiotic piglets can be obtained without significant levels of maternal antibody by depriving them of colostrum. They are then useful for research in the field of immunity, since such piglets are capable of responding to several classes of antigens (Binns, 1968).

The production of monospecific antisera is possible in gnotobiotic piglets and there are indications that these animals produce higher levels of antibodies than conventional animals (Trexler and Betts, 1975).

d) Physiological and Nutritional Research

Gnotobiotic piglets are good subjects in which to study important aspects of production, for example the effect of nutrition and genetics on growth rate. The absence of an associated microflora and pathogenic organisms simplify the assessment of such experiments. Bywater and Wood (1980) have used gnotobiotic piglets challenged with rotavirus to demonstrate the efficacy of a glucose-glycine electrolyte solution for oral rehydration in piglet diarrhoea. Most work on the physiology and nutrition of gnotobiotes has however been performed on laboratory mammals (Coates, 1968; Gordon and Pesti, 1971).

e) Studies on the Aetiology and Pathogenesis of Infectious Disease

The principal use that has been made of gnotobiotes in biomedical research is in the study of the pathogenesis of disease. The pig species is particularly useful in this field because of the large number of piglets in each litter, the relative ease of production and rearing, and the comparative low cost (Trexler and Betts, 1975).

Smith and Hayward (1969) used gnotobiotic pigs to investigate the pathogenesis and pathology of sterile

inhalation pneumonia. Most other researchers have studied infectious diseases of bacterial, viral, mycoplasmal or mixed aetiology.

The pathogenesis of Escherichia coli infections of piglets has been studied by Kohler and Bohl (1966), Kohler and Cross (1969), Miniats, Mitchell and Barnum (1970), Meyer and Simon (1972), Waxler and Britt (1972) and Corley, Staley and Jones (1973). The pathogenesis of strains of pasteurellae and bordetellae has been investigated by Smith (1971; cited by Trexler and Betts, 1975), whilst Geissinger, Miniats and Quinn (1969) used germ-free pigs to study infections with Erysipelothrix insidiosa.

Hodges, Betts and Jennings (1969) used gnotobiotic pigs to investigate the pathogenesis of Mycoplasma hyopneumoniae infection, whilst Gois et al., (1972) and Poland (1973; cited by Trexler and Betts, 1975) have used them to study the pathogenicity of strains of Mycoplasma hyorhinis.

Gnotobiotic pigs have been used extensively for studies on the pathogenesis of viral infections. They have been used to study porcine enteroviruses inducing polioencephalomyelitis (Betts and Jennings, 1960; Edington, Christofinis and Betts, 1972); transmissible gastro-enteritis (TGE) virus (Olsen, Waxler and Roberts, 1973); adenoviruses of porcine origin (Shadduck, Koestner and Kasza, 1967; Kasza et al., 1969); and the

pathogenesis of rotavirus infections, using strains of porcine (Crouch and Woode, 1978; Theil et al., 1978; McAdaragh et al., 1980; Torres-Medina and Underdahl, 1980), bovine (Hall et al., 1976) and human (Bridger et al., 1975; Torres-Medina et al., 1976a, 1976b) origin.

Gnotobiotic piglets have also proved useful for the study of mixed infections of the respiratory or intestinal tract. Thus Kasza et al. (1969) studied mixed respiratory infections of adenovirus and M. hypopneumoniae, whilst Smith et al. (1973b) compared infections of gnotobiotic pigs with Pasteurella septica alone or in combination with M. hypopneumoniae. Smith et al. (1973a) also studied the effects of combined infection with P. septica and adenovirus or enterovirus in gnotobiotic swine. Mixed infections of enteropathogens in gnotobiotic piglets have been described by Underdahl et al. (1972) who studied infections of transmissible gastroenteritis virus-like particles and E. coli. Tzipori et al. (1980d) have used gnotobiotic piglets to study the effect of mixed rotavirus and E. coli infections.

The use of gnotobiotic piglets in the study of the pathogenesis of diarrhoea associated with rotaviral strains of human origin has already been mentioned (vide supra). Gnotobiotic piglets have also been of value in the study of human respiratory viruses and human adenoviruses (Betts et al., 1962; Trexler and Betts, 1975).

THE USE OF GNOTOBIOTIC PIGS FOR ATTEMPTED TRANSMISSION OF PIA

(i) Known Immunological Status

The possible effects of maternally-acquired immunity on the establishment of *mucosalis* in the gut of baby pigs has been discussed more fully in Chapter 4. The presence of specific antibodies in the colostrum and milk of the dam may inhibit colonisation of the gut by *mucosalis* either by preventing infection of the lumen of the gut or by interfering with adherence to or penetration of enterocytes. The difficulties of rearing colostrum-deprived piglets in a non-sterile environment have been discussed in Chapter 4. Gnotobiotic piglets, due to the stringent microbiological barriers employed in derivation and rearing would be likely to thrive better and live longer, thus allowing a longer time interval post-dosing in which adenomatous change could occur. Clearly however adenomatous mucosa would not be a suitable inoculum for such piglets, since the contaminating microflora would remove the 'gnotobiotic' status of the piglets and would probably result in severe enteric upset and death as happened with the colostrum-deprived piglets. For these reasons the use of gnotobiotic piglets in this chapter is confined to the study of the behaviour of laboratory-cultured *mucosalis*.

(ii) Known Microbiplogical Status

Conventional animals, by definition, have an

associated microflora the members of which are largely unknown, vary between individuals and may include pathogenic as well as non-pathogenic organisms. This heterogenous flora may influence the results of an experimental exposure to any agent under investigation. The use of gnotobiotes allows the study of the effect of the agent in question in a defined environment and eliminates an important uncontrolled variable between groups of experimental animals.

Roberts (1978), reviewing the mechanisms that might inhibit or prevent the establishment of mucosalis in the gut lumen (and logically mucosalis must establish in the lumen of the alimentary canal before parasitising host enterocytes) listed the production of volatile fatty acids, the maintenance of a low oxidation-reduction potential, or competition for available nutrients by resident microflora as important factors. The absence of enteric bacteria in germ-free animals would thus eliminate such possible inhibitory effects on mucosalis.

(iii) A Study of the Ability of Campylobacter sputorum subspecies mucosalis in Isolation to Produce Lesions of PIA.

The disadvantages of using an inoculum of adenomatous mucosa in gnotobiotes and the possible inhibitory effects of a conventional enteric flora have been mentioned in (i) and (ii) above. The use of a pure inoculum of C. sputorum ss mucosalis in gnotobiotic

piglets has several advantages. In the absence of competitive bacteria mucosalis would have every opportunity to establish in high numbers in the lumen of the gut and in such circumstances a study could be more easily made of the relationship between mucosalis and the mucosa of the gut. In essence the ability of mucosalis to exert a pathogenic effect could be studied without the complications of maternal immunity or the presence of other, possibly inhibitory organisms.

The problem encountered in Chapter 4 in isolating mucosalis, accurately assessing the numbers which establish, and interpretation of light and electron - microscopic observations should in theory be reduced, since in gnotobiotes there are no contaminating bacteria to interfere with plate counts and no other morphologically similar bacteria to confuse the light and EM studies.

Consideration of the points discussed in (i), (ii) and (iii) indicated that oral exposure of germ-free piglets to mucosalis could answer several important questions:

a) Does mucosalis establish readily and in high numbers in the alimentary tract of such piglets and if so at what sites, e.g. throughout the gut or only in certain areas; in the lumen or only in the mucosa?

b) If mucosalis establishes in the mucosa in high numbers, is this due to infection of host enterocytes and does this result in development of lesions of PIA?

c) If mucosalis establishes and persists in

gnotobiotes, is this reflected by the production of specific circulating antibodies - possibly an indication of pathogenicity or the penetration of the mucosa by mucosalis?

PIG ROTAVIRUS AS A PREDISPOSING AGENT IN ATTEMPTED TRANSMISSION OF PIA IN GNOTOBIOTIC PIGLETS.

Several authors have discussed the possible involvement of a virus in the pathogenesis of members of the PIA complex (Biester, Schwarte and Eveleth, 1939; Korpassy and Tiboldi, 1957; Goodwin and Jennings, 1959; Smith and Jones, 1963; Rowland and Rowntree, 1972; Rowland and Lawson, 1974). Some have attempted to isolate viral agents from or demonstrate their presence in adenomatous mucosa. Although rotavirus (Bergeland et al., 1975), transmissible gastroenteritis virus (Goodwin and Jennings, 1959) and even swine fever virus (Smith and Jones, 1963) have been considered as candidates there has been no convincing evidence of the involvement of a virus in the disease process.

Since intracellular bacteria were demonstrated in adenomatous cells (Rowland, Lawson and Maxwell, 1973) few workers have pursued the question of viral aetiology. However the frequent unsuccessful attempts to reproduce PIA using either mucosalis in pure culture or adenomatous mucosa as inocula (Roberts, 1978) indicate that the conditions necessary for consistent transmission are not

being fulfilled and that perhaps it is worth reconsidering the role which a virus could play in initiating adenomatous proliferation. Such an agent could act by increasing the susceptibility of host cells to infection by mucosalis or, given that some host cells are already susceptible, by increasing the number and availability of susceptible cells to infection. This hypothetical agent may only act in the early stages of the disease and although absent once lesions of PIA have developed yet be essential for successful reproduction of the disease. The failure to demonstrate a virus in developed lesions of PIA does not necessarily disprove the theory of a virus initiator.

Adenomatous epithelial cells closely resemble undifferentiated crypt cells. It has been suggested that in the initial stages of adenomatous change crypt cells become infected by mucosalis which persist intracellularly by multiplying in dividing host cells, and that adenomatous cells containing mucosalis then spread within the epithelium (Lawson, Rowland and Roberts, 1976). In theory an initiating agent could enhance the process of cell infection by altering the surface membrane of crypt cells in such a way as to encourage bacterial adherence and intracellular penetration, or, if crypt cells are susceptible per se, by simply increasing the number and availability of crypt cells for infection.

The first possibility is examined in Chapter 7 where cryptosporidia are used in an attempt to enhance bacterial attachment to host cells. The second possibility is examined in Experiment 2 of this chapter, using rotavirus to increase the exposure of crypt cells to mucosalis.

Rotavirus was selected as a possible predisposing agent for a number of reasons, the most obvious being that it fulfills the necessary qualification of producing an increase, albeit transient, in the number and availability of crypt cells in the small intestine (Snodgrass et al., 1979). Rotavirus effects this change by attacking villar epithelial cells causing extensive atrophy of villi and hyperplasia of crypt cells (Crouch and Woode, 1978). Hence the number of crypt cells are temporarily increased and also appear clothing the villous remnants, in which position they would logically be more susceptible to infection by luminal bacteria.

Another important reason for choosing to investigate the role of rotavirus as a predisposing factor in PIA is the ubiquity of this virus (Bridger, 1980). Like PIA rotavirus has a world-wide distribution and so would be universally 'available' as a predisposing factor. Roberts (1978) discarded transmissible gastroenteritis virus as a possible initiator of PIA because transmissible gastroenteritis virus does not occur in Scotland, yet the proliferative enteropathies of the pig are not uncommon.

Both weaned and unweaned piglets from less than 1 week to 8 weeks of age can develop rotavirus-associated diarrhoea (Rodger, Craven and Williams, 1975; Woode and Bridger, 1975; Lecce, King and Mock, 1976; McNulty *et al.*, 1976; Woode *et al.*, 1976; Bohl *et al.*, 1978; Lecce and King, 1978), thus there is a long time period for possible rotavirus / host-cell / *mucosalis* interactions. This time interval includes the period between the fall in maternally-acquired circulating antibodies to *mucosalis* (by 30 days of age) and the appearance of actively-produced circulating antibodies to *mucosalis* (70-80 days of age) (Lawson *et al.*, 1982). The interval between the fall in passively-acquired antibodies and the later rise in actively-produced circulating antibodies emcompasses the period when infection with *mucosalis* is likely to occur in the field. Rotavirus is known to occur in this age group of pig (*vide supra*) and so could play a role in the initiation of PIA, provided the time sequence of infection with rotavirus and infection with *mucosalis* were such that crypt cells became available for *mucosalis* when there were *mucosalis* present in the gut lumen.

The failure to induce early signs of adenomatous change in weaned pigs exposed to rotavirus and *mucosalis* (see Chapter 6) may have been associated with the difficulty in establishing infection with *mucosalis* in weaned pigs. Roberts (1978) found that weaned pigs

were more refractory to infection than younger piglets sucking their dam. Clearly adenomatous hyperplasia is unlikely to occur if there are no *mucosalis* to infect susceptible cells, no matter how numerous or exposed the susceptible cells. A suitable experimental model to study this problem would require the presence of rotavirus and *mucosalis* 'in the right places at the right times'. If *mucosalis* establishes only transiently in the lumen of the gut of weaned piglets then the likelihood of rotavirus preceding this infection at just the correct time to 'predispose' to *mucosalis* is remote.

In order to minimise these possible problems gnotobiotic piglets were used. Under these circumstances it was expected that:

a) There would be no antibodies present and hence no inhibition of either rotavirus or *mucosalis*.

b) *Mucosalis* would be more likely to establish and persist in high numbers than in conventional, weaned pigs (see Experiment 1, Chapter 5). This would allow the maximum opportunity for infection of crypt cells with the bacteria.

c) There would be no other enteric flora present, hence the specific interactions, if any, between rotavirus, host cells and *mucosalis* could be studied and their possible role in the pathogenesis of PIA assessed.

MATERIALS AND METHODS

GENERAL PROCUREMENT AND MAINTENANCE OF GNOTOBIOTIC PIGLETS

(i) Source of Dams

One pregnant gilt (Experiment 1) and one pregnant sow (Experiment 2) were bought from Easter Bush Piggery, the 'Minimal Disease' herd described in Chapter 2. The good health status of this herd was considered an advantage as transplacental infections would be less likely.

(ii) Derivation of Piglets - Hysterotomy

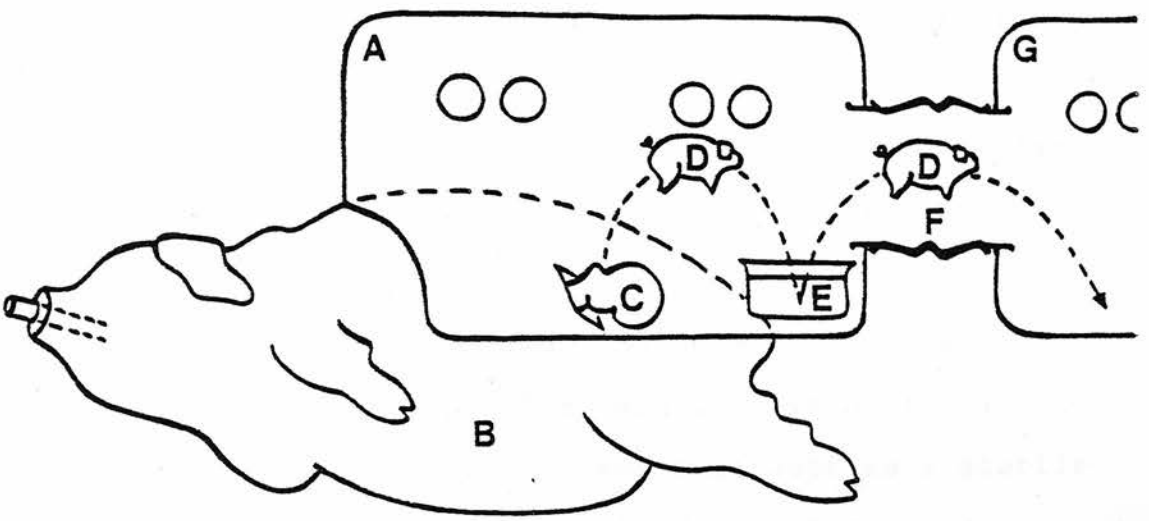
The hysterotomies were performed at Moredun Research Institute by Mr. Ben Mitchell and his staff. The anaesthetist was Mr. Martyn Camburn, Royal (Dick) School of Veterinary Studies.

At 112 days of gestation the dam was transported to Moredun's gnotobiotic unit and restrained in a wooden crate. Confinement was such that the animal could neither turn round nor move backwards and forwards. The panels of the crate were solid in order to minimise distressing visual stimuli to the pig. No premedication was used. General anaesthesia was induced by the administration, *via* a close-fitting mask, of 50% nitrous oxide and 50% oxygen passed through a halothane vaporiser. A cuffed endotracheal tube was inserted and anaesthesia was maintained by 2% halothane in nitrous oxide and oxygen, administered by closed circuit.

The sow was then placed in right lateral recumbency and a large area of the left flank clipped, shaved and flooded with an aqueous solution of 1% Cetavlon (ICI Ltd., Pharmaceuticals Division, Alderly Park, Macclesfield, Cheshire, SK10 4TF). The area was wiped with sterile gauze swabs. Next an iodophor skin cleaning liquid ('Wescodyne Skin Scrub', Ciba-Geigy Agrochemicals, Whittlesford, Cambridge, CB2 4QT) was poured undiluted on the skin which was scrubbed vigorously with a stiff brush. The suds were washed off with the Cetavlon solution and the skin swabbed dry. The area was then sprayed with 10% tincture of iodine and dried with a hair drier. When dry the surface was sprayed with a sterile surgical adhesive ('VI-DRAPE Adhesive', Parke, Davis and Co., Usk Road, Pontypool, NP4 87H).

The sterile surgical isolator was then lowered onto this area and stuck on firmly by means of a sterile adhesive panel in the floor of the isolator (vide infra). Using diathermy an oblique cranio-ventral incision was made through the floor of the surgical isolator, the sow's skin and the oblique muscles. The transversalis muscle and the peritoneum were incised using scissors and one uterine horn exteriorised. The piglets were removed through one incision in the antimesometrial border. The second cornu was dealt with similarly (Figure 5.1). The piglets were first washed briefly in a 10% aqueous solution of 'Wescodyne' and then passed through to an adjoining transport isolator (Figure 5.1).

Figure 5.1: Closed hysterotomy.



The surgical isolator (A) is attached to the flank of the anaesthetised sow (B) by an adhesive. The uterus (C) is exposed through an incision made from within the isolator. The piglets (D) are removed, washed in disinfectant (E) and passed through a transfer sleeve (F) into the transport isolator (G).

Here mucus and amniotic fluid were removed from the nose and mouth of each piglet and respiration was encouraged by rubbing the chest gently with a dry towel. Polypropylene umbilical clips ('Hallister Umbilical Clamps', Chas. F. Thackray Ltd., P.O. Box 171, Park Street, Leeds) were attached to stem navel bleeding, and the umbilici were cut below the clips.

When all the piglets were in the transport isolator the connection between the isolators was sealed by covering the inner entry-port of the transport isolator with a polyvinylchloride (PVC) cap (vide infra) and the isolators were separated by cutting the surgical isolator at its junction with the outer entry-port of the transport isolator. The surgical isolator was then split longitudinally and the base used as a sterile drape during the routine closure of the uterus and flank.

The dam was hospitalised until removal of the skin sutures and then sent for slaughter. Both animals made an uneventful recovery.

(iii) Design and Construction of the Isolators (Figures 5.2, 5.3, 5.4, 5.5).

The equipment was based on the flexible-film isolators described originally by Trexler and Reynolds (1957), and adapted for use with gnotobiotic lambs. The isolators were assembled at Moredun Research Institute by the present author and the staff of the gnotobiotic unit, under the supervision of Mr. R. McVitie.

a) The Surgical Isolator consisted of a commercially produced clear PVC flexible film canopy (150cm X 75cm X 75cm) with 2 sets of frosted PVC sleeves on two opposite sides (Vickers Ltd., Priestly Road, Basinstoke, Hampshire). Surgical gloves were attached to 2 opposite sets of sleeves and rubber household gloves ('Marigold Lightweight Gloves', LRC Products Ltd., London, EC4 8QA) to 3 of the remaining sleeves. All gloves were attached by the method of Trexler (1971) using stainless steel rings and rubber 'O-rings' (Edwards High Vacuum Ltd., Crawley, Sussex). The remaining sleeve at the corner of the isolator was cut out and the diameter of the circle widened to 45cm, in order to attach the entry-port of the transport isolator (vide infra). A rectangular panel 30cm wide by 60cm long was cut from the base of the canopy so that its centre was between the sleeves with the surgical gloves. Double-sided adhesive tape was applied to cover the panel and external to this was applied a 30cm wide panel of sterile surgical drape ('Vi-drape Film', Parke,Davis and Co., Warner-Lambert (UK) Ltd., Usk Road, Pontypool, Gwent NP4 0YH). The outer layer of protective paper remained on the 'Vi-drape' and was removed immediately before application of the isolator to the flank of the sow.

b) The Transport Isolator was supported by a wood and metal frame ('Dexion Speedframe', Dexion House, Empire Way, Wembly, Middlesex) and consisted of a moulded fibre-glass bin 70cm deep, 45cm wide and 120cm

long, surmounted by a PVC canopy of similar size and specifications as the surgical isolator. Household rubber gloves were attached to all four sets of sleeves. A rigid PVC pipe (45cm in diameter X 50cm long) served as an entry-port and was taped in place at one end of the canopy. The part was also attached to the surgical isolator so that the two isolators were linked. One inlet and 2 exit air-filters, consisting of several layers of fibre-glass wool enclosed in perforated metal dinner plate covers were fixed into opposite ends of the transport isolator canopy with the two exit filters on either side and above the entry-port. An electric fan attached to the inlet air-filter supplied both surgical and transport isolators with sufficient air to keep both canopies inflated and under positive pressure relative to the outside.

All necessary equipment for both surgical and transport isolators plus the peracetic acid spray for sterilisation of the interior of both isolators was placed in the transport isolator prior to attaching it to the surgical isolator.

c) The Maintenance Isolators were of similar design and construction as the transport isolator. In addition each tank was fitted with a perforated metal false-bottom 5cm above the fibre-glass floor. A 5cm diameter plastic pipe drained waste from the tank into a 5 litre plastic container. The maintenance isolators in Experiment 2 were provided with a meshed-metal lid

which attached by means of metal clips just inside and below the lips of the fibre-glass bins. Each entry-port also served as a disinfectant lock and could be sealed by inner and outer PVC caps, 46cm diameter (Vickers Ltd.). The outer caps had 2 spray tubes sealed with rubber stoppers.

Figure 5.2 shows a maintenance isolator used in Experiment 1. Other details shown are the electric fan and inlet air-filter (Figure 5.3), the interior of the isolator (Figure 5.4) and the entry-port/ disinfectant lock and exit air-filters (Figure 5.5).

(iv) Sterilisation Procedures

Prior to assembly the floors, lids, drains, fibre-glass bins and entry-ports were scrubbed with a 0.25% solution of an iodophor detergent ('FAM 30', Evans Vanodine International Ltd., Brierly Road, Walton Summit Centre, Bamber Bridge, Preston, PR5 8AH).

All heat-resistant equipment such as air-filters, surgical instruments, towels, feeding utensils and sampling materials were packed in autoclavable nylon film ('Portex Lay-Flat Tubing', C gauge thickness, 10cm or 30cm wide, supplied by Mackay and Lynn Ltd., 2 West Bryson Road, Edinburgh, EH11 1EH) and autoclaved (121°C, 15 minutes, 1.05kg/cm^2). All equipment was double-wrapped except for the air-filters which were single-wrapped and stored thus until immediately before use when the 'Portex' tubing was discarded.

Once an isolator was assembled as much necessary equipment as possible was placed inside the isolator discarding, in the case of heat resistant items, the outer layer of 'Portex' tubing. The isolator canopies were then inflated and the air inlets and outlets sealed. Complete sterilisation of the interior of the isolators was achieved by spraying every surface with a fine mist of 3% peracetic acid solution. The isolator was left overnight after which the seals on the air-inlets and outlets were removed and the air-flow resumed.

Items to be introduced subsequently were placed in the entry-port, sprayed with 3% peracetic acid and left for 30 minutes with inner and outer-entry port caps in place before being taken inside the isolator proper. The reverse procedure applied for items removed from the isolator.

(v) Detection of Contaminants

(a) During the Hysterotomy:

Swabs were taken at various points during the derivation in order to determine when and where any contamination occurred. These samples were taken as follows:

A. Swab of the floor of the surgical isolator prior to the skin incision.

B. Swab of the skin incision.

C. Peritoneal swab.

D. Swab of the surface of the uterus prior

to the incision.

E. Swab of the incision of the uterus.

F. Swab of the skin of the first piglet born, prior to washing in 'Wescodyne'.

Each swab was dipped in nutrient broth (NB), then broken off by flaming into thioglycollate broth (TB). One swab, picked at random, was retrieved from the TB and inoculated onto a Sabouraud's Dextrose Agar (SDA) slope after dipping in Sabouraud's Broth (SB).

(b) <u>During the Experiment</u>

(i) <u>Experiment 1</u>: Rectal swabs were taken from each piglet at 48 hours of age and thereafter twice weekly. It was estimated that each swab absorbed approximately 0.2g faeces (see Chapter 2). Each swab was broken by flaming into 2ml PBS (0.1M, pH 7.2) and then rotated on a Mattburn Mixer for 5 minutes. From the resultant suspensions 0.01ml loopfuls were inoculated onto BA and into TB. Once a week 0.01ml loopfuls from that day's faecal suspensions were inoculated onto SDA slopes and into SB.

Skin swabs were taken weekly and used first to inoculate SDA slopes before being broken by flaming into SB.

(ii) <u>Experiment 2:</u> Rectal swabs, taken from surviving piglets at 5, 7, 8, 11, 14, 21, 26, 33 and 40 days of age, were treated as described for Experiment 1.

Skin swabs were taken weekly and treated as described for Experiment 1.

(c) Isolation and Identification of Contaminants.

Blood agar plates inoculated with faecal suspensions were incubated aerobically at room temperature and at 37°C and examined daily for 5 days. Any colonies suspected of being isolator contaminants were subcultured and identified according to the methods of Cowan and Steel (1974).

Thioglycollate broths were incubated at room temperature and at 37°C and examined daily for 5 days. Broths showing turbidity were subcultured onto BA plates, incubated anaerobically at 37°C and colonies suspected of being isolator contaminants identified as described above.

All SDA slopes were left at room temperature and examined daily for 21 days. Sabouraud broths were incubated at 37°C and examined daily for 21 days. No fungal contaminants were detected in either experiment.

(vi) Management of Gnotobiotic Piglets

Once all the piglets were passed into the transport isolator the inner entry-port cap was placed in position. The piglets were revived, their umbilici clamped and cut, and each piglet was sexed and identified with a numbered ear-tag. To prevent damage to the PVC canopy the piglets' teeth were cut level with the gums using surgical bone forceps. The air inlets

and exits of the transport isolator were then sealed. The entry-port was sterilised by spraying with 3% peracetic acid and the outer entry-port cap attached. The isolator was then transported by lorry to heated (approximately 25°C) rooms at the Edinburgh University Centre for Laboratory Animals, Bush Estate, Penicuik.

The piglets were transferred into the maintenance isolators by linking the transport isolator with each maintenance isolator in turn, using a cylindrical PVC transfer sleeve (60cm long, Vickers Ltd.). The sleeve was disinfected by spraying with 3% peracetic acid through 2 spray ports. The tunnel was left sealed for 30 minutes after spraying and then the inner entry-port caps of the isolators were removed and the piglets passed from one isolator to the other.

The diet, consisting of evaporated cows' milk and water/mineral mixture, was based on the feeding schedule for gnotobiotic piglets at the Institute for Research on Animal Diseases, Agricultural Research Council, Compton. This schedule was kindly supplied by Dr. R. Lysons (Appendices 5.1 and 5.2). The piglets were bottle-fed three times daily (Figure 5.6). Initial attempts to encourage trough-feeding were frustrated by the absence of fixed troughs and the number of piglets in each isolator. Bottle-feeding had the added advantage that milk intake could be accurately assessed.

Oral and rectal swabs were taken as described in Chapter 2. Skin swabs were taken from the axillary

region. Swabs were immediately broken into sterile bijou bottles inside the isolator. The bottles were capped tightly, labelled and brought out of the isolator *via* the entry-port/disinfectant lock (Experiment 1) or *via* a smaller rigid PVC tube 5cm in diameter fitted in the isolator canopy and sealed with rubber bungs at either end (Experiment 2). The use of a smaller tube for removal of samples was considered to reduce the risk of isolator contamination.

EXPERIMENT 1: ORAL EXPOSURE OF GNOTOBIOTIC PIGLETS TO CAMPYLOBACTER SPUTORUM SUBSPECIES MUCOSALIS.

(i) Design of Experiment:

Eight piglets were obtained from the gilt used in Experiment 1. These were separated into 2 isolators (M and C) with 4 piglets in each isolator. The four piglets in isolator M were orally dosed at 24 hours of age with a pure culture of *mucosalis*, leaving the four piglets of isolator C as uninfected controls.

It was planned that the establishment and excretion of *mucosalis* would be monitored as closely as possible while the piglets remained alive and that the sites sampled at necropsy would be selected to give as much information as possible about the behaviour of *mucosalis* in gnotobiotes. Necropsies were to be performed on two piglets (1 control and 1 exposed) every week; the first piglets were to be killed at 7 and 8 days of age.

Due to the unexpected death of control piglets from drenching pneumonia and suffocation (see Results), only one control was necropsied as planned. This control was killed at 8 days of age. The infected piglets were killed at 7, 15, 22 and 28 days of age respectively.

(ii) Source of Inoculum:

The strain of mucosalis used was 1075/78 A-F, derived from adenomatous lesions in the small intestine of a pig slaughtered at Gorgie abattoir in 1978 (see Chapter 2).

(iii) Preparation of Inoculum and Introduction into Isolator:

A fresh aliquot of 1075/78 A-F was removed from the -80°C store, thawed at room temperature and a few drops inoculated onto CBA plates.

These were incubated microaerophilically for 48 hours and then checked grossly for contaminants. Growth from one of the inoculated plates was also checked for purity by examination of Gram-stained smears and its identity as a serotype A strain confirmed by a slide agglutination test. Heavy loopfuls of growth from the remaining plates were inoculated onto two 30ml CBA slopes each overlaid with 30ml TPB.

The inoculated diphasic slopes were incubated microaerophilically for 48 hours and the cultures checked

for purity by examination of Gram-stained smears, and for motility by examination using phase contrast microscopy. One of the diphasic cultures, which were assessed by the above criteria as containing only vigorously motile mucosalis bacteria was decanted aseptically into a McCartney bottle held in a sterile bag. A small amount was retained in the diphasic bottle for assessment of numbers of mucosalis (vide infra). The McCartney bottle was sealed with a sterile rubber bung and the neck of the bottle and protruding area of the bung wiped with 10% tincture of iodine followed by absolute alcohol. The bottle was aseptically transferred to a fresh sterile bag, the bag sealed and placed in a vacuum flask at 37°C. In the isolator room the bag was opened, the bottle placed in the entry-port/disinfectant lock of isolator M and the lock and bottle sprayed with 3% peracetic acid. After 30 minutes the culture was taken into the isolator and each piglet dosed orally with 2ml. The remainder of the culture was returned to the laboratory.

(iv) Assessment of Inoculum: Quantification of Mucosalis and checking for Contamination.

(a) Quantification of Mucosalis in the Inoculum

Each piglet received 6.1×10^8 (8.78 log 10) mucosalis, estimated by the method of Miles and Misra (1938).

The unused culture which was returned from the piglets was left overnight on the bench in the laboratory

and then a few drops inoculated onto a BA plate. This was incubated microaerophilically at 37°C for 48 hours. Growth occurred and was confirmed as mucosalis.

(b) Checking the Inoculum for Contaminants

Drops of 0.1ml from the aliquot used to quantify mucosalis were plated conventionally onto a BA plate, and inoculated into 2 NB's and 2 TB's. All were incubated at 37°C, the BA plates and the NB's aerobically, and examined daily for 7 days for the presence of contaminants. No contaminants were detected.

(v) Monitoring of Mucosalis During the Experiment

Oral and rectal swabs were taken at intervals and cultured for mucosalis as described in Chapter 2.

Rectal swabs were taken from each piglet within 24 hours of birth, prior to oral exposure to mucosalis, and from surviving piglets on post-exposure days 1, 3, 6, 9, 11, 14, 17, 19, 24 and 26. Oral swabs were taken from surviving piglets on days post-exposure 11, 14, 17, 19, 24 and 26.

(vi) Necropsy Procedures and Sites Sampled

An oral swab was taken from the selected piglet immediately prior to its removal from the isolator via the entry-port. The piglet was carried to the post-mortem room in a sterile bag, shot with a humane-killer and bled from the neck. The procedure described in Chapter 2 was then followed. Particular attention was paid to asepsis in order to avoid contamination e.g.

samples of gut for bacteriological examination were ligated with sterile string, taken to the laboratory in sterile trays, and examined immediately.

Alimentary sites were sampled for bacteriological examination, histology, transmission electron microscopy and immunofluorescence and were as follows: (see Figure 2.1).

Stomach

Duodenum (site 1, US1)

Jejunum (site 2)

Upper Ileum (site 3, MS1)

Terminal Ileum (site 5, TS1)

Caecum (site 6, Caec)

Spiral colon (site 7, LB)

Portions of lung, liver, kidney, spleen and mesenteric lymph nodes were processed for histology as described in Chapter 2.

In addition spleen, lung and liver were examined bacteriologically. The surface of the tissue was seared, a 0.005ml loopful obtained from beneath the seared surface, plated out conventionally on CBA and NBGT plates which were incubated microaerophilically and examined for mucosalis.

The mucosal samples for bacteriological examination were processed and examined for mucosalis as described in Chapter 2. Four 1 in 20 dilutions were prepared and CBA and NBGT plates were inoculated from each dilution.

Occasional deviations from these procedures are mentioned in the Results.

EXPERIMENT 2: DUAL INFECTION OF GNOTOBIOTIC PIGLETS WITH CAMPYLOBACTER SPUTORUM SUBSPECIES MUCOSALIS AND PIG ROTAVIRUS.

(i) Design of Experiment:

Twelve piglets were derived from the sow used in Experiment 2. These were divided into 3 isolators (RMA, RMB, AND MC) with 4 piglets in each isolator. All twelve piglets were orally dosed at 7 days of age with a pure culture of mucosalis. One piglet from Isolator RMA was killed 24 hours post-exposure to mucosalis.

The remaining 7 piglets in isolators RMA and RMB were orally dosed with pig rotavirus at 14 days of age, 7 days after the exposure to mucosalis.

The establishment and excretion of mucosalis was monitored as in Experiment 1 by frequent oral and rectal swabs (vide infra) while the establishment and excretion of rotavirus was assessed by clinical signs and electron-microscopic examination of faecal material from rectal swabs (vide infra).

Two piglets died 2 days and a third was killed 5 days after exposure to rotavirus. One mucosalis-only infected piglet was killed at 20 days of age to provide control material of the same age range as those dual-

infected piglets.

The remaining 7 piglets were killed between 34 and 54 days of age in order to encompass the period when it was considered adenomatous change might occur.

(ii) Sources of Inocula: Rotavirus and Campylobacter sputorum ss mucosalis.

(a) Strain of Mucosalis. The strain of mucosalis used was 1075/78 A-F, as for Experiment 1.

(b) Pig Rotavirus. The strain used was P5 SW9/11 - C308 which was derived from strain P4SW9/11, full details of which are given in Chapter 6.

(iii) Preparation of Inocula, Introduction to Isolators and Assessment of Inocula.

(a) Mucosalis strain 1075/78 A-F: The inoculum was prepared and quantified as described for Experiment 1, except that the final inoculum was divided into 3 sterile glass vials which were sealed by flaming. The vials were opened once in the isolators by snapping the necks. Each piglet received 0.5ml orally containing 8.0×10^8 (8.93 log 10) mucosalis.

(b) Pig Rotavirus P5SW9/11 - C308: Strain P4SW9/11 was used to infect 2 colostrum-deprived piglets (See Chapter 6) C308 and C309. Piglet C308 was killed 24 hours later and the gut contents harvested and pooled aseptically. The contents were stored at -70°C for 13 days. A bacteria-free filtrate (strain P5SW9/11-C308) was prepared from the thawed gut contents as

follows:

The contents were mixed well and filtered through sterile gauze to remove gross debris. This filtrate was then filtered through successive millipore filters (1.2μm, 0.8μm and finally 0.45μm). A small aliquot of the final filtrate was examined by direct electron-microscopy and assessed as containing abundant (3+) virus (Campbell, personal communication, 1979). Further aliquots were transferred aseptically to sterile glass vials which were sealed by flaming and stored at -70°C until required. The piglets which were dosed with P5SW9/11-C308 received 0.5ml orally.

(iv) Monitoring of Mucosalis and Rotavirus.

(a) Mucosalis: Oral and rectal swabs were taken from surviving piglets at 4, 11, 21, 22, 26, 33 and 40 days of age. These were cultured for mucosalis as described in Chapter 2.

(b) Rotavirus: Daily rectal swabs were taken from all surviving piglets from 14 days of age, just prior to exposure of some piglets to rotavirus, until 22 days of age, 8 days after exposure to rotavirus. These were examined for the presence of rotavirus by Dr. D. Snodgrass using an enzyme-linked immunosorbent assay (ELISA) similar to that described by Ellens and de Leeuw (1977).

(v) Necropsy Procedures and Sites Sampled.

These were largely as described for Experiment 1, except that the alimentary sites sampled were as follows:

Duodenum (Site 1, US1).

Upper Ileum (Site 3, MS1).

Terminal Ileum (Site 5, TS1).

Caecum (Site 6, Caec).

Spiral Colon (Site 7, LB).

In addition portions of the tongue, tonsils, gingival margin and upper lip were processed for histological examination.

Occasional deviations from these procedures are mentioned in the Results.

(vi) Immunofluorescence Technique for Detection of Rotavirus Antibody in Piglet Sera.

This test was performed on selected sera by the author at Moredun Research Institute using reagents and methods provided by Dr. Snodgrass.

Two cell types were used, bovine embryonic kidney (BEK) cells in monolayer and African green monkey kidney (VERO) cells in monolayer. These monolayers, grown in wells of microtitre plates, were infected with a calf strain of rotavirus (CRV/C BEK/9) (Campbell, personal communication, 1979). Infected monolayers were incubated at 37°C for 24 hours, then fixed in cold acetone for 10 minutes and dried in air. Dilutions of pig sera under test were added to individual wells, the plates incubated at 37°C for 30 minutes, washed 3 times in PBS (pH 7.2, 0.01M), drained, and then stained with fluorescein-isothiocyanate-(FITC) conjugated rabbit anti-swine γ-globulin (Nordic Immunological

Laboratories, Langestraat 57-61, P.O. Box 22, The Netherlands) diluted 1 in 60 in PBS (pH 7.2, 0.01M). The plates were reincubated then washed as above, drained, dried with a hair-drier and examined using incident blue light from a Leitz Ortholux Microscope.

Positive controls were wells prepared as above but incubated with lamb serum B209 (1 in 20 dilution in PBS, pH 7.2, 0.01M), known to have a high titre of rotavirus antibodies, and finally stained with FITC-conjugated pig anti-sheep γ-globulin (1 in 30 dilution in PBS, pH 7.2, 0.01M, Wellcome Laboratories, Cheshire, CW1 1UB).

Negative controls were uninfected monolayers treated as described for the positive controls.

(vii) Detection of IgA in Cryostat Sections.

Selected sections from the oral cavities and alimentary sites were examined for IgA using an immunofluorescent staining method similar to that described in Chapter 2.

Sections were overlaid with rabbit anti-porcine IgA (diluted 1 in 30 in PBS, pH 7.2, 0.01M; Nordic Immunological Laboratories), incubated and washed as described in Chapter 2 and stained with FITC-conjugated sheep anti-rabbit γ-globulin (diluted 1 in 40 in PBS, pH 7.2, 0.01M; Wellcome Laboratories Ltd. Control sections were overlaid with PBS (pH 7.2, 0.01M) instead of rabbit anti-porcine IgA but otherwise treated as above.

RESULTS; EXPERIMENT 1.

(i) Clinical Findings/Daily Observations.

All 8 piglets were healthy and vigorous until 4 days of age when the piglets infected with mucosalis (M1-M4) began to pass liquid yellow faeces. The diarrhoea persisted for 3 days at which time piglet M1, being unable to stand, was removed and killed (Table 5.1). The other 3 infected piglets improved until by 9 days of age they were and thereafter remained clinically normal.

The control piglets G1-G4 (Table 5.1) remained healthy until 5 days of age when piglet G1 was observed in respiratory distress shortly after the last feed of the day. This piglet died the next day at 6 days of age.

Controls G2, G3 and G4 remained healthy until they died or were killed. Control G2 was killed at 8 days of age. Controls G3 and G4 jumped down the sleeves of the isolator when they were 14 days old and suffocated.

(ii) Bacteriological Results.

(a) Procurement of Piglets - No bacterial or fungal contaminants were detected at any stage during the derivation of the piglets and both maintenance isolators remained "germ-free" until the piglets were 4 days of age (vide infra).

TABLE 5.1

EXPERIMENT 1: TREATMENT, CLINICAL SIGNS AND WHEN KILLED OR DIED.

Piglet Number	Treatment	Clinical Signs	Age (days) when killed or died
M1 (BA 367/78)	Oral exposure to *mucosalis* at 24 hours of age	Diarrhoea from 4-7 days of age	7 (K)*
M2 (BA 376/78)	"	Diarrhoea from 4-9 days of age	15 (K)
M3 (BA 387/78)	"	"	22 (K)
M4 (BA 391/78)	"	"	28 (K)
G1 (BA 366/78)	Control, unexposed	Respiratory distress at 5 days of age	6 (D)**
G2 (BA 368/78)	"	Healthy throughout	8 (K)
G3 (BA 374/78)	"	Healthy until jumped down isolator sleeve and suffocated	14 (D)
G4 (BA 375/78)	"	"	14 (D)

* K = piglet killed

** D = piglet found dead

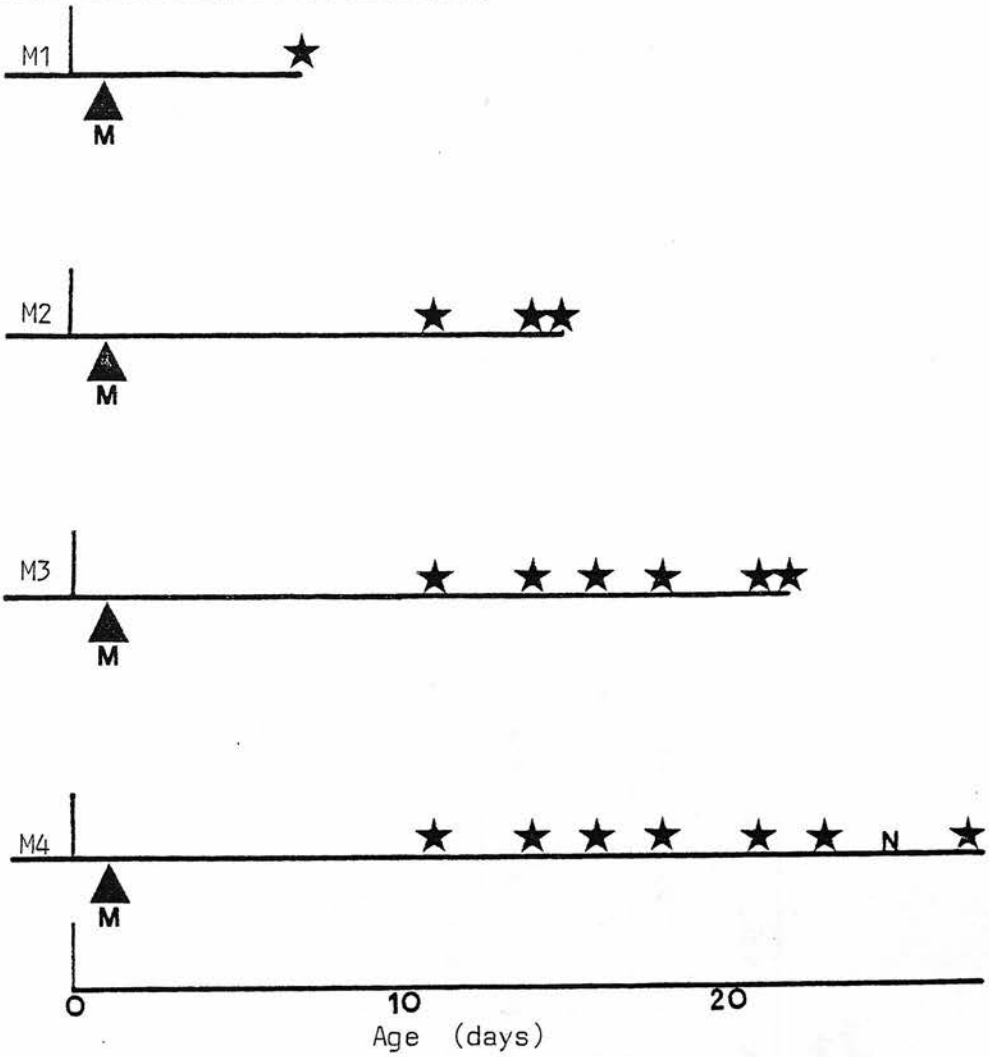
(b) Isolator Contaminants. Both isolators became contaminated by bacteria of the genus Bacillus during the first 15 days of the piglets' life. Isolator C (control piglets G1-G4) was contaminated by a Bacillus stearothermophilus-like organism, detected when the piglets were 6 days old. Isolator M (infected piglets M1-M4) was eventually contaminated by three bacillary species. At 4 days of age a Bacillus stearothermophilus-like organism was detected. At 7 days of age a second bacillary species was detected which biochemical tests indicated was either Bacillus coagulans or Bacillus polymixa.

A third dysgonic bacillary species was detected in Isolator M when the piglets were 15 days of age. This organism was difficult to identify further due to its poor growth.

(c) Isolations of Mucosalis. At no time was mucosalis isolated from any of the control piglets G1-G4. Mucosalis was recovered from all of the exposed piglets by 3 days post-exposure and from the majority of samples thereafter. The numbers of mucosalis and the sites from which they were recovered are summarised in Figure 5.7 and Tables 5.2 - 5.3.

The bacillary contaminants grew readily on all CBA plates and made isolation of mucosalis from these plates difficult or impossible. For example all isolations of mucosalis from the oral cavity were

Figure 5.7: Experiment 1: Isolation of Mucosalis from the Oral Cavities of Piglets M1-M4*.



★ = successful isolation

N = unsuccessful isolation

▲ M = oral exposure to mucosalis

* - all isolations from NBGT plates

TABLE 5.2: EXPERIMENT 1: ESTIMATES OF NUMBERS OF MUCOSALIS ISOLATED FROM RECTAL SWABS OF PIGLETS M1–M4 (NUMBERS EXPRESSED AS LOG 10/g FAECES)

PIGLET NUMBER	DAYS POST-EXPOSURE TO MUCOSALIS													AVERAGE FAECAL EXCRETION (NBGT counts)
	1		3	6	8	10	13	15	17	20	22	24	27	
	CBA*	** NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	CBA NBGT	
M1 (BA 367/78)	3.40	2.65	NR 3.70	NR 5.70										4.0
M2 (BA 376/78)	NR***	NR	NR 4.74	NR 5.60	NR 5.00	NR 2.00	NR NR							2.9
M3 (BA 387/78)	NR	NR	NR 6.00	NR NR	NR 3.08	NR 5.30	NR 5.52	4.70 4.00	NR NR	NR 2.70				3.0
M4 (BA 391/78)	NR	1.70	NR 3.70	NR NR	NR NR	NR 2.30	NR 2.70	NR NR	NR NR	NR NR	NR NR	NR NR	NR 3.00	1.1

* CBA's inoculated at 2nd, 3rd and 4th dilutions only

** NBGT's inoculated at 1st, 2nd and 3rd dilutions only

*** NR = mucosalis not isolated

TABLE 5.3.

EXPERIMENT 1: ESTIMATES OF NUMBERS OF MUCOSALIS ISOLATED FROM MUCOSAL SAMPLES AT NECROPSY OF PIGLETS M1 - M4 (NUMBERS EXPRESSED AS LOG 10/g MUCOSA).

Piglet Number	MUCOSAL SITES SAMPLED						Average (NBGT Counts)
	STOMACH	DUODENUM	JEJUNUM	MSI	TSI	LB	
	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT	
M1 (BA 367/78)	NR* / 2.30	5.50 / 4.86	6.68 / 4.90	NR / 5.68	6.50 / 4.30	NR / 6.90	4.8
M2 (BA 376/78)	NR / 4.90	NR / 2.60	NR / 4.90	NR / 4.02	NR / 4.38	7.05 / 7.13	4.6
M3 (BA 387/78)	NR / 4.90	NR / 3.90	NR / 5.20	NR / 4.90	NR / 6.20	NR / 6.68	5.3
M4 (BA 391/78)	NR / NR	NR / NR	NR / 3.38	NR / NR	NR / 3.08	NR / 4.30	1.8
Average (NBGT counts)	3.0	2.8	4.6	3.6	4.5	6.2	4.1

* NR = mucosalis not isolated

TABLE 5.4

EXPERIMENT 1: COMPARISON OF THE FAECAL EXCRETION OF MUCOSALIS JUST PRIOR TO DEATH OF PIGLETS M1-M4 WITH THE NUMBERS ISOLATED FROM THE LARGE BOWEL MUCOSA. (NBGT COUNTS)

Piglet Number	Faecal Excretion of *Mucosalis*		Numbers of *Mucosalis* in LB mucosa	
	Days post-infection last faecal sample taken	log 10/g faeces	Killed (days post-infection)	log 10/g mucosa
M1 (BA 367/78)	6	5.70	6	6.90
M2 (BA 376/78)	13	NR*	14	7.13
M3 (BA 387/78)	20	2.70	21	6.68
M4 (BA 391/78)	27	3.00	27	4.30
Average		2.8		6.2

* NR = *mucosalis* not isolated.

made on NBGT plates (Figure 5.7). The CBA plates inoculated in parallel were overgrown with bacilli.

The situation was similar with isolations of mucosalis from rectal swabs. The majority of isolations were made from NBGT plates with the exception of the samples taken 1 day post-exposure before Isolator M became contaminated with bacillary species. In fact only one isolation of mucosalis was achieved from a CBA plate inoculated with a faecal dilution after bacillary contamination of the infected piglets. This isolation was made 15 days post-exposure from the faeces of piglet M3 on the CBA plate of the 4th dilution (Table 5.5).

The isolations of mucosalis from the mucosal samples at necropsy followed a similar pattern to rectal and oral isolations in that bacillary contamination largely prevented isolation of mucosalis from CBA plates (Table 5.6). Occasional isolations of mucosalis were made from CBA plates at the 3rd or 4th dilution but accurate colony counts were not possible as spreading bacillary growth occurred over most of the rest of the surface area of the plates thus obscuring the true number of mucosalis colonies.

TABLE 5.5

EXPERIMENT 1: FAECAL RECOVERY OF MUCOSALIS; MEDIA AND DILUTIONS FROM WHICH ISOLATIONS MADE.

Days Post-Exposure	PIGLET NUMBER							
	M1 (BA 367/78)		M2 (BA 367/78)		M3 (BA 387/78)		M4 (BA 391/78)	
	CBA	NBGT	CBA	NBGT	CBA	NBGT	CBA	NBGT
1*	2	1	NR	NR	NR	NR	NR	1
3***	NR**	3,2,1	NR	3,2,1	NR	3,2,1	NR	3,2,1
6	NR	4,3,2,1	NR	4,3,2,1	NR	NR	NR	NR
8			NR	3,2,1	NR	1	NR	NR
11			NR	1	NR	4,3,2,1	NR	1
13			NR	NR	NR	3,2,1	NR	2,1
15					4	3,2,1	NR	NR
17					NR	NR	NR	NR
20					NR	2,1	NR	NR
22							NR	NR
24							NR	NR
27							NR	2,1

* On day 1 post-exposure CBA plates were inoculated only from the 2nd, 3rd and 4th dilutions.
*** On day 3 post-exposure NBGT plates were inoculated only from the 1st, 2nd and 3rd dilutions.
** NR = mucosalis not isolated. 1 = isolation from 1st dilution (10^1).
2 = isolation from 2nd dilution (10^2). 3 = isolation from 3rd dilution (10^3).
4 = isolation from 4th dilution (10^4).

TABLE 5.6.

EXPERIMENT 1: MUCOSAL RECOVERY OF MUCOSALIS; MEDIA AND DILUTIONS FROM WHICH ISOLATIONS MADE.

Mucosal Site	PIGLET NUMBER							
	M1 (BA 367/78)		M2 (BA 376/78)		M3 (BA 387/78)		M4 (BA 391/78)	
	CBA	NBGT	CBA	NBGT	CBA	NBGT	CBA	NBGT
Stomach	NR	1	NR	3,2,1	NR	3,2,1	NR	NR
Duodenum	3	2,1	NR	1	NR	2,1	NR	NR
Jejunum	4	3,2,1	NR	3,2,1	NR	3,2,1	NR	1
MSl	NR	3,2,1	NR	1	NR	3,2,1	NR	NR
TSl	4	1	NR	2,1	NR	4,3,2,1	NR	1
LB	NR	4,3,2,1	4	3,2,1	NR	4,3,2,1	NR	1

NR = mucosalis not isolated.
1 = isolation from 1st dilution (20^1)
2 = isolation from 2nd dilution (20^2)
3 = isolation from 3rd dilution (20^3)
4 = isolation from 4th dilution (20^4)

There were samples from which mucosalis was not isolated (Tables 5.2 - 5.3), and most of these samples were from piglet M4. Mucosalis was isolated from 31 of 36 samples (86%), faecal and mucosal, from piglets M1, M2 and M3 but from only 8 of 18 (44%) of samples, faecal and mucosal, from piglet M4. The average numbers of mucosalis isolated from faecal and mucosal samples of piglet M4 were much lower than the average numbers from piglets M1, M2 and M3. Piglet M4 excreted on average 2.2 less logs of mucosalis than piglet M1, M2 and M3 and on average 3.1 logs less were isolated from mucosal sites at necropsy.

The average faecal excretion of mucosalis (all samples, expressed as log 10/g faeces) was 2.3 and the average number of mucosalis isolated from the mucosa at necropsy (all sites, expressed as log 10/g mucosa) was 4.1, suggesting that higher numbers became established in mucosal sites than were excreted in the faeces. Whether the mucosa contained higher numbers of mucosalis than the luminal contents at any particular site is unknown since luminal contents were not cultured for mucosalis. A comparison between the average number of mucosalis isolated from the LB mucosa (expressed as log 10/g) which was 6.2, with the average faecal excretion just before necropsy (Table 5.4) which was 2.8 shows that the ratio between mucosal-associated mucosalis and contents-associated

mucosalis was large, again suggesting that the majority of mucosalis which established were associated with the mucosa. The numbers of mucosalis which established were particularly high in the mucosa of the TS1 and LB (Table 5.3). At these two sites the average numbers (expressed as log 10/g) were 4.5 and 6.2 respectively.

(iii) Pathology

(a) Gross Findings:

Control Piglets G1-G4: Piglets G1, G3 and G4 died during the experiment (vide supra). Piglet G1 had gross lesions indicative of a drenching pneumonia. There were no gross abnormalities of the alimentary tract. Changes were absent from piglets G3 and G4 other than those which might have been brought about by the presumed cause of death, suffocation.

Piglet G2 was killed at 8 days of age. The carcase was anatomically normal, moderately well-nourished and without gross pathological lesions.

Infected Piglets M1-M4: Piglet M1, killed at 7 days of age, was in poor condition. The coat was rough, the perineal region, hocks and tail showed loss of hair, erythema and were stained with soft yellow faecal matter. Other abnormalities were confined to the lower alimentary tract. The caecum and large bowel contained foetid fluid yellow material.

Piglets M2, M3 and M4 killed at 15, 22 and 28 days respectively had no gross abnormalities at necropsy with the exception of M3 which had necrosis of the tip of its tail. There was no gross evidence of intestinal mucosal change in any of the infected piglets.

(b) Histopathology.

Control Piglets G1-G4: The alimentary sites sampled from piglets G1, G3, and G4 showed extensive autolytic changes but no pathological abnormalities. Lung sections from these piglets had changes consistent with a drenching pneumonia (G1) and suffocation (G3 and G4).

The samples from piglet G2 showed no pathological changes and all tissues examined were consistent with those of a healthy gnotobiote. The histological appearance of the tissues differed from conventional animals, the most prominent of these features are described below.

Stomach, Spleen, Liver, Kidney, Lung and Mesenteric Lymph Nodes:

In general these organs conformed to published descriptions of "conventional" tissues except that lymphoid nodules in the spleen and mesenteric lymph nodes were less mature than would have been expected from a conventionally-reared animal. The liver lacked a well-defined lobular structure and the lung was notably devoid of peribronchiolar lymphoid cells.

Small Intestine:

The general architecture was similar to that of conventional piglets (Moon, 1972) except that the muscle layers and connective tissue components were finer and there were fewer cells in the interstitium. As in conventional piglets the main cell types in the interstitium were lymphocytes and eosinophils. The lymphoid follicles of the lower ileum were immature. The crypts were uniformly short and simple, while the villi were up to five times the crypt length. Villi, particularly in the mid and lower small intestine were lined by heavily vacuolated tall columnar epithelial cells. The overall impression was of regularity and of morphology unblemished to an extent rarely observed in conventional animals (Figures 5.8 - 5.10).

Caecum and Spiral Colon:

As in the small intestine the muscle and connective tissue moieties were finer than in conventional gut, and the lymphoid component less prominent. The crypts were uniformly simple, straight and mucus-secreting. The periluminal epithelium was tall columnar and covered a surface which tended to be thrown up in villus-like projections between the neck of the crypts (Figures 5.11 - 5.12).

Infected Piglets M1-M4:

There was no histological evidence of adenomatosis in any of the gut sections examined. In all respects

the tissues examined from the infected piglets closely resembled that of control piglet G2.

Argyrophilic strains (Levaditi and Young's).

Silver stains were used in attempts to visualise bacteria associated with the mucosa.

Control Piglet G2:

No campylobacter-like bacteria (CLO's) were observed in any of the gut sections examined. It was not possible to demonstrate the bacillary contaminants of this piglet using the silver stains.

Infected Piglets M1-M4:

The silver-staining techniques demonstrated the presence of both bacillary and campylobacter-like bacteria in luminal areas throughout the gut. In the small intestine bacterial forms were mostly confined to the luminal debris and were rarely present deeper than the crypt-villus junctions although occasionally a few campylobacter-like organisms were seen in the lumina of basal crypts. In the large intestine both bacillary bodies and CLO's were observed in clumps near the surface of the mucosa and less frequently in the upper and basal crypt lumina. There was no convincing evidence at any site of intracellular parasitism by campylobacters although in every piglet there were occasional cells in basal crypts of either the small or large intestine which contained argyrophilic bodies resembling campylobacters.

(c) Immunofluorescence:

Sections of US1, MS1, TS1 and LB were examined from piglets G2, M1, M2, M3 and M4, in parallel with control sections from adenomatous cases and "gelatin control material" (See Chapter 2). "Gelatin control material" was used as the evidence of the light microscope suggested that most of the mucosalis bacteria were scattered in the luminal debris of the gut and it was thought that organisms prepared in gelatin would be a more suitable control than the densely-packed intracellular organisms found in field cases of PlA. The gelatin control material fluoresced quite well when stained for mucosalis using the immuno-fluorescence technique described in Chapter 2. The organisms were scattered throughout the gelatin sections and appeared as tiny spots of particulate fluorescence or larger brighter foci, probably clumped organisms.

Results of Immunofluorescence:

The success of the "gelatin control material" was not reflected in the results when the cryostat sections from the piglets were examined. Sections from control and infected piglets appeared similar. All sections displayed scattered particulate fluorescence in the luminal areas around villi, even the sections from piglet G2, uninfected with mucosalis. Hence it was not possible to attribute the scattered luminal fluorescence in piglets M1-M4 to the presence of mucosalis at this site.

None of the sections examined showed the bright particulate intracellular fluorescence of the adenomatous glands in control field cases and in all respects the sections examined from uninfected piglet G2 did not differ from infected piglets M1, M2, M3 and M4.

(d) Electron Microscopy.

Tissues from piglets G2, M1, M2, M3 and M4 were examined by electron microscopy.

Piglet G2 US1, MS1, TS1 and LB.

In general the tissues examined did not differ from published descriptions of the neonatal pig (Kenworthy, Stubbs and Syme, 1967; Moon, 1972) or other species (Pink, 1970; Toner, Carr and Wyburn, 1971; Threadgold, 1976; Ghadially 1977), except for the absence of Paneth's cells in the pig.

Featuring prominently in the small intestinal villar enterocytes were the cytoplasmic vacuoles observed by light microscopy. Ultrastructurally it appeared that small invaginations of the apical cytoplasmic membrane were pinched off to become intracytoplasmic vesicles and that fusion of these vesicles resulted in large cytoplasmic vacuoles (Figures 5.13 and 5.14). Occasionally apical vacuoles contained aggregations of coated vesicles and resembled multivesicular bodies (Ghadially, 1977) (Figure 5.15).

There was no evidence of close bacterial

association with the mucosa and the bacillary contaminants were not observed at any site examined.

Piglets M1, M2, M3 and M4:

The following tissues were examined from the infected piglets:

M1 - US1, TS1

M2 - US1, site 2, MS1, TS1 and LB

M3 - US1, site 2, TS1

M4 - US1, site 2, MS1, TS1 and Caec.

The tissues examined did not differ markedly from the tissues of control piglet G2. There was no evidence of the presence of bacteria resembling either campylobacters or bacilli in any close association with the mucosa and no forms recognisable as bacteria were found in intracellular situations.

RESULTS: EXPERIMENT 2.

(i) Clinical Findings/Daily Observations.

The piglets at birth were anatomically normal but of low average weight (Appendix 5.3). Navel-sucking was frequently observed during the first week. All piglets remained healthy and vigorous until 4 days of age. Between 4 and 9 days of age all piglets

except numbers 10, 11 and 12 experienced a mild diarrhoeic episode and passed fluid faeces, although all piglets maintained enthusiasm for bottle-feeding (Table 5.7). Piglet 11 gradually weakened from 4-8 days of age due to loss of blood from the umbilicus. Exposure to mucosalis at 7 days of age was not associated with signs of ill-health in any piglet. Within 24 hours of rotavirus exposure at 14 days, all the rotavirus-exposed piglets began to pass copious fluid faeces, and piglet 9 was observed to vomit. There was rapid weight loss accompanied by depression and inappetence (Figures 5.16 - 5.17, Appendix 5.4). Piglets 2 and 6 died 2 days after rotavirus infection and piglet 12 was moribund when killed at 19 days of age. The remaining rotavirus-infected piglets made a gradual recovery although never becoming as heavy or vigorous as littermates not exposed to rotavirus (Figures 5.16 - 5.17). The strength and vigour of the piglets exposed only to mucosalis are demonstrated by piglet 10 which had to be killed at 46 days of age as the isolator was not strong enough to contain the animal. Piglets which recovered from the acute diarrhoeic episode due to rotavirus became stunted in appearance, pot-bellied (Numbers 5 and 8) and were observed on occasions to retch after feeding (Numbers 9 and 8) (Table 5.7).

Piglets exposed to mucosalis only remained healthy throughout except for number 11 which was killed after

TABLE 5.7.

EXPERIMENT 2: EXPERIMENTAL PROCEDURES, CLINICAL SIGNS AND AGE AT DEATH.

Piglet Number	Age-related clinical signs Pre-exposure/s	Age-related Experimental Exposures	Age-related clinical signs Post-exposures	Age at death (days)
11 (BA 408/79)	Umbilical bleeding from 4 - 8 days	Oral *mucosalis* at 7 days	Further blood loss	8 (K)*
3 (BA 416/79)	Diarrhoea from 5 - 7 days	Oral *mucosalis* at 7 days	Diarrhoea at 18 days Lame from 16-20 days	20 (K)
1 (BA 431/79)	None	Oral *mucosalis* at 7 days	None	34 (K)
7 (BA 441/79)	Diarrhoea from 5 - 7 days	Oral *mucosalis* at 7 days	None	41 (K)
10 (BA 451/79)	None	Oral *mucosalis* at 7 days	None	46 (K)
2 and 6 (BA 413/79)	Diarrhoea from 4-6 days	Oral *mucosalis* at 7 days Oral rotavirus at 14 days	Diarrhoea, inappetence and weight loss from 15-16 days. Perineal erythema. Death at 16 days	16 (D)**
12 (BA 414/79)	None	Oral *mucosalis* at 7 days Oral rotavirus at 14 days	Diarrhoea, inappetence and weight loss from 15 - 19 days	19 (K)

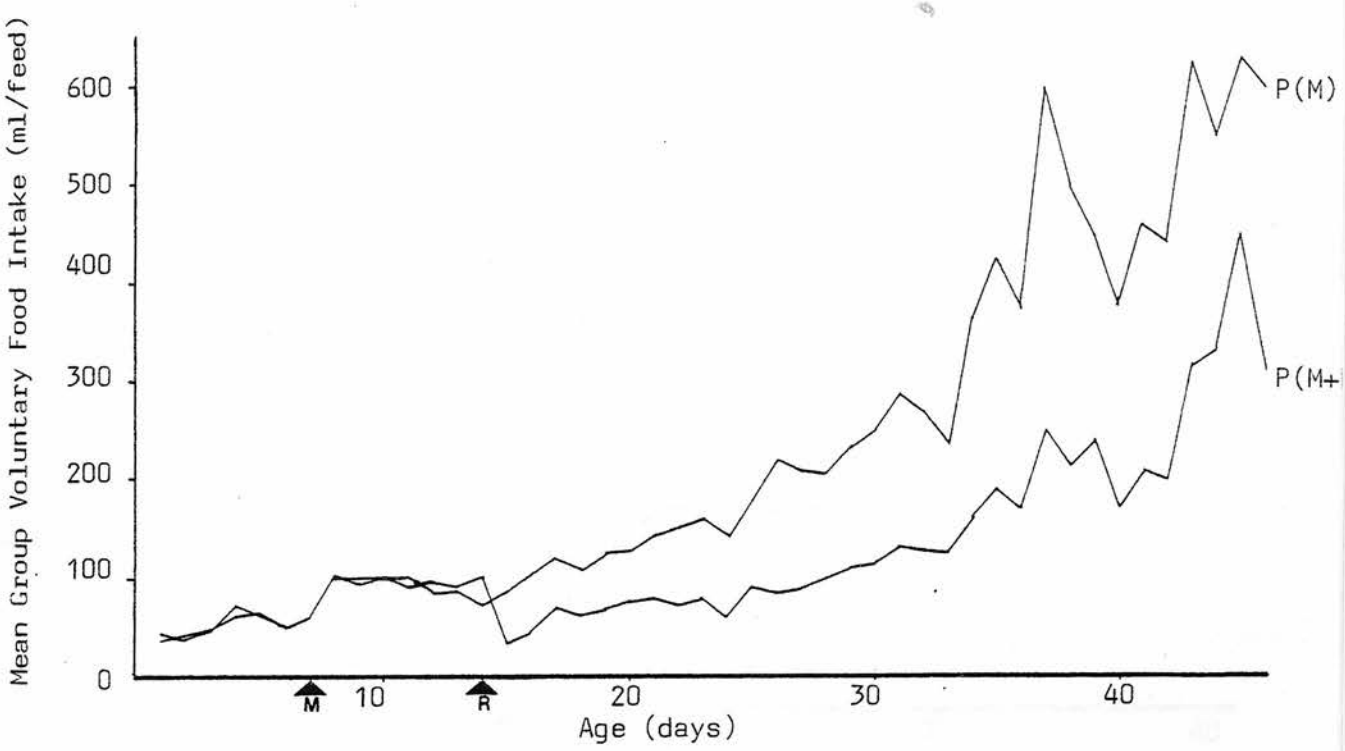
Table continued overleaf/...

TABLE 5.7
(CONTINUED)

Piglet Number	Age-related clinical signs Pre-exposure/s	Age-related Experimental Exposures	Age-related clinical signs Post-exposures	Age at death (days)
4 (BA 435/79)	Diarrhoea from 4 - 7 days	Oral mucosalis at 7 days Oral rotavirus at 14 days	Diarrhoea, inappetence and weight loss from 15 - 19 days	35 (K)
9 (BA 445/79)	Diarrhoea from 5 - 9 days	Oral mucosalis at 7 days Oral rotavirus at 14 days	Vomiting at 15 days. Diarrhoea, inappetence and weight loss from 15 - 17 days. Retching after feeding at 36 days	43 (K)
5 (BA 457/79)	Diarrhoea from 4 - 7 days	Oral mucosalis at 7 days Oral rotavirus at 14 days	Diarrhoea, inappetence and weight loss from 15-19 days. Pot-belly from 25-48 days	48 (K)
8 (BA 467/79)	Diarrhoea from 5 - 6 days	Oral mucosalis at 7 days Oral rotavirus at 14 days	Diarrhoea, inappetence and weight loss from 15 - 19 days. Retching after feeding at 46 days. Pot-belly from 30-54 days	54 (K)

* K = piglet killed ** D = piglet died

Figure 5.16: Experiment 2: Effect of Exposure to Rotavirus on Voluntary Food Intake.



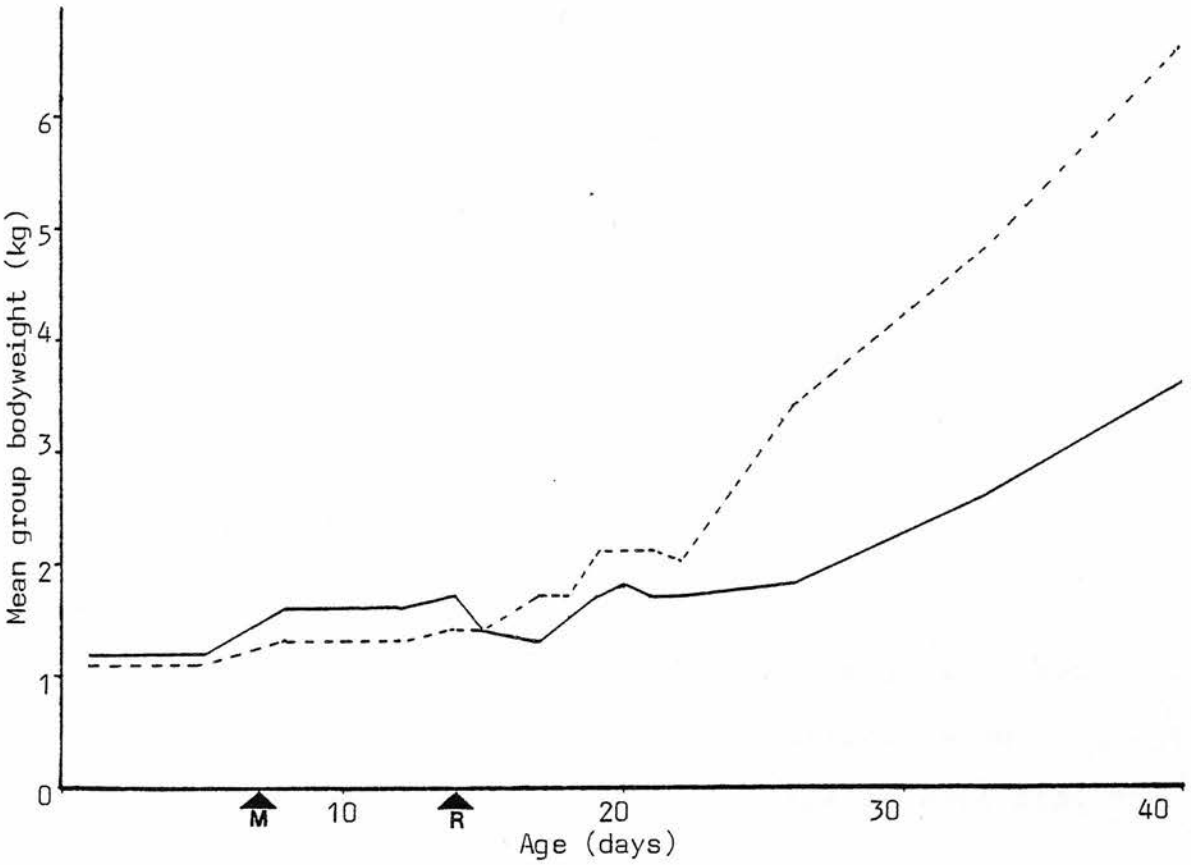
M = oral mucosalis

R = oral rotavirus

P(M) = piglets exposed to mucosalis only

P(M+R) = piglets exposed to mucosalis and rotavirus

Figure 5.17: Experiment 2: Effect of Exposure to Rotavirus on Bodyweight.



M = oral exposure to mucosalis

R = oral exposure to rotavirus

——— = piglets exposed to mucosalis and rotavirus

----- = piglets exposed to mucosalis only

prolonged navel bleeding, and number 3 which became lame and was killed when the lameness did not improve.

At 49 days of age piglet 8, the sole surviving piglet escaped from its isolator by forcing open the metal lid, jumping down a sleeve and tearing its way out. The piglet was caught and placed in Isolator MC until it was killed a few days later.

(ii) Bacteriology and Virology.

(a) Isolator Contaminants:

Isolator MC containing piglets 1, 3, 7 and 10 became contaminated with a streptococcus, detected when the piglets were 14 days old. No other contaminants were detected.

Isolator RMA containing piglets 2, 4, 5 and 6 became contaminated with a yeast, detected when surviving piglets (Numbers 4 and 5) were 26 days old. No other contaminants were detected.

Isolator RMB containing piglets 8, 9, 11 and 12 became contaminated with a bacillus (*Bacillus coagulans*-like), detected when surviving piglets (Numbers 8, 9 and 12) were 11 days old. Piglet 8 escaped from the isolator when 49 days old and was considered to be multiply contaminated thereafter. No attempt was made to identify these later contaminants.

(b) Isolations of Mucosalis:

The numbers of *mucosalis* isolated and the sites are summarised in Figure 5.18 and Tables 5.8-5.9.

FIGURE 5.18: Experiment 2: Isolations of Mucosalis from the oral cavities of Piglets 1–12

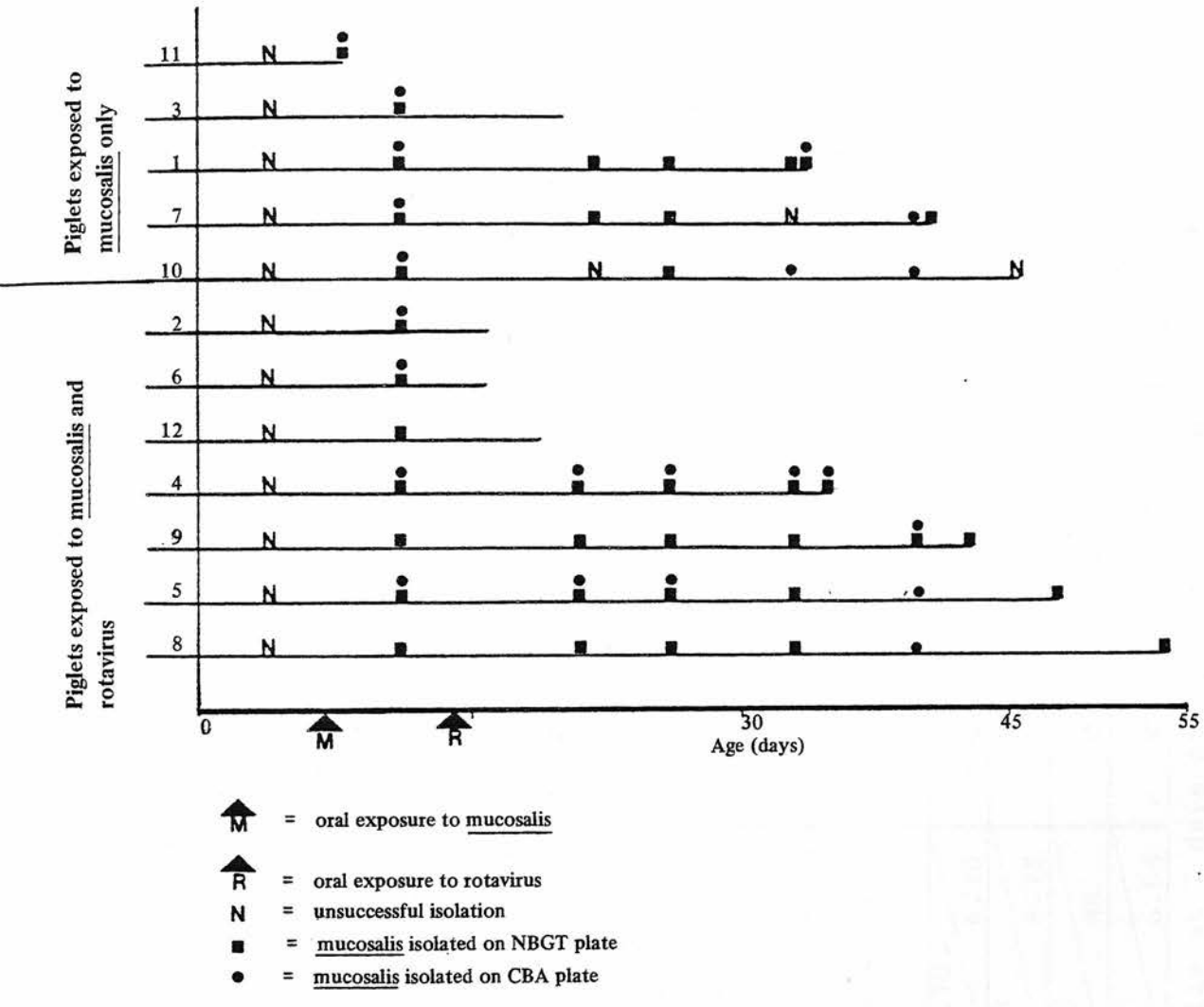


TABLE 5.8.

EXPERIMENT 2: ESTIMATES OF NUMBERS OF MUCOSALIS IN FAECES (log 10/g).

Piglet Number	AGE (DAYS) *** 11	21	22	26	33	40
	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT	CBA / NBGT
3 (BA 416/79)	5.48 / 1.70					
1 (BA 431/79)	5.00 / 4.70	ND*	6.18 / 7.00	NR / 4.70	NR / 4.70	
7 (BA 441/79)	6.40 / 4.00	ND	NR / NR	NR / 2.40	NR / NR	NR / 4.78
10 (BA 451/79)	4.00 / NR**	ND	NR / NR	NR / NR	NR / 2.70	4.70 / 3.70
2 (BA 413/79)	6.75 / 4.70					
6 (BA 413/79)	6.40 / NR					
12 (BA 414/79)	4.70 / 3.81					
4 (BA 435/79)	5.81 / NR	4.70 / 4.70	ND	6.00 / 6.02	4.70 / 3.30	
9 (BA 445/79)	6.40 / 4.70	NR / 6.78	ND	6.00 / 5.95	4.70 / 6.13	6.00 / 6.70
5 (BA 457/79	6.31 / NR	NR / NR	ND	6.00 / 5.28	4.70 / NR	5.90 / NR
8 (BA 467/79)	4.70 / 3.60	NR / 6.06	ND	6.00 / 6.40	NR / 5.70	7.00 / 5.70

*** Piglets exposed to mucosalis at 7 days of age. **NR = mucosalis not isolated.
* ND = piglet not sampled.

TABLE 5.9.

EXPERIMENT 2: ESTIMATES OF NUMBERS OF MUCOSALIS IN MUCOSAL SAMPLES AT NECROPSY (log 10/g).

Piglet Number	US1 CBA / NBGT	MS1 CBA / NBGT	TS1 CBA / NBGT	CAEC CBA / NBGT	LB CBA / NBGT
11 (BA 408/79)	5.20 / 4.90	6.20 / 6.20	6.68 / 6.68	ND*	7.35 / 6.98
3*** (BA 416/79)	NR** / NR	NR / NR	NR / NR	NR / NR	NR / NR
1 (BA 431/79)	NR / 4.08	NR / 4.68	NR / NR	NR / 6.81	ND
7 (BA 441/79)	NR / NR	NR / 3.60	NR / 3.00	NR / 5.68	ND
10 (BA 451/79)	NR / 2.90	NR / 2.30	NR / 2.30	7.50 / 3.20	ND
2 (BA 413/79)	6.50 / 5.38	6.50 / 6.28	6.50 / 6.50	ND	6.20 / 6.11
6 (BA 413/79)	5.50 / 4.90	6.68 / 6.20	3.90 / 3.26	ND	7.11 / 6.81
12 (BA 414/79)	6.81 / 2.78	4.90 / 4.90	4.81 / 5.20	ND	4.30 / 6.50
4 (BA 435/79)	NR / 3.90	NR / 4.08	6.20 / 4.90	6.20 / 6.50	ND
9 (BA 445/79)	NR / 3.08	4.90 / 3.90	NR / NR	6.32 / 6.33	6.68 / 6.20
5 (BA 457/79)	NR / NR	NR / 2.30	4.90 / 3.90	5.68 / 6.20	ND
8 (BA 467/79)	NR / 2.60	NR / NR	NR / NR	NR / NR	ND

Table continued overleaf/...

TABLE 5.9.
(CONTINUED)

* ND = site not sampled

** NR = *mucosalis* not isolated

*** Piglet 3 - plates left at room temperature and atmosphere overnight

N.B. Piglets 11, 2 and 6 uncontaminated

Piglets 3, 1, 7 and 10 contaminated with a streptococcus

Piglets 12 and 9 contaminated with a bacillus

Piglets 4 and 5 contaminated with a yeast

Piglet 8 multiply contaminated

Mucosalis was not isolated from samples of lung, liver or spleen except for the spleen of piglet 4 from which mucosalis was isolated. Excluding these viscera mucosalis was recovered from the majority of post-exposure samples throughout the experiment; from 42/45 oral swabs (93%), 32/37 faecal swabs (86%) and, excluding piglet 3, 38/45 (84%) of mucosal samples. Mucosalis was not isolated from piglet 3 at necropsy but this may have been due to failure to incubate the samples until the day after necropsy.

As in Experiment 1 isolator contaminants when they occurred reduced the effectiveness of CBA plates. Piglets 11, 2 and 6 were killed before contamination of their isolators occurred and in these piglets CBA plates were significantly better at isolating mucosalis than NBGT plates (Appendix 5.5), behaving largely as expected from the in vitro tests performed on plates from the same batches (Appendix 5.6). For contaminated piglets overall NBGT plates were significantly better than CBA plates for the isolation of mucosalis from mucosal samples at necropsy (Appendix 5.7). Columbia blood agar plates were significantly more successful than NBGT plates in the faecal recovery of mucosalis from uncontaminated piglets but there was no significant difference between CBA and NBGT plates in contaminated piglets overall (Appendices 5.8-5.9). Taking the contaminating species individually, in piglets with streptococci, NBGT plates were significantly better

than CBA plates, both for faecal mucosalis and mucosal mucosalis (Appendices 5.10 - 5.11).

For yeast and bacillary contaminants there was no significant difference between CBA and NBGT plates in their ability to isolate mucosalis (Appendices 5.12 - 5.15).

There was no significant difference in the numbers of mucosalis isolated at necropsy from piglet 11 (uncontaminated and not exposed to rotavirus) and those isolated from piglets 2 and 6 (uncontaminated and exposed to rotavirus) (Appendix 5.16).

The presence of bacterial contaminants in other piglets prevented accurate assessment of the numbers of mucosalis, and in any case may have influenced the numbers in vivo, and so, comparing only successful or unsuccessful isolations of mucosalis it was found that there was no significant difference in the number of positive sites at necropsy or the number of positive faecal samples between the group exposed to mucosalis only and the group exposed to mucosalis and rotavirus (Appendices 5.17 - 5.18).

Comparing piglets exposed only to mucosalis in Experiments 1 and 2, there was no significant difference in the number of successful isolations at necropsy or from faecal swabs (Appendices 5.19 - 5.20).

(c) Detection of Rotavirus (Table 5.10).

No rotavirus was found in piglets not exposed to rotavirus. Rotavirus was detected in every piglet

TABLE 5.10

EXPERIMENT 2: DETECTION OF ROTAVIRUS IN FAECES BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

Piglet Number	DAYS OF AGE 14	15	16	17	18	19	20	21	22
3 (BA 416/79)	-*	-	-	-	-	-	-		
1 (BA 431/79)	-	-	-	-	-	-	-	-	-
7 (BA 441/79)	-	-	-	-	-	-	-	-	-
10 (BA 451/79)	-	-	-	-	-	-	-	-	-
2 (BA 413/79)	-	+**	-						
6 (BA 413/79)	-	-	+						
12 (BA 414/79)	-	-	-	+	+	-			
4 (BA 435/79)	-	+	-	+	-	-	-	-	-
9 (BA 445/79)	-	-	+	+	+	+	+	-	-
5 (BA 457/79)	-	+	-	-	-	-	-	+	-
8 (BA 467/79)	-	-	+	-	+	+	-	-	-

* - = rotavirus not detected. ** + = rotavirus detected.

exposed, the last positive faecal sample being 7 days post-exposure.

(iii) Pathology

(a) Gross Findings:

I Piglets Exposed to Mucosalis Only: (Numbers 11, 3, 1, 7 and 10). There were no gross abnormalities relating to the alimentary canal in any of these piglets. The stomachs generally contained clotted milk, the small intestines normal fluid digesta, and the large bowel soft yellowish faeces. The lymphatics of the upper small intestine were easily seen as they contained white milk fat. The mucosal surfaces throughout were normal, having a pink velvety appearance particularly in the small intestine. There was no evidence of mucosal thickening at any site.

The carcase of piglet 11, which was killed after prolonged navel bleeding, was extremely pale and emaciated but not dehydrated. Piglets 3, 1, 7 and 10 had small uncomplicated umbilical hernias, possibly a result of the navel sucking of the first week. Piglet 3 had a swollen left fore coronet.

II Piglets Exposed to Mucosalis and Rotavirus: (Numbers 2, 6, 12, 4, 9, 5 and 8).

Piglets 2, 6 and 12: Gross abnormalities in these three piglets were consistent with a severe diarrhoeic episode. The carcases were emaciated and dehydrated. There was scalding of the perineal areas.

The serosal surfaces of the alimentary tracts were dry and tacky. The stomachs were empty (numbers 2 and 6) or contained a small amount of clotted milk (number 12). The small and large intestines contained abnormally fluid digesta and yellow foetid clots. The mucosal surfaces distal to the duodenum were thin and yellow. Piglet 12 had a small uncomplicated umbilical hernia.

Piglet 4: The carcase was that of a poorly grown animal. There were no gross abnormalities detected in the alimentary tract except that the contents of the large bowel were particularly fluid. There was a small uncomplicated umbilical hernia.

Piglet 9: The carcase was that of a poorly grown animal. Gross abnormalities were restricted to the alimentary tract. The serosal surface of the caecum and large bowel was reddened and oedematous, and on opening these organs the wall was seen to be thickened and the mucosa dotted with several intensely reddened plaques (3-4mm in diameter), some of which had necrotic centres. On close inspection the increase in thickness of the wall was seen to be due to submucosal oedema (Figures 5.19 - 5.20).

Piglets 5 and 8: Both carcases were poorly grown and pot-bellied. There were no gross abnormalities associated with the alimentary tracts. The lungs of piglet 8 had small scattered foci (3-4mm diameter) which were intensely reddened and firm in consistency.

(b) Histopathology.

I Piglets Exposed to Mucosalis Only:

(Numbers 11, 3, 1, 7 and 10; necropsied between 8 and 46 days of age).

H & E Stains: The tissues examined fell within the bounds of normality for gnotobiotic piglets. There was no evidence in the alimentary canal of adenomatous change. In older piglets (numbers 7 and 10) there was some slight reduction in the height of villi particularly in the terminal ileum, a reduction in the vacuolation of villar enterocytes and a slight increase in the number of cells in the lamina propria. Although much reduced, vacuolation of enterocytes at the tips of villi in the terminal ileum was present even in piglet 10, killed at 46 days of age (Figure 5.21).

Silver Stains: (Piglets 11, 3, 1, 7 and 10).

(a) Tongue, Tonsils, Buccal Mucosa and Gingival Margins: Silver-stained organisms many of which resembled campylobacters (CLO's) were frequently observed close to these mucosal surfaces and occasionally in tonsillar crypts. (Table 5.11). No bacteria appeared to be intracellular, although both streptococcal-like and campylobacter-like forms sometimes were present deep in the tonsillar crypts and were observed occasionally in the lumen of buccal gland ducts.

TABLE 5.11

EXPERIMENT 2: SILVER-STAINS OF ORAL SITES: OBSERVATIONS OF CAMPYLOBACTER-LIKE (CLO'S) OR OTHER BACTERIA.

Piglet Number	TONGUE		TONSILS		BUCCAL MUCOSA		GINGIVAL MARGIN	
	CLO'S	OTHERS	CLO'S	OTHERS	CLO'S	OTHERS	CLO'S	OTHERS
11	+	-	+	-	+	-	+	-
3	+	S	+	S	-	-	ND	ND
1	+	-	ND	ND	+	-	ND	ND
7	+	S	-	-	ND	ND	-	-
10	+	S	-	-	+	-	+	-
12	+	-	+	-	ND	ND	ND	ND
4	ND	ND	ND	ND	+	-	+	-
9	+	-	ND	ND	+	B	ND	ND
5	+	-	ND	ND	-	-	+	-
8	+	M	+	M	+	M	+	-

+ = campylobacter-like organisms observed. S = streptococcal forms observed.

B = bacillary-like forms observed. M = mixed bacterial forms observed.

- = bacteria not observed. ND = tissue not examined.

(b) Alimentary Sites (Numbers 11, 3, 1, 7 and 10).

Piglet 11: (Uncontaminated; killed within 24 hours of exposure to mucosalis).

The numbers of CLO's observed were small and there was no evidence at any site of widespread intracellular parasitism or adherence of bacteria to enterocytes. No adenomatous glands were observed and all crypts were of uniform size and shape and normal in appearance. In the small intestine CLO's were observed in scattered groups of 3 or 4, sometimes apparently adherent to villar enterocytes but more often free in the inter-villous spaces. Where luminal contents were not lost from the section in preparation CLO's appeared among the debris in larger groups. CLO's were infrequently observed below crypt-villus junctions but occasional crypt lumina of the TS1 contained aggregations of CLO's. On rare occasions isolated CLO's were observed in the cytoplasm of crypt epithelial cells.

In the large bowel the crypt lumina were largely free of bacteria. CLO's were observed in small numbers either closely associated with the mucosal surface or free in the lumen. There was no indication that any of the observed bacteria were intracellular.

Piglets 3, 1, 7 and 10 (contaminated with streptococci; necropsied between 13 and 39 days post-mucosalis-exposure). In these piglets there was no

evidence of intracellular parasitism by bacteria at any site. Both CLO's and streptococcal forms were observed at all the sites examined. In the small intestine these bacteria were not present below crypt-villus junctions and were mostly confined to inter-villous spaces and luminal contents, only occasionally were bacteria observed in close association with the surface of villar enterocytes.

In the caecum and large intestine bacteria were more numerous than in the small intestine. Both streptococci and CLO's were closely adherent to the surface of the mucosa, and were also found deep within the lumina of crypt glands. No intracellular bacteria were observed.

II Piglets Exposed to Mucosalis and Rotavirus. (Numbers 2, 12, 4, 9, 5 and 8; necropsied between 16 and 54 days of age).

H & E Stains (Alimentary Sites):

Piglets 2 and 12 (necropsied at 16 and 19 days of age respectively).

The abnormalities observed were consistent with rotavirus infection and were more severe in piglet 2 than in piglet 12. The changes were largely restricted to the small intestine and included severe, extensive stunting and fusion of villi; congestion of the mucosal surfaces and subepithelial aggregations of neutrophils, macrophages and necrotic debris. The surface enterocytes throughout were flattened or

cuboidal and in many areas did not form a continuous sheet over the villous remnants thus leaving the lamina propria exposed.

The crypts throughout appeared depleted of goblet cells and in the MS1 and TS1 were sometimes distended with inflammatory cells and eosinophilic debris. In the large intestine the surface epithelium was largely intact and columnar in shape although there were occasional subepithelial accumulations of pyknotic debris. The large intestinal mucosa as a whole appeared thinner and less well-architectured than that of the comparable control piglet 3.

In piglet 12 there were some areas of the US1 resembling those described for piglet 2 but otherwise the changes were of a less severe nature. The villi throughout the small intestine were attenuated but fusions between villi were less common than in piglet 2. There was almost complete absence of vacuolation of villous enterocytes in the MS1 and TS1, with increased cellularity of the lamina propria, the predominant cell types being lymphocytes, eosinophils and neutrophils. The crypts of the small intestine contained abundant goblet cells and were moderately hyperplastic throughout. The caecum and spiral colon were without abnormality.

Piglet 4 (necropsied at 35 days of age).

The tissues were broadly similar to those of the

piglets not exposed to rotavirus, except for an absence of vacuoles within villar enterocytes, increased cellularity of the lamina propria and the presence of a few areas in the submucosa of the TSl which featured mild dilation of lymphatics and aggregations of proliferating granulation tissue. The caecum and spiral colon were unremarkable. There was no evidence of adenomatous change at any site.

Piglet 9 (necropsied at 43 days of age)

Sections of the small intestine were without abnormality and similar to those of control piglet 7. In the caecum and spiral colon the mucosal glands appeared normal but were surrounded by an oedematous lamina propria and surmounted a vastly oedematous submucosa, containing greatly dilated lymphatics and a cellular infiltrate of eosinophils, macrophages, fibroblasts, lymphocytes and plasma cells. In the spiral colon large cyst-like structures were present in both the submucosa and mucosa. There was a moderate degree of vasculitis of the submucosal vessels (Figures 5.22 - 5.24) and substantial subserosal oedema. There was no evidence at any site of adenomatous change.

Piglets 5 and 8 (necropsied at 48 and 54 days of age respectively).

The small intestinal sites examined did not differ markedly from those of control piglet 10 except that vacuoles were absent from villar enterocytes of piglets 5 and 8. In the caecal and spiral colon sites were

changes similar to but milder than those observed in piglet 9. There were areas of substantial submucosal oedema and poorly organised granulation tissue containing scattered aggregates of eosinophils, lymphocytes and macrophages. Dilated lymphatics and inflamed capillaries also featured in these areas. The serosal linings were thickened with fibrous tissue but the crypt glands were largely normal and presented no evidence of adenomatous change.

Silver stains (Piglets 2, 12, 4, 9, 5 and 8; contaminated with yeasts [Numbers 4 and 5] and bacilli [Numbers 12, 9 and 8]).

(a) Tongue, Tonsils, Buccal Mucosa and Gingival Margins:

CLO's were observed close to the surface of these tissues, largely as described for piglets not exposed to rotavirus (Table 5.11). No bacteria were observed in intracellular sites.

(b) Alimentary Sites:

There was no evidence at any site of widespread intracellular parasitism by CLO's. CLO's had a similar distribution to the contaminating organisms i.e. they were restricted to the lumina of sections, were rarely present in small intestinal crypts, had no greater association with the surface of enterocytes than other bacteria, and in the caecum and spiral colon were frequently adherent to the mucosal surface and could be

observed deep in the crypt lumina. The number and distribution of CLO's did not differ markedly from the equivalent areas of pigs not exposed to rotavirus.

Other tissues examined: (H & E stains; piglets 12, 4, 9, 5 and 8):

Examination of liver, lung, spleen, kidney and mesenteric lymph nodes did not reveal the presence of abnormalities apart from the lung of piglet 8 and the mesenteric lymph nodes of piglet 9.

The reddened foci observed at necropsy in the lungs of piglet 8 were found to consist of dense aggregations of red blood cells and neutrophil polymorphs.

The mesenteric lymph nodes of piglet 9 were composed of hyperplastic lymphoid follicles in which there were increased numbers of apoptotic cells and scattered aggregations of necrotic debris. There were increased numbers of eosinophils, neutrophils, macrophages and plasma cells in peripheral areas and cuffing of blood vessels with mature lymphocytes.

(c) Immunofluorescence

(i) Detection of Mucosalis by Immunofluorescence.

Tissues Examined:

Piglet	Sites Examined
11	Gingival Margin (GM), Tonsil, USl, MSl, LB.
3	GM, USl, MSl, Caec, LB.
1	GM, Tongue, Tonsil, USl, MSl, TSl, Caec, LB.
7	GM, Tongue, USl, MSl, TSl, Caec, LB.
10	Tonsil, USl, MSl, Caec, LB.
12	USl, MSl, TSl, LB.
4	GM, Tonsil, USl, TSl, LB.
9	GM, Tongue, USl, MSl, TSl, Caec, LB.
5	Tonsil, TSl, Caec, LB.
8	GM, Tonsil, USl, MSl, TSl, Caec, LB.

There was no evidence at any site of the presence of intracellular mucosalis. Occasional scattered particulate fluorescence was observed in the intervillous spaces of small intestinal sites, on the surface and in gland lumina of the caecal and spiral colon sites or close to the surface of the oral mucosae (Table 5.13).

The positive gelatin controls showed scattered particulate fluorescence throughout.

(ii) Detection of IgA by Immunfluorescence and Correlation with Distribution of Mucosalis.

Sections of Tonsil, TSl, Caec and LB were examined from all the piglets except numbers 2 and 6. With the exception of piglets 11 and 12 where little or no IgA was detected, the distribution of IgA was closely

TABLE 5.12

EXPERIMENT 2: INDIRECT IMMUNOFLUORESCENCE TEST TO DETECT ROTAVIRUS ANTIBODIES.

Piglet Number	SERUM DILUTIONS							
	$^1/_{10}$	$^1/_{20}$	$^1/_{40}$	$^1/_{80}$	$^1/_{160}$	$^1/_{320}$	$^1/_{640}$	$^1/_{1280}$
7 (a)*	-***	-	-	-	-	-	-	-
5 (a)	+****	+	+	+	+	+	-	-
8 (a)	+	+	+	+	+	+	-	-
7 (b)**	-	-	-	-	-	-	-	-
5 (b)	+	+	+	+	+	-	-	-
8 (b)	+	+	+	+	+	+	-	-

* (a) = rotavirus grown in VERO cells
** (b) = rotavirus grown in BEK cells
*** - = fluorescence not detected
**** + = fluorescence detected.

TABLE 5.13

EXPERIMENT 2: DETECTION OF MUCOSALIS BY IMMUNOFLUORESCENCE.

Piglet Number	GM*	Tongue	Tonsil	USl	MSl	TSl	CAECUM		LB	
							Surface	Crypts	Surface	Crypts
11	-***	ND****	-	-	-	+**	ND	ND	-	-
3	+	ND	ND	+	+	-	+	+	-	-
1	-	+	-	+	-	+	+	+	-	-
7	-	+	ND	-	-	+	+	+	+	+
10	ND	ND	-	-	+	ND	+	+	+	+
12	ND	ND	ND	-	-	+	ND	ND	+	-
4	-	ND	-	+	ND	+	ND	ND	+	+
9	-	+	ND	-	-	-	+	-	+	+
5	ND	ND	-	+	+	-	+	+	+	+
8	+	ND	-	+	+	-	+	+	+	+

** + = a small amount of extracellular scattered particulate fluorescence

*** - = no fluorescence

**** ND = not examined

*GM = gingival margin.

similar to that of conventional pigs, i.e. positive fluorescence was detected in the lymphoid follicles of the tonsils and the TSl, in plasma cells in the lamina propria of gut tissues and to a lesser extent in the apical cytoplasm of cells at the neck of crypts of the TSl, Caec and LB. Plasma cells in the lamina propria were easily recognised by their cytoplasmic fluorescence (Allen, Smith and Porter 1973, 1976).

There was no obvious correlation between the distribution of IgA and the distribution of mucosalis when the fluorescence results were compared.

(d) Electron Microscopy:

A selection of tissues from piglets 11, 3, 1, 10 (exposed to mucosalis only) and piglets 12, 4, 9 and 8 (exposed to mucosalis and rotavirus) were examined ultrastructurally. The results are summarised below:-

Piglet 11 TSl and Caecum:

Ultrastructurally the tissues did not differ markedly from those of piglet G2 (Control Gnotobiote, Experiment 1, not exposed to mucosalis). Mucosalis organisms were rarely observed, and were not found in the cytoplasm of enterocytes (Figures 5.25 and 5.26). Occasional mucosalis were observed in the intervillous spaces of the TSl but showing no close association with the microvilli of host cells (Figure 5.27). Tuft cells (Lee and Toner, 1980) were particularly prominent on the villi of the TSl (Figures 5.28 - 5.30). One tuft

cell contained a supranuclear polyphagolysosome whose contents resembled the debris surrounding luminal mucosalis bacteria (Figure 5.31). Although neighbouring cells contained a variety of apical bodies, which may have represented secondary lysosomal digestion of mucosalis no intracytoplasmic bodies recognisable as mucosalis were observed (Figure 5.31). The apical tubular system was prominent and active among villar enterocytes (Figure 5.32).

Particular attention was paid to the cytoplasm of crypt enterocytes but although a variety of bodies were observed some of which may have represented degenerate forms of mucosalis (Figure 5.33) and others which resembled apical granules (Figure 5.34), no bodies were firmly recognisable as mucosalis.

In the lamina propria at the base of one group of crypt glands there were a number of bacterial forms indistinguishable ultrastructurally from mucosalis (Figure 5.35 - 5.38). They appeared to lie free in the intercellular spaces and showed no signs of degenerative change. These bacteria were not observed associated with phagocytic vacuoles although the area was rich in granulocytic leucocytes. Despite the presence of mucosalis in the lamina propria neighbouring crypt enterocytes were apparently free of bacteria (Figure 5.34).

Further examination of mucosal samples from the TS1 did not result in more observations of mucosalis

although membranous debris in the intervillous spaces may have represented degenerate forms.

Piglet No. 3 (TS1 and Caecum).

Piglet No. 1 (LB).

Piglet No. 10 (TS1).

The above tissues did not exhibit ultrastructural abnormalities and were in all respects typical of young pigs of good health status. There was no evidence of intracellular parasitism by mucosalis. Bacteria with the ultrastructural morphology of mucosalis were not observed at any site except occasionally in debris associated with the mucosal surface of the LB of piglet 1 (Figures 5.39 and 5.40).

Piglet No. 12 (MS1 and TS1).

Ultrastructurally there was evidence that cells lining the villi were less mature than piglets not exposed to rotavirus. There were large numbers of free ribosomes, numberous apical granules, an irregular microvillous border and a poorly developed apical tubular system (Figure 5.41). In short the villar cells resembled undifferentiated crypt cells. The crypt areas appeared relatively normal although the tissues were more susceptible to artefactual changes such as fissuring of the cytoplasm and disruption of mitochondria. There was also evidence of increased apoptotic activity (Figures 5.42 and 5.43).

No bacteria were observed at any site, nor were

any viral bodies observed.

Piglet No. 4 (MS1 and TS1).

Ultrastructurally the tissues appeared relatively normal. No bacterial or viral forms were observed at any site.

The villar enterocytes appeared mature in every respect but did not have a well-developed apical tubular system (still present in piglet 1, not exposed to rotavirus and killed within 24 hours of piglet 4). The lamina propria surrounding crypt glands was well-populated with a variety of cells among which neutrophils, macrophages and plasma cells were noted. There was no evidence of intracellular parasitism by mucosalis.

Piglet No. 9 (Caecum and LB).

Piglet No. 8 (Caecum and LB).

The mucosal glands appeared relatively normal apart from minor irregularities of the microvilli and slight goblet cell hyperplasia. Both the surrounding lamina propria and submucosal tissues were characterised by oedema, increased amounts of poorly-organised collagen fibrils and a cellular infiltrate in which granular leucocytes, lymphocytes, plasma cells and fibroblasts were prominent. There was no evidence of viral infection or of intracellular parasitism by mucosalis. The surfaces of the mucosal sites of piglet 8 were associated with a mixed bacterial flora

among which CLO's were not prominent (Figure 5.44).

(iv) Serology

Detection of Mucosalis Antibodies:

Sera taken at necropsy from piglets 1, 3, 7, 10 (exposed to mucosalis-only) and piglets 4, 5, 8 and 9 (exposed to mucosalis and rotavirus) were examined by the tube agglutination test (See Chapter 2) using strain 1075/78 A-F mucosalis as the antigen. All the sera examined were negative (all titres $< \frac{1}{2}$).

Detection of Rotavirus Antibodies:

Sera taken at necropsy from piglet 7 (not exposed to rotavirus) and piglets 5 and 8 (exposed to rotavirus) were examined by the indirect immunofluorescence test (see Materials and Methods, Chapter 5) using rotavirus grown in cell cultures as the antigen. Serum from piglet 7 was negative (titre $< {}^{1}/10$) while sera from piglets 5 and 8 were positive (titres $> {}^{1}/160$ and $> {}^{1}/320$ respectively) (Table 5.12).

The positive and negative controls were uniformly positive and negative respectively.

DISCUSSION

The experiments presented in this chapter have shown that mucosalis establishes readily in gnotobiotic piglets and persists in the majority of exposed

animals throughout the alimentary canal for a considerable period of time. Infection persisted for at least 27 days post-exposure in Experiment 1 and for 47 days post-exposure in Experiment 2. The numbers of mucosalis isolated were often within the range expected from adenomatous mucosa (mean = 6.718 $\pm$ 1.3, Lawson, personal communication, 1982). However despite prolonged exposure of the mucosa of these piglets to substantial numbers of mucosalis there was no evidence of adenomatous change, little evidence of intracellular parasitism by mucosalis, and considerable evidence from the light, fluorescence and electron microscope that the majority of mucosalis remained in extracellular sites in the lumen of the gut.

No bacteriological examination of gut contents was performed in either experiment. This was an unfortunate omission as a comparison between the numbers of mucosalis isolated from gut contents with the numbers isolated from the mucosa at the same site would have provided useful information about the predilection or otherwise of mucosalis for the mucosa. Further experiments in gnotobiotic piglets where this comparison was performed have shown that mucosalis is present in the gut contents in numbers up to 3 log 10/g greater than in the mucosa, behaving in a similar way to other non-pathogenic enteric bacteria (McCartney, Lawson and Rowland, unpublished results).

In gnotobiotic pigs the mucosa is finely constructed and consists of a thin basal area containing simple crypt glands surmounted by long delicate villi. Adenomatous mucosa, in contrast, consists of an enormously thickened, turgid basal component containing hyperplastic crypt glands and a relatively even surface devoid of villi. It is therefore extremely difficult to remove all traces of gut contents from gnotobiotic mucosa by washing since the contents become trapped between villi, whereas it is relatively easy to remove the gut contents from adenomatous mucosa by washing as the surface is flat and the tissue is robust. Hence one can be reasonably sure that cultures of adenomatous mucosa reflect the numbers of mucosalis in the mucosa. This is not the case with gnotobiotic mucosal samples and the high numbers of mucosalis recovered from these in Experiments 1 and 2 may have been due to mucosalis present in gut contents trapped between villi.

Faecal swabs were probably unsatisfactory samples from which to accurately estimate the numbers of mucosalis. Although swabs absorb 0.2ml of water it was noted that the amount of faecal matter absorbed depended on the consistency of the faeces which varied considerably especially in Experiment 2. The assumption that 0.2g of faeces was collected by rectal swabbing was probably erroneous. A more accurate method would have been to collect faeces per rectum

and use weighed amounts for assessing counts of mucosalis. This however would have resulted in practical difficulties since especially in diarrhoeic piglets it is often difficult to obtain sufficient faeces to handle easily or weigh accurately. The added difficulties of prolonged handling of piglets within isolators resulted in the decision to use rectal swabs.

Roberts (1978) exposed 6 litters of conventional piglets to mucosalis in culture and examined mucosal sites at necropsy up to 58 days post-exposure. Mucosalis was recovered in relatively small numbers (2-4 log 10/g) from a minority of sites in a minority of piglets. Thus gnotobiotic piglets are a promising tool for further studies on the relationship between mucosalis and the pig enterocyte since infection can be readily achieved and maintained for at least 47 days.

Roberts (1978) found that in experimental reproduction of the disease increasing numbers of mucosalis isolated from the mucosa at necropsy was related to the development of adenomatous change. The results of Experiments 1 and 2 suggest that in gnotobiotics it is the ratio of mucosalis in the mucosa to mucosalis in the contents which is an important parameter. Presumably a large shift in this ratio in favour of mucosal mucosalis could indicate the

change from extracellular commensalism to intracellular parasitism.

The apparent susceptibility of gnotobiotic piglets to mucosalis infection does not appear to be simply related to the absence of antibodies or non-specific inhibitory systems in sow colostrum and milk. When colostrum-deprived piglets (Chapter 4) were exposed to adenomatous mucosa, mucosalis was recovered from only 1 piglet and in relatively small numbers. It is perhaps the absence of other bacterial flora in gnotobiotes which enables infection by mucosalis to occur so readily. It is interesting that in piglets 2, 6, 11 whch were free of bacterial contaminants there were no failures to isolate mucosalis at necropsy. In piglets contaminated with other bacteria there were occasional failures to isolate mucosalis. This is particularly noticeable in piglet 8 which became multiply contaminated a few days before death, and yet at 40 days of age prior to the multiple contamination this piglet was still excreting large numbers of mucosalis (Tables 5.8 and 5.9). It is clear that bacterial contamination prevents the isolation of mucosalis from the sample cultured e.g. by overgrowing CBA plates. What is not clear is whether the contaminating bacteria depress the numbers of mucosalis in vivo. The problem is complicated by the occasional

failure of NBGT plates to isolate *mucosalis* from a sample known to contain *mucosalis*. Examination of Table 5.8 shows that there were occasions when despite the isolation of high numbers of *mucosalis* from CBA plates, NBGT plates inoculated in parallel did not grow *mucosalis*. *In vitro* tests have shown that minor differences in the gaseous environment of the jars in which the plates are incubated may result in significant depression of the growth of some strains of *mucosalis* on NBGT plates relative to CBA plates (Lawson, personal communication, 1981). This may in part explain occasional NBGT "failures" *in vivo*.

Hence it is not possible to say with absolute certainty that contaminating bacteria reduce the *in vivo* counts of *mucosalis*, since when contaminating bacteria are present the CBA plates may be overgrown and the NBGT plates may have "failed". However there is some evidence (piglet 8) that increasing bacterial contamination reduces the *in vivo* counts of *mucosalis*. The mechanisms by which this depression could occur are discussed more fully elsewhere (Introduction, Chapter 5; Roberts, 1978).

There was evidence of limited penetration of the mucosa by *mucosalis* in the early period post-exposure. In piglet 11 of Experiment 2 *mucosalis* organisms were observed in the lamina propria surrounding basal crypts of the TSl. Several bacteria appeared between the

base of a crypt gland and the lamina propria (Figure 5.36). Despite this early penetration of the mucosa there was no evidence of a prolonged period of intracellular existence and it seems that little if any intracellular multiplication occurred. The fate of the mucosalis observed in the lamina propria is unknown. The organisms were apparently lying in the intercellular spaces and although in an area rich in granulocytic leucocytes were not observed in phagocytic vacuoles.

Limited uptake of mucosalis by enterocytes particularly of young piglets in the early period post-exposure has been suggested by Duncan (1974) and Roberts (1978). The ultrastructural observations reported here provide further evidence that this uptake occurs. However the extent of mucosal penetration in the experiments reported here was probably very limited. Ultrastructural examination of a wide range of mucosal sites from the exposed piglets did not result in further observations of mucosalis other than in extracellular sites and the light microscopic observations suggested that the presence of intracellular bacteria was indeed a rare occurrence.

Further evidence that mucosalis remained largely extracellular is provided by the serological results. None of the piglets infected with mucosalis produced serum antibodies to mucosalis and yet the piglets were immunocompetent, demonstrated by the fact that piglets

which experienced rotavirus infection produced serum antibodies to rotavirus.

There was no indication that rotavirus influenced the numbers of mucosalis present in the gut or affected the isolation of mucosalis from sites throughout the gut and if the two agents behave in relation to one another in conventional circumstances as they did in Experiment 2 it seems improbable that rotavirus is involved in the pathogenesis of PIA (See Chapter 6).

The failure to produce adenomatous change in gnotobiotic piglets persistently infected with mucosalis suggests that the requisite conditions for the development of PIA were not met. The incubation period of the disease is unknown. Roberts (1978) found developed lesions of PIA in experimental piglets 50 days after exposure. As all the gnotobiotic piglets in Experiment 2 were killed by 47 days post-exposure it is possible that the incubation period was not long enough. Love and Love (1977) in studies concerning a natural outbreak of PHE have suggested that the incubation period may be as short as 3 weeks.

The lesions of PIA are associated with the presence of an intracellular campylobacter and there is considerable evidence that this bacterium is mucosalis (Lawson and Rowland, 1974; Rowland and Lawson, 1974; Roberts, Rowland and Lawson, 1977; 1980b; Roberts, 1978; Rajasekhar, 1981; and others).

Recently workers in America have implied that mucosalis is a commensal which cross-reacts antigenically with a catalase-positive campylobacter, the cause of PIA (Chang et al, 1981; Gebhart, Kurtz and Ward, 1981).

These results, based on bacteriological culture and immunofluorescent staining of affected tissues, have not as yet been published in a primary journal and so a proper estimation of their validity should await this event. Nevertheless it would be foolish to discount the possibility that mucosalis is not the intracellular organism of PIA.

One of the well-documented sequelae of rotavirus infection is the increase in the rate at which crypt cells divide and migrate up the villi in order to cover the recently denuded mucosal surface (Crouch and Woode, 1978; Snodgrass et al., 1979). There is little doubt that in Experiment 2 there was an increase in the number and availability of immature enterocytes for infection by mucosalis and yet large scale infection of these cells did not occur. The control piglets of Experiment 2 also provided a large population of villar enterocytes with active apical tubular systems. Both immature crypt cells and the apical tubular system of villar enterocytes have been suggested as likely entry-ports for mucosalis during the initial stages of PIA (Roberts, 1978; Rowland et al., 1980) but in the experiments reported here it seems that mucosalis was not attracted by either possibility to any great extent.

The mechanisms by which mucosalis gains entry into pig enterocytes are unknown but gnotobiotic piglets infected with mucosalis may be a useful in vivo model in which to study possible mechanisms of attachment and entry of the organism into the cell and factors which promote this. Rajasekhar (1981) has studied the behaviour of mucosalis in various cell culture systems, work which is continuing in this department. Useful developments of these in vitro systems could possibly be tested in vivo using the gnotobiotic pig model. It is interesting to note however that transmissible ileal hyperplasia of hamsters cannot be transmitted using cell-cultured campylobacter-like organisms antigenically indistinguishable from those associated with natural lesions (Jacoby and Johnson, 1981).

The presence of tuft cells in the TSl of piglet 11, Experiment 2 is intriguing. This is the first record of such cells in the pig. Tuft cells have been recorded in a variety of laboratory animals and occur in both respiratory and alimentary mucosa. Their function is as yet unknown but is believed to be absorptive (Isomäki, 1973; Nabeyama and Leblond, 1974; Lee and Toner, 1980). It is unknown if the presence of these cells in the TSl of piglet 11 was in any way related to the appearance of mucosalis in the lamina propria at this site.

The lesions in the large bowel of piglets 8 and 9

of Experiment 2 are puzzling. There is little published evidence of rotavirus-associated damage to the large bowel and so it is difficult to attribute them as sequelae of rotavirus. Both piglets 8 and 9 were observed to retch after feeding in the weeks following rotavirus infection and piglet 9 was observed to vomit in the acute stages of rotavirus infection. There may have been gross physical damage to the large bowel due to the violent abnormal bowel motility at the time of the severe enteritis and this possibly resulted in chronic disturbance of the vascular and lymphatic bed. The large numbers of globular leucocytes observed associated with the lesions may, on the other hand, indicate an allergic reaction to some dietary constituent. There was no evidence that mucosalis or the bacterial contaminants were involved in the lesions.

CHAPTER 6

PILOT STUDIES IN WEANED PIGS: DUAL EXPOSURES TO 1) ROTAVIRUS AND MUCOSALIS, 2) 987 P+ ESCHERICHIA COLI AND MUCOSALIS.

INTRODUCTION

Much of the work described in this thesis deals with the behaviour of *mucosalis* in piglets exposed early in life while on a milk diet. Although PIA is clinically recognised as a condition of the post-weaned pig the evidence of Roberts (1978) suggests that neonatal piglets are more suceptible to infection with *mucosalis* than post-weaned pigs, and that therefore exposure of neonatal piglets is possibly more likely to result in the development of lesions of adenomatosis. In Roberts' apparently successful transmission experiment a litter of piglets was exposed within 24 hours of birth. Lesions of adenomatosis were observed in some of this litter when the piglets were about 8 weeks of age, some 5 weeks after weaning (Roberts, Rowland and Lawson, 1977).

Although records of the haemorrhagic form of the disease (PHE) exist in unweaned piglets (Rowntree, unpublished; Rowland and Rowntree, 1972), the common appearance of PIA in the weeks following weaning suggests that the weaning process may be involved in the induction of the disease. While it is unknown which, if any, factors associated with weaning are important in this context, some consideration is given here to the effects of weaning on the young pig.

Weaning is a period of drastic change for the

pig. The sucking litter is removed from the sow, may be introduced into a different environment and may be mixed with other newly-weaned litters. There is an abrupt dietary change due to the removal of sow milk from the diet. Passive humoral immunity is already waning and added to this there is the sudden withdrawal of the protective antibodies and non-specific antibacterial systems present in sow milk (discussed in Chapter 4). The new environment may be heavily contaminated, resulting in exposure to previously unencountered organisms, both pathogenic and non-pathogenic. The newly-weaned pig is subjected then to considerable environmental stress, and it is hardly surprising that enteric disturbances are frequently encountered at this time.

Kenworthy (1976) describes an inflammatory response in the gut after weaning associated with a physiological diarrhoea and resulting from the change in diet. There is an increase in the metabolic activity of the gut microflora at weaning (Porter and Kenworthy, 1969); this may be responsible for the degenerative changes seen in the enterocytes of newly-weaned pigs (Kenworthy, Stubbs and Syme, 1967). The practice of mixing litters of pigs at weaning may result in the spread of pathogens between individuals and litters, and in further alterations of the enteric microbial flora (Lee and Gemmell, 1972). Environmental

and dietary stress has also been associated with changes in the gastro-intestinal flora (Tannock and Savage, 1974). Tzipori *et al*. (1980d)have suggested that piglets are more susceptible to both rotavirus and *E. coli* immediately after a dietary change from milk to dry food.

Clearly weaning is a time of considerable physiological and microbial change. It would not seem unreasonable that some of these events have a part to play in the induction of PIA.

Rotavirus as a Predisposing Agent in Weaned Pigs.

During the past decade rotavirus has emerged as an ubiquitous and important enteric pathogen of young animals and man (Flewett and Woode, 1978; McNulty, 1978; Woode and Crouch, 1978; Bartz, Conklin and Steele, 1979; Bridger, 1980). Although the majority of reports concerning field infections of rotavirus in pigs have concentrated on sucking piglets there is increasing evidence that rotavirus infection occurs in and is associated with diarrhoeic episodes of post-weaned pigs (Woode *et al*., 1976; Askaa and Bloch, 1981; Bridger, 1980, Lecce and King, 1980).

The enteric changes induced by rotavirus, those of villous atrophy and crypt hyperplasia (see Chapter 5), might logically be expected to predispose to infection of crypt cells by *mucosalis* by increasing

the number and availability of crypt enterocytes to a luminal population of mucosalis. The first pilot study described in this chapter concerns dual exposure of post-weaned pigs to mucosalis and rotavirus. It was hoped that if the factors operating around the time of weaning are important in the pathogenesis of PIA the experiment would allow their influence and it would be possible to determine whether early lesions of PIA are initiated under these circumstances.

987P+ Escherichia coli as a Predisposing Agent in Weaned Pigs.

Pathogenic strains of Escherichia coli have long been considered a cause of enteritis in domestic animals and man (Sojka, 1965, 1971; Moon, 1974). In recent years much progress has been made in elucidating the mechanisms whereby certain strains of E. coli induce disease. The pathogenesis of enteric diseases caused by E. coli has been described in detail by Moon (1974), who classified 4 different syndromes as enterotoxic, enterotoxaemic, local-invasive and septicaemic colibacillosis. Strains of E. coli designated as enterotoxic (ETEC) proliferate in the lumen of the small intestine where they produce enterotoxins which cause the small intestine to secrete fluid, resulting in watery diarrhoea. This form of the disease is known to occur spontaneously in calves, lambs and man, and is considered the predominant

syndrome in pigs (Moon, 1974). The ability of ETEC to colonise the small intestine has been shown to be greatly facilitated by the presence of certain virulence attributes, known as pili, which promote adherence of the E. coli to host enterocytes (Smith and Linggood, 1971; Jones and Rutter, 1972; Nagy, Moon and Isaacson, 1976; Isaacson, Nagy and Moon, 1977; Moon et al., 1977).

Three antigenically distinct pili, designated K88, K99 and 987P have been identified in ETEC strains pathogenic for the pig (Isaacson, 1980). In vitro tests using small intestinal epithelial cells or brush borders have provided strong evidence that pilus receptors exist on enterocytes of many strains of pigs, and that these receptors recognise only a single pilus type (Isaacson et al., 1978; Isaacson, 1980).

Enterotoxic colibacillosis in pigs has three ages of peak incidence - neonatal, 3 weeks, and immediately post-weaning (Moon, 1974). However experimental reproduction of the disease has proved easier in neonatal piglets than in any other age group (Sojka, 1971) and there is some evidence that the susceptibility of post-weaned pigs to ETEC wanes rapidly after the change of diet (Tzipori et al., 1980d).

The second study in this chapter describes dual exposure of post-weaned pigs to mucosalis and ETEC, in order to investigate:

(a) Whether enteric disturbance could result from exposure of weaned pigs to ETEC.

(b) Whether such enteric disturbances in the post-weaned pig, if it occurred, would enhance the establishment of mucosalis in the gut and initiate lesions of PIA. As the aim of the experiment was to produce enteric disturbance and to avoid mortality, an ETEC strain was selected to this end. In experimentally produced post-weaning colibacillosis an ETEC strain possessing the K88 pilus resulted in $^{6}/6$ deaths when the piglets were exposed immediately after weaning (Tzipori et al., 1980d). In natural outbreaks of neonatal scours mortality was much less (11.5%) when attributed to an ETEC strain possessing the 987P pilus than when attributed to an ETEC strain possessing the K88 pilus (74.2%) (Sellwood, 1979).

Unfortunately little is known of the pathogenicity of 987P possessing (987P+) strains of ETEC in young weaned pigs (Sellwood, personal communication, 1980). However a 987P+ strain was chosen as there seemed to be less likelihood of mortality and useful information could be accrued concerning the behaviour of 987P+ E. coli in weaned pigs.

MATERIALS AND METHODS

Exposure of Weaned Pigs to Rotavirus and Mucosalis (Pilot Study 1).

(i) Source of Experimental Animals.

A pregnant sow was purchased from Easter Bush Piggery (see Chapter 2) and introduced into a clean, freshly-fumigated isolation room one week prior to the expected date of farrowing. She was fed a proprietary cubed feed ("Sowlac" pellets, Seafield Mill) and offered *ad libitum* water. On the 114th day of gestation she was injected intramuscularly with 184 µg Cloprostenol Sodium BP (Vet) ("Planate", ICI Ltd.). Labour commenced the following day and 10 healthy piglets were born. There were no still-births and the sow remained healthy throughout the experiment.

(ii) Management of Piglets.

Using aseptic techniques two piglets (C308 and C309) were caught at birth, placed in a sterile box and transported immediately to Moredun Research Institute where they were placed in a germ-free flexible-film isolator (see Chapter 5 for design of isolator). Thereafter these two piglets were managed largely as described for the gnotobiotic piglets of Chapter 5, except that the feeding regime was 25ml of a 1:1 mixture of evaporated cows' milk and mineral mixture offered every hour between 9.00 hours and 23-00 hours.

The remaining piglets were allowed to suck the sow until weaning. A proprietary creep-feed ("Finisher Creep", Seafield Mill) was introduced at 7 days and the piglets were weaned (i.e. removed from the sow) at 16 days of age, divided into two groups of four and housed in separate clean fumigated rooms.

(iii) Sources of Inocula and Experimental Procedures.

(a) Rotavirus: A bacteria-free faecal filtrate containing virulent pig rotavirus, strain number P4 SW9/11, was obtained from Dr. C. Crouch, Institute for Research on Animal Diseases, Compton, Berkshire. This strain had been passed 4 times in gnotobiotic piglets, producing typical lesions of rotavirus infection throughout the small intestine and resulting in high mortality in such piglets exposed in the first week of life (Crouch, personal communication 1979). After the 4th passage the faeces were diluted 1 in 18 in PBS (pH 7.2, 0.01M), passed through a 0.45 μm filter and stored at -70°C. At 5 days of age piglets C308 and C309 were dosed orally with 1ml of a thawed aliquot of P4 SW9/11 (Snodgrass, personal communication, 1979).

Faeces samples from piglets C308 and C309 were taken at 5 days of age, just prior to rotavirus exposure, and at 6 days of age, and examined by direct electron-microscopy. Piglet C308 was killed at 24 hours and

piglet C309 at 30 hours after exposure to rotavirus. The gut contents of each were harvested, the presence of rotavirus confirmed by direct electron microscopy, and stored at -70°C until used.

Four of the weaned littermates of piglets C308 and C309 were dosed orally at 19 days of age with 1g of chalk in 5ml sterile distilled water followed immediately by 5ml of a 1 in 10 dilution in PBS (pH 7.2, 0.01M) of unfiltered gut contents from piglet C308 (Table 6.1).

(b) Mucosalis: The infective strain was 1075/78 A-F (see Chapter 2). The inoculum was prepared as described in Chapter 2 and the weaned littermates were orally dosed at 20 and 22 days of age with 1g of chalk in 5ml sterile distilled water followed immediately by 5ml of a 24 hour diphasic culture of mucosalis 1075/78 A-F. The numbers of mucosalis in each piglet inoculum were as follows:

Inoculum at 20 days - 8.90 log 10 (per piglet).
Inoculum at 22 days - 9.44 log 10 (per piglet).

(iv) Other Procedures

(a) Body Weights: Piglets were weighed at 9 days of age, at weaning (16 days of age) and thereafter daily.

(b) Oral and Rectal Swabs were taken at 12, 22, 26, 33 and 39 days of age. These were

TABLE 6.1.

PILOT STUDY 1: MANAGEMENT AND EXPERIMENTAL PROCEDURES.

Piglet Number	Management of piglets	EXPERIMENTAL EXPOSURES		Age (days) when killed
		Rotavirus	Mucosalis	
C308 (BA 285/79)	Caught at birth. Colostrum-deprived	Oral exposure at 5 days of age	None	6
C309 (BA 286/79)				
Y11 (BA 315/79)	Sow-suckled until 16 days of age. Creep feed introduced at 7 days of age	None	Oral exposure at 20 days of age.	21
Y17 (BA 333/79)			Oral exposure at 20 and 22 days of age	28
Y13 (BA 345/79)				35
Y15 (BA 360/79)				42
Y16 (BA 316/79)	Sow-suckled until 16 days of age. Creep feed introduced at 7 days of age	Oral exposure at 19 days of age	Oral exposure at 20 days of age	21
Y14 (BA 334/79)			Oral exposure at 20 and 22 days of age	28
Y12 (BA 346/79)				35
Y18 (BA 361/79)				42

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cultured for mucosalis as described in Chapter 2. Additional rectal swabs were taken at 19 days of age, just prior to rotavirus exposure, and thereafter daily until 28 days of age. These were examined for the presence of enteric viruses by direct electron microscopy, performed at Moredun Research Institute by Dr. Snodgrass.

(v) Necropsy Procedures and Sites Examined.

(a) Pigs were necropsied under terminal anaesthesia as described in Chapter 2. Samples were taken from the USl, MSl, TSl, Caec and LB and cultured for mucosalis as described in Chapter 2. Parallel samples from these sites were processed for histology, immunofluorescence and transmission electron microscopy.

Piglets C308 and C309 were not cultured for mucosalis; bacteriological examination was restricted to routine culture of the small intestinal contents for pathogenic E. coli and Salmonella species.

(b) Enzymology. Fresh unfixed portions of USl, MSl and TSl were taken from piglets Y11 and Y16 and assayed for thymidine kinase activity and lactase activity by Dr. Snodgrass of Moredun Institute, using methods described by Dahlqvist (1964) and Davidson et al. (1977).

(c) Histological Measurements: Haematoxylin and eosin stained 5µm sections of USl, MSl and TSl from piglets Y11 and Y16 were examined, and villus

heights and crypt depths measured using an ocular micrometer on 10 properly orientated villi and crypts at each site. This examination was carried out by Dr. Snodgrass of Moredun Institute.

(d) Immunofluorescent Staining for Rotavirus: Cryostat sections from the USl, MSl and TSl of piglets Y11 and Y16 were stained by the author at Moredun Research Institute using reagents and control sections of small intestine from a rotavirus-infected lamb (8596/86), all provided by Dr. Snodgrass. The method was as follows:

Sections were cut at 6µm, air-dried for 5 minutes and then fixed for 5 minutes in cold acetone. Fixed sections were overlaid with a few drops of sheep anti-rotavirus antiserum (195/205) diluted 1 in 20 in PBS (pH 7.2, 0.01M), and then incubated for 30 minutes in a moist atmosphere at 37°C. The sections were washed gently in PBS (pH 7.2, 0.01M) for 15 minutes with 3 changes of PBS. Excess PBS was blotted off and the sections were then overlaid with a few drops of FITC-conjugated rabbit anti-sheep immunoglobulin (Wellcome Reagents, Wellcome Laboratories Ltd.) diluted 1 in 20 in PBS (pH 7.2, 0.01M), incubated and washed as described above and finally air-dried. The stained sections were examined immediately using a Leitz-Ortholux microscope and incident blue light.

Exposure of Weaned Pigs to 987P+ Escherichia coli and Mucosalis (Pilot Study 2).

(i) Source of Experimental Animals.

A pregnant sow was purchased from ABRO Skedsbush Piggery (see Chapter 2) and introduced into a clean, freshly-fumigated isolation room one week prior to the expected date of farrowing. She was fed a proprietary cubed feed ("Sowlac" pellets, Seafield Mill) and offered water ad libitum. On the 114th day of gestation she was injected intramuscularly with 184µg Cloprostenol Sodium B.P. (Vet) ("Planate", ICI Ltd.). Labour commenced the following day and 10 healthy piglets were born. There were no stillbirths and the sow remained healthy throughout the experiment.

(ii) Management of Piglets.

The litter was allowed to suck the sow for 12 days. A proprietary creep-feed ("Finisher Creep", Seafield Mill) was offered from 2 days of age. At 12 days of age the piglets were divided into 2 groups of 5 and introduced into 2 separate, cleaned, freshly-fumigated rooms. Between 12 and 14 days of age each piglet was offered 200ml of a 1:1 mixture of evaporated cows' milk and mineral mixture (see Appendices 5.1 and 5.2) 3 times daily. From 15 days of age the diet consisted of creep feed and water ad libitum.

(iii) Sources of Inocula and Experimental Procedures.

(a) 987P+ Exchericia coli: A strain of 987P+ E. coli was obtained from Dr. R. Sellwood, Institute for Research on Animal Diseases, Agricultural Research Council, Compton. The strain had been serotyped as 09: K(A) 103: (987P+) (Sellwood, personal communication 1980) and was a stable toxin producer (Sherwood, personal communication, 1980). Ultrastructural examination confirmed the presence of pili (Figure 6.1).

At 14 days of age one group of 5 piglets was orally dosed with 50ml of a 24 hour culture of this strain grown aerobically at 37°C in trypticase soy broth. Immediately prior to exposure to 987P+ E. coli the piglets were dosed with 1g chalk powder in 5ml sterile distilled water (Table 6.2).

The numbers of E. coli which each piglet received were 10.79 log 10.

(b) Mucosalis: The infective strain was 209/80 L1 - 4 (see Chapter 2). The inoculum was prepared as described in Chapter 2. Piglets which remained were dosed orally at 18 and 25 days of age with 1g chalk in 5ml of sterile distilled water followed immediately by 25ml (18 days of age) or 30ml (25 days of age) of 48 hour diphasic cultures of mucosalis 209/80 L1 - 4. The numbers of mucosalis which each piglet

TABLE 6.2.

PILOT STUDY 2: MANAGEMENT AND EXPERIMENTAL PROCEDURES.

Piglet Number	Management	Experimental Exposures		Age (days) when killed
		987P + ETEC	Mucosalis	
BA 120/80	Sucked sow for 12 days, with creep feed offered from 2 days of age. Evaporated cows' milk and mineral mixture offered from 12-14 days of age. Creep feed and water ad libitum from 15 days of age.	None	Oral exposure at 18 days of age	21
BA 125/80				22
BA 146/80			Oral exposure at 18 and 25 days of age	29
BA 150/80				34
BA 167/80				40
BA 115/80		Oral exposure at 14 days of age	None	16
BA 121/80			Oral exposure at 18 days of age	21
BA 126/80				22
BA 157/80			Oral exposure at 18 and 25 days of age	35
BA 163/80				39

received were as follows:

Inoculum at 18 days of age - 10.11 log 10 (per piglet).

Inoculum at 25 days of age - 10.65 log 10 (per piglet).

(iv) Other Procedures.

(a) Oral and vaginal swabs were taken from the sow on the 115th day of gestation. These were cultured for mucosalis as described for oral swabs in Chapter 2.

(b) Oral swabs were taken from the piglets which remained, at 12, 20, 26 and 35 days of age, and cultured for mucosalis as described in Chapter 2.

(c) Rectal swabs were taken from all piglets at 19 days of age and cultured for ETEC as follows:

Swabs were inoculated conventionally onto SBA and McC plates and incubated aerobically for 24 hours at 37°C. Five isolated coliform colonies and any haemolytic coliform colonies were subcultured from SBA plates onto BA and Minca-1S plates and incubated as above. If spreading growth prevented selection from SBA plates colonies were picked instead from McC plates. Each subculture was examined by slide agglutination test using in turn polyvalent pig OK antisera, unabsorbed 987P+ antiserum (BA subcultures) and absorbed K99+ antiserum (Minca-1S plates). Any colonies agglutinated by unabsorbed 987P+ antiserum were also examined using absorbed 987P+ antiserum (see Chapter 2).

(v) Necropsy Procedures and Sites Sampled.

(a) Pigs were necropsied under terminal anaesthesia as described in Chapter 2.

The sites sampled for mucosalis are listed in Table 6.3. Parallel portions from the USl, MSl, TSl, Caec and LB were processed for histology, immunofluorescence and transmission electron-microscopy as described in Chapter 2.

Small aliquots (0.05ml) of gut contents taken aseptically from the USl, MSl, TSl, Caec and LB of 7 piglets (Table 6.3) were cultured for ETEC as described for rectal swabs (vide supra).

(b) 987P+ Immunofluorescence: Fresh segments, approximately 2 inches long of USl, MSl and TSl from piglet BA115/80 were filled with "Tissue-Tek II"*, frozen in an isopentone/dry ice slurry and stored at -80°C until examined.

Sections were cut at 6µm on a Slee Cryostat, fixed for 5 minutes in cold acetone and then stained in a similar manner to that described in Chapter 2 for mucosalis except that unabsorbed 987P+ antiserum, diluted 1 in 5 in PBS (ph 7.2, 0.01M) or inactivated rabbit serum (Wellcome Laboratories Ltd., diluted 1 in 5 in PBS [pH 7.2, 0.01M]) absorbed with pig small intestinal mucosa, were used instead of mucosalis antiserum.

* O.C.T. Compound, Lab-Tek Products, Miles Laboratories Inc., Naperville, Illinois 60540.

TABLE 6.3.

PILOT STUDY 2, SITES CULTURED AT NECROPSY FOR MUCOSALIS AND 987P+ETEC.

Piglet Number	Mucosal sites cultured for *mucosalis*	Gut contents cultured for 987P+ETEC	Age (days) at Necropsy
BA 115/80*	USl, MSl, TSl Caec, LB	USl, MSl, TSl Caec, LB	16
BA 120/80+	MSl, TSl, Caec	USl, MSl, TSl Caec, LB	21
BA 121/80*+	MSl, TSl, Caec	USl, MSl, TSl Caec, LB	21
BA 125/80+	MSl, TSl, Caec	USl, MSl, TSl Caec, LB	22
BA 126/80*+	MSl, TSl, Caec	USl, MSl, TSl Caec, LB	22
BA 146/80+	USl, MSl, TSl Caec, LB	N.D.**	29
BA 150/80+	USl, MSl, TSl, Caec, LB	USl, MSl, TSl Caec, LB	34
BA 157/80*+	USl, MSl, TSl, Caec, LB	USl, MSl, TSl Caec, LB	35
BA 163/80*+	USl, MSl, TSl, Caec, LB	N.D.	39
BA 167/80+	USl, MSl, TSl, Caec, LB	N.D.	40

* = piglet exposed to 987P+ETEC only. *+ = piglet exposed to 987P+ETEC and *mucosalis*.
+ = piglet exposed to *mucosalis* only. ** N.D. = not sampled.

RESULTS

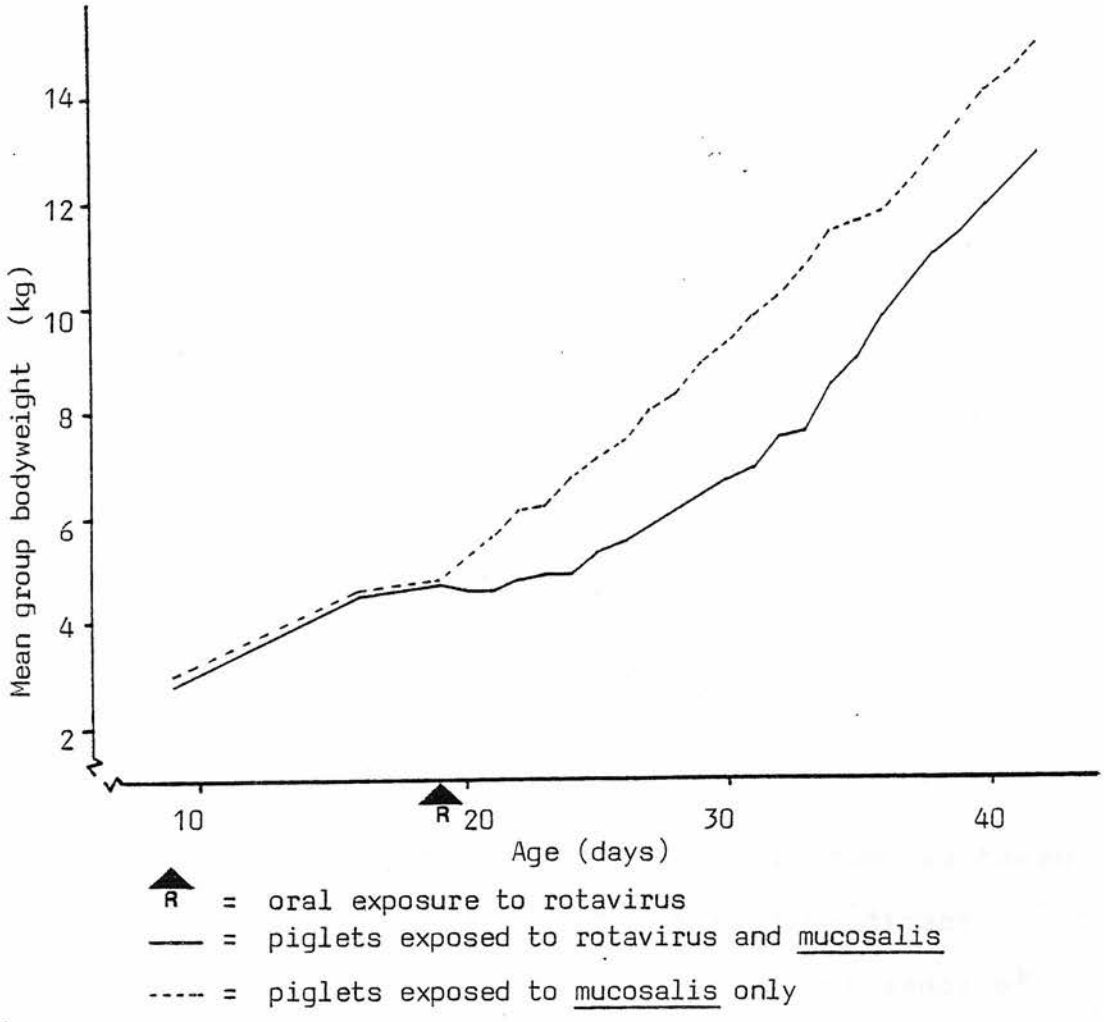
Exposure of Weaned Pigs to Rotavirus and Mucosalis (Pilot Study 1).

(i) Clinical Findings/Daily Observations.

Piglets C308 and C309: These piglets adapted well to bottle-feeding and were initially vigorous. Both experienced a mild diarrhoeic episode between 3 and 5 days of age but this did not result in depression of appetite or energy. By 22 hours after exposure to rotavirus both piglets had staring coats and were lethargic although still drinking readily the 25ml offered at each feed. At this stage both piglets had begun to excrete copious quantities of yellow, fluid diarrhoea. Piglet C308 was killed 24 hours post-exposure. By 27 hours post-exposure piglet C309 was severely depressed and in-appetent, drinking only 5-10mls at each feed. Piglet C309 was killed 30 hours post-exposure to rotavirus.

Piglets Y11, Y12, Y13, Y14, Y15, Y16, Y17, Y18: All the piglets in both groups remained healthy throughout. Exposure to rotavirus and/or mucosalis was not associated with signs of ill-health, however, the piglets which had been exposed to rotavirus experienced a significant period of retarded growth from the time of exposure until about 8 days after exposure (Figure 6.2). There was no significant

Figure 6.2: Pilot Study 1: Mean group bodyweights of rotavirus-exposed and non-exposed piglets.



difference in weight gains between the 2 groups in the period prior to rotavirus exposure (Appendices 6.1 - 6.2).

(ii) Bacteriology.

Piglets C308 and C309: No pathogenic E. coli or Salmonellae species were isolated from the gut contents at necropsy. Culture for mucosalis was not attempted.

Piglets Y11, Y12, Y13, Y14, Y15, Y16, Y17, Y18: Mucosalis was isolated from the oral cavity of most piglets after experimental exposure (Figure 6.3). Mucosalis was not isolated from rectal swabs or from the gut at necropsy of any piglet.

(iii) Virology.

Piglets C308 and C309: Faecal samples taken prior to rotavirus exposure and examined by direct electron microscopy were negative for the presence of enteric viruses. Both faeces and gut contents of C308 and C309 at necropsy contained rotavirus particles (Snodgrass, personal communication, 1979).

Piglets Y11, Y12, Y13, Y14, Y15, Y16, Y17, Y18: The results of direct electron microscopic examination of faecal material from rectal swabs are summarised in Figure 6.4. Rotavirus was detected in 3 of the 4 experimentally-exposed piglets and in one animal (Y13) not experimentally-exposed. The rotavirus was detected from 3-5 days post-exposure. A coronavirus was detected in the faeces of 7 of the 8 piglets,

FIGURE 6.3: Pilot Study 1: Isolations of Mucosalis from the oral cavities of Piglets Y11–Y18

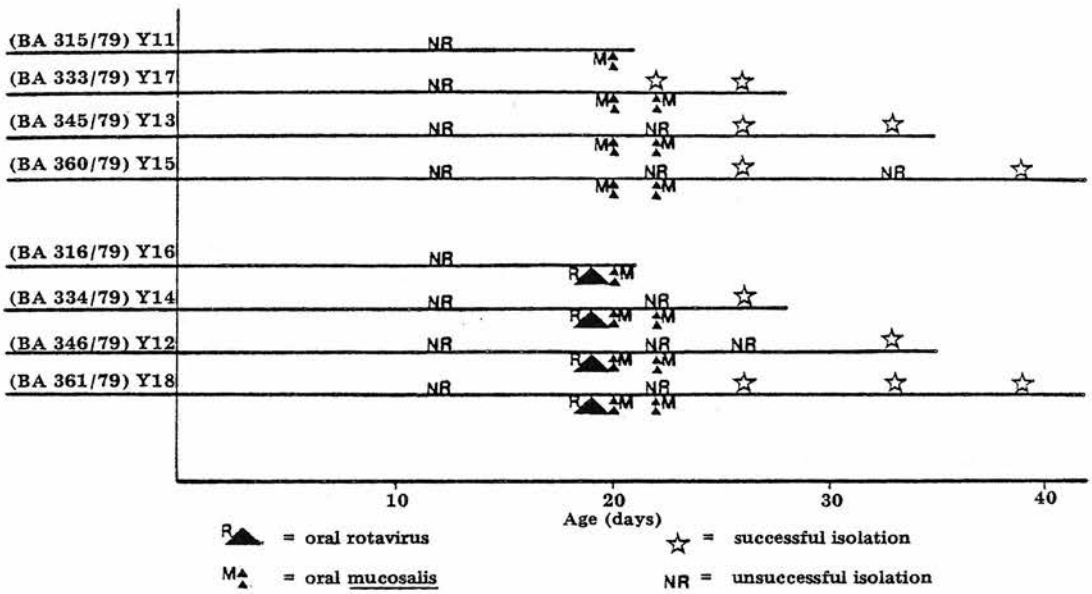
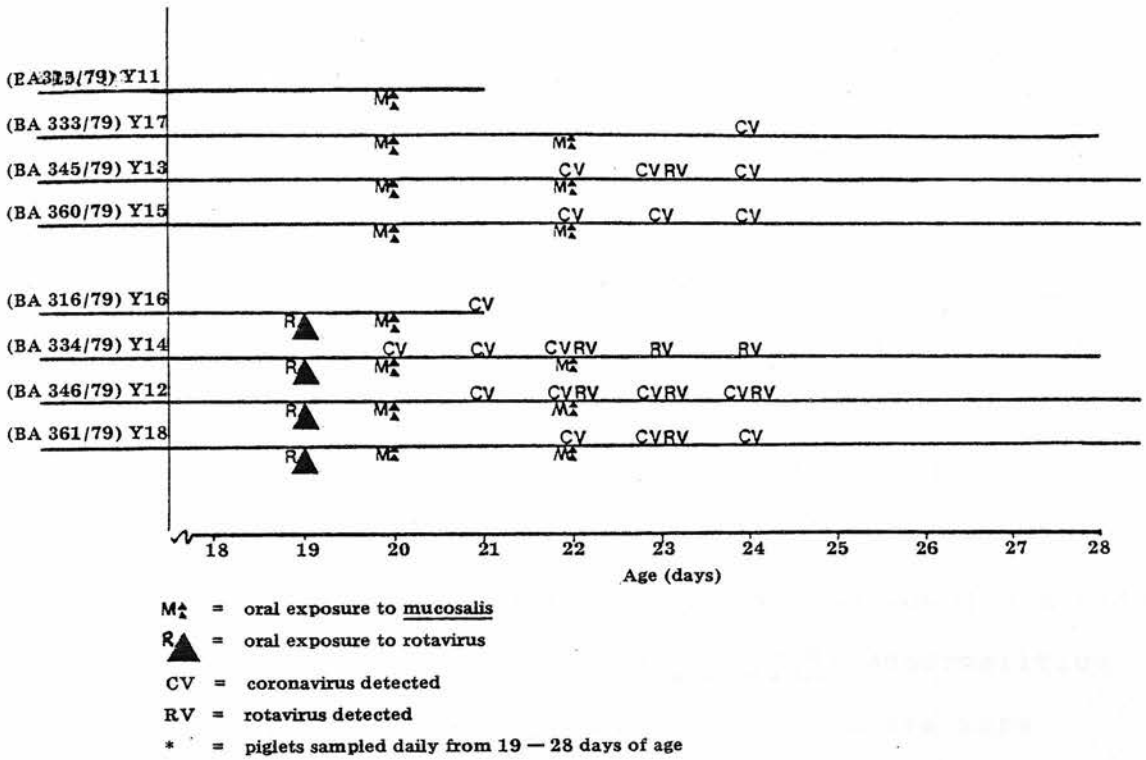


FIGURE 6.4: Pilot Study 1: Viruses, detected by direct electron microscopy, in faeces of Piglets Y11–Y18*



M▲ = oral exposure to mucosalis

R▲ = oral exposure to rotavirus

CV = coronavirus detected

RV = rotavirus detected

* = piglets sampled daily from 19 — 28 days of age

appearing between 20 and 24 days of age (Figure 6.4).

(iv) Pathology.

(a) Gross Findings at Necropsy.

Piglets C308 and C309: Abnormalities were confined to the alimentary canal. There was slight distension of the small intestine with watery fluid. The contents of the large bowel were yellow and abnormally fluid.

Piglets Y11, Y12, Y13, Y14, Y15, Y16, Y17, Y18: There were no gross abnormalities except for ectopic kidneys (Y17), an inspissated colonic abscess (Y15) and slightly fluid contents of the large bowel (Y16).

(b) Histopathology (H&E and Young's stains)

Piglets C308 and C309: Abnormalities were confined to the small intestine. There were changes consistent with rotavirus infection, of blunting and fusion of villi, attenuation of surface epithelium and subepithelial aggregations of necrotic and inflammatory cell debris. The damage was most severe in the US1 of piglet C308 and the MS1 of piglet C309 respectively.

Piglets Y11, Y17, Y13, Y15 (exposed to mucosalis only): There was no evidence of abnormality in any tissue. The sections from the alimentary tract were typical of young weaned piglets of good health status. The salient features of the small

intestine were well-formed villi clothed in columnar epithelial cells and surmounting short crypts abundantly populated with goblet cells. The lamina propria was moderately cellular, lymphocytes and eosinophils predominating.

The mucosa of the caecum and spiral colon consisted of regular crypt glands lined almost entirely by goblet cells and opening onto a smooth surface clothed in columnar epithelium. The lamina propria was populated mainly with lymphocytes and eosinophils. There was no evidence of adenomatous change at any site nor any evidence of intracellular bacterial parasitism.

Piglets Y16, Y14, Y12, Y18 (exposed to rotavirus and mucosalis): With the exception of piglet Y16 there were no histological abnormalities and all tissues examined were similar to comparable controls.

In piglet Y16 there were moderate changes, mostly confined to the USI, of stunting, fusion and occasionally loss of villous elements together with mild crypt hyperplasia and subepithelial aggregations of necrotic and inflammatory debris. In the MSI villi were of irregular shape and there was evidence of increased cell extrusion at the tips of villi. The appearance of the TSI was similar to that of the relevant control piglet Y11. There was no evidence of adenomatous change in any piglet nor any evidence of intracellular

bacteria.

(c) Immunofluorescence.

I. Detection of Rotavirus by Immunofluorescence: Acetone fixed cryostat sections of USl, MSl and TSl of Piglets Y11 and Y16 were examined in parallel with known positive control sections of lamb gut (see Materials and Methods, this Chapter). Sections from Piglet Y11 were uniformly negative while control lamb sections were positive, showing bright diffuse fluorescence of villar enterocytes, particularly those at the tips of villi (Figure 6.5).

The MSl and TSl of Piglet Y16 showed positive fluorescence, more extensive in the MSl than in the TSl (Figure 6.6).

II. Detection of Mucosalis by Immunofluorescence: Sections of USl, MSl, TSl, Caec and LB from Piglets Y11 and Y16 were examined.

There was no evidence at any site of fluorescence attributable to mucosalis.

(d) Electron Microscopy.

Sites examined:

Y11 - USl, MSl, TSl

Y16 - USl, MSl, TSl.

Limited ultrastructural examination of these tissues did not reveal the presence of abnormalities. Rotavirus was not detected and there was no evidence of intracellular parasitism by bacteria.

TABLE 6.4.

PILOT STUDY 1. VILLUS HEIGHTS AND CRYPT DEPTHS (μM) IN SMALL INTESTINAL SITES OF PIGLETS Y11* and Y16**

US1				MS1				TS1			
Villus heights		Crypt depths		Villus heights		Crypt depths		Villus heights		Crypt depths	
Y11	Y16	Y11	Y16	Y11	Y16	Y11	Y16	Y11	Y16	Y11	Y16
355	200	218	243	546	510	125	179	355	346	136	168
537	273	161	286	510	464	164	179	328	237	118	168
400	155	179	286	501	355	161	161	273	209	139	86
473	155	143	303	537	410	268	161	273	355	114	171
419	191	164	278	491	300	250	139	282	328	125	139
364	164	173	179	437	408	179	186	282	382	125	161
419	100	200	196	464	500	179	221	228	300	126	129
282	209	179	232	446	474	214	196	319	300	120	146
445	246	161	232	419	345	157	203	300	382	134	150
382	170	159	271	510	420	203	180	264	315	130	186

* Piglet Y11 - not exposed to rotavirus

** Piglet Y16 - exposed to rotavirus

(e) Other Observations.

I. Enzymology: The results of thymidine kinase and lactase activity of small intestinal sites from piglets Y11 and Y16 are summarised in Table 6.6. The results indicated that lactase activity was severely depressed in Piglet Y16 while thymidine kinase activity, at least in the MS1 was more than doubled.

II. Histological Measurements: Measurements of villus heights and crypt depths of small intestinal sites from Piglets Y11 and Y16 are summarised in Table 6.4. Statistical analysis of these figures (Appendix 6.3) indicated that in Piglet Y16 there was villous stunting in the US1 and MS1, and crypt hyperplasia in the US1 and TS1 - results which were in broad agreement with the subjective assessment of the histological sections examined (vide supra).

TABLE 6.6.

PILOT STUDY 1, ASSAY OF SELECTED ENZYMES IN SMALL INTESTINAL SITES OF PIGLETS Y11 AND Y16.

Piglet Number	Thymidine kinase (μmol/minute/mg)		Lactase (μmol/minute/g protein)	
Y11 ** (BA 315/79)	US1	16.6	US1	211
	MS1	24.7	MS1	95
	TS1	ND*	TS1	ND
Y16*** (BA 316/79)	US1	19.1	US1	0
	MS1	61.8	MS1	0
	TS1	56.7	TS1	0

* ND = sample lost.
** Y11 = piglet not exposed to rotavirus.
*** Y16 = piglet exposed to rotavirus.

Exposure of Weaned Pigs to 987P+ Excherichia coli and Mucosalis (Pilot Study 2).

(i) Clinical Findings/Daily Observations.

All piglets remained healthy throughout. Exposure to 987P+ ETEC and/or mucosalis did not result in signs of ill-health in any piglet.

(ii) Bacteriology.

(a) Isolations of Mucosalis

Sow Oral and Vaginal Swabs: Mucosalis was not isolated from these.

Piglet Oral Swabs: Isolations of mucosalis from the oral cavities are summarised in Figure 6.7. Mucosalis was recovered from most piglets although never from BA 120/80 nor BA 167/80 despite 2 exposures of the latter to mucosalis (Figure 6.7).

Sites at Necropsy: There were no isolations of mucosalis from the alimentary sites sampled at necropsy (see Table 6.3 for sites cultured).

(b) Isolations of 987P+ E. coli.

Piglet Rectal Swabs: 987P+ ETEC were isolated from 2 (BA 157/80 and BA 163/80) of the piglets 5 days post-exposure but not from the other 8 piglets.

Sites at Necropsy: 987P+ ETEC was not isolated from the three control piglets (BA 120/80, BA 125/80, and BA 150/80) which were sampled at necropsy.

FIGURE 6.7: Pilot Study 2: Isolations of Mucosalis from oral cavities of Piglets exposed to mucosalis only or to 987 P+ETEC and Mucosalis

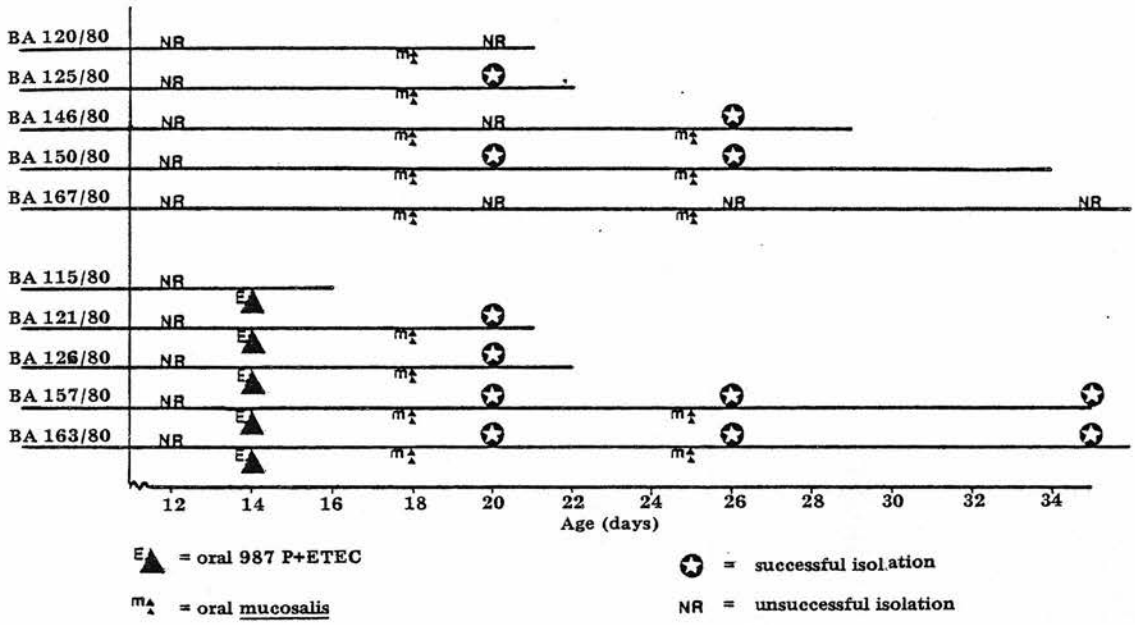


TABLE 6.5.

PILOT STUDY 2, ISOLATIONS OF 987P+ETEC FROM ALIMENTARY SITES AT NECROPSY OF EXPOSED PIGLETS.

Piglet Number	Days post-ETEC-exposure when killed	Alimentary Sites Cultured				
		USl	MSl	TSl	Caec	LB
BA 115/80	2	NR*	NR	+**	+	+
BA 121/80	7	+	+	+	NR	+
BA 126/80	8	+	+	NR	NR	+
BA 157/80	21	NR	NR	NR	NR	NR
BA 163/80	25	ND***	ND	ND	ND	ND

* NR = 987P+ETEC not isolated

** + = 987P+ETEC isolated

*** ND = site not cultured for 987P+ETEC

Isolations of 987P+ ETEC from the exposed group are summarised in Table 6.5. 987P+ ETEC was isolated from exposed piglets killed up to 8 days post-ETEC-exposure but was not isolated from BA 157/80 killed 21 days after exposure (Table 6.5).

(c) Other ETEC were not isolated from rectal swabs or alimentary sites at necropsy of any piglet.

(iii) Pathology

(a) Gross Findings at Necropsy: There were no gross abnormalities in any piglet at necropsy except BA 115/80. This piglet had a small fibrous adhesion between the apex of the spiral colon and the abdominal wall.

(b) Histopathology (H&E, Giemsa and Young's stains): There were no histological abnormalities detected in any of the tissues examined. The alimentary sites were typical of young weaned pigs of good health status. Giemsa stains revealed the presence of a few adherent coliforms to the villi of the US1 and TS1 of BA 115/80, killed 2 days after exposure to 987P+ ETEC, but these were not associated with mucosal abnormality at these sites. There was no evidence of adenomatous change in any piglet and no evidence of intracellular parasitism by bacteria.

(c) Immunofluorescence:

I. Detection of 987P+ ETEC: The US1, MS1 and TS1 of BA 115/80 were examined. In sections overlaid with 987P+ antiserum there were no fluorescing organisms in the US1 or MS1 but a few were observed in the lumen of the TS1, and occasional groups of bacteria were found in mucus around the villi. No fluorescing bacteria were observed in sections overlaid with inactivated rabbit serum.

II. Detection of Mucosalis: Sections of MS1, TS1 and Caec from BA 146/80, BA 150/80, BA 157/80, BA 163/80 and BA 167/80 were examined. There was no evidence of fluorescence at any site which could be attributed to mucosalis.

(d) Electron Microscopy: No ultrastructural examination was undertaken.

DISCUSSION.

There was no evidence, under the circumstances of these experiments, that either rotavirus or 987P+ ETEC enabled establishment of mucosalis in the gut of weaned pigs or in any way initiated intracellular parasitism by mucosalis.

Neither agent produced serious enteric disturbance although exposure to rotavirus did appear to retard growth for about 7 days, results which are consistent with the findings of other workers (Woode and Bohl, 1981).

Mucosalis did establish in the oral cavity of the majority of weaned pigs in these studies, and in most animals was isolated from more than one sampling, and yet no isolations of mucosalis were made from mucosal sites at necropsy. These results are consistent with those of Roberts (1978) who found weaned pigs particularly refractory to mucosalis infection unless benzetimide, an anti-cholinergic agent, was used to reduce gut motility. Roberts (1978) was however able to demonstrate mucosalis in the oral cavities of exposed piglets for up to 8 weeks post-exposure.

The reasons why it is difficult to establish mucosalis infection in post-weaned pigs are unclear. Roberts (1978) considered that the greater surface area of weaned pig gut relative to the size of the inoculum could be a factor; also the possible greater inhibitory effect of gastric acidity in the weaned pig compared to milk-fed pigs.

Alterations in the activity of or the species of gut flora may possibly have prevented the establishment of mucosalis in these piglets. There is some

evidence from the gnotobiotic piglets (piglet 8, Chapter 5) that the establishment of a varied bacterial flora suppresses mucosalis in vivo.

The difficulty in establishing experimental mucosalis infection in weaned pigs suggests that mixing of newly-weaned litters may not be an important factor in the spread of mucosalis infection in the field, although further investigation is necessary to clarify this point. In PHE outbreaks in SPF herds the evidence indicates that older weaned pigs are susceptible to and become infected when introduced into the breeding herd (Love and Love, 1977; Love, Love and Edwards, 1977).

Both rotavirus and 987P+ ETEC established in weaned pigs exposed to these agents (Figure 6.4 and Table 6.5) but neither agent produced a diarrhoeic episode. The management of both litters was, by commercial standards, very good - the litters were reared in isolation and introduced into clean, fumigated accommodation. Hence there was an absence of factors, such as poor hygiene, fierce competition for food and overcrowding, which in commercial situations would tend to exacerbate the effect of exposure to potential pathogens (Alexander, 1981). These two pilot studies highlight the difficulties inherent in the study of pathogenic organisms using conventional animals. It is not practical to reproduce the field situation and

it is not possible to simulate field situations in experimental studies. The necessity of good isolation facilities is in direct conflict with the desirability of recreating commercial pressures and stresses.

One control piglet in the rotavirus pilot study became infected with rotavirus, despite maintaining the groups in separate rooms with different attendants. This may have been a natural infection but was more likely to have been cross-contamination from the exposed group. Rotavirus may be carried either actively or passively by humans or other animals such as rodents (Woode and Bohl, 1981).

The presence of coronavirus in 7 of the 8 weaned piglets in Pilot Study 1 is interesting. The coronavirus of transmissible gastro-enteritis (TGE) is not present in Scotland. However coronaviruses serologically distinct from TGE have been reported in pigs elsewhere (Pensaert and De Bouck, 1978).

The pathogenic significance of the coronavirus detected in the pigs of Pilot Study 1 is unknown but there were no overt clinical signs.

The enzyme assay results from piglets Y11 and Y16 (Table 6.6) of the rotavirus pilot study confirmed the effect of rotavirus infection on the types of cell present at different levels of the mucosa. Mature villous cells, rich in lactase, are lost as a result

of rotavirus infection, while crypt cells, rich in thymidine kinase, divide and migrate upwards to cover the denuded mucosa (Weiser, 1973; Davidson *et al.*, 1977; Snodgrass *et al.*, 1979). The reduction in digestive enzymes such as lactase in piglets exposed to rotavirus and the resultant disruption of normal digestion due to their absence probably contributed to the retarded growth of these piglets in the period after exposure.

The results of the pilot studies reported here suggest that mild or inapparent rotavirus or entero-toxigenic *E. coli* infection of weaned conventional pigs do not enhance colonisation of the gut by *mucosalis* and are unlikely to act as initiating agents of PIA in such pigs.

CHAPTER 7

EXPERIMENTAL EXPOSURE OF PIGLETS TO CRYPTOSPORIDIA WITH OBSERVATIONS ON THE ESTABLISHMENT OF MUCOSALIS IN CRYPTOSPORIDIAL-INFECTED PIGLETS.

Page Number

INTRODUCTION

The failure to induce the widespread intracellular *mucosalis* infection associated with adenomatous change would seem to be not simply related to failure to establish infection in the lumen of the gut, since in exposed gnotobiotic piglets large numbers of *mucosalis* established without penetrating the enterocytes to any great extent (see Chapter 5). This observation suggests that one of the factors preventing intracellular parasitism may be resistance of the gut epithelial cells themselves to infection. Extensive interactions between the surfaces of host cells and *mucosalis* leading to bacterial attachments and eventual widespread penetration seem logical steps in the establishment of the considerable intracellular infection observed in adenomatous mucosa. In this chapter attempts were made, using cryptosporidia, to alter the surface of gut epithelial cells in a manner which would enhance bacterial penetration.

THE GENUS CRYPTOSPORIDIUM.

Members of the genus Cryptosporidium are protozoan parasites classified by Levine (1973) in the family Cryptosporidiidae, suborder Eimeriorina. This suborder also contains the family Eimeriidae which encompasses most enteric coccidia of domestic animals and man in the genera Eimeria and Isospora (Levine, 1973).

HISTORICAL ASPECTS

In 1907 Tyzzer described a protozoan parasite which he observed attached to the surface of cells in the gastric glands of domestic mice. He named this organism *Cryptosporidium muris*, the first report of the occurrence of a member of the genus Cryptosporidium (Tyzzer, 1907). Tyzzer later extended these observations and succeeded in transmitting infection to other mice but failed to transmit infection to a rat (Tyzzer, 1910). In 1912 Tyzzer described another species, *Cryptosporidium parvum*, which he found parasitising the microvilli of enterocytes in the small intestine of mice (Tyzzer, 1912). In feeding experiments Tyzzer found that *C. muris* infected only the stomach of susceptible mice, while *C. parvum* infected only the small intestine. He described both sexual and asexual endogenous cycles and postulated that oocysts passed in the faeces were the infective form, although he also suggested that in the case of *C. muris* the entire life cycle could take place in the gastric glands (Tyzzer, 1910, 1912). Both *C. parvum* and *C. muris* were found throughout the year in mice of all ages and either species was sometimes present in enormous numbers in apparently healthy mice (Tyzzer, 1912).

Tyzzer also observed cryptosporidia in rabbits (Tyzzer, 1912) and in chickens (Tyzzer, 1929). He

considered those in chickens to be C. parvum although Levine (1973) disagreed with this classification and considered the chicken parasite to be C. tyzzeri.

Interestingly the first report associating cryptosporidia with enteric disease came from Scotland when Slavin (1955) described a diarrhoeic episode of young turkeys resulting in moderate morbidity and mortality. From Tyzzer's earlier work it appeared that cryptosporidia were species and site specific, hence Slavin named the turkey parasite C. meleagridis.

Similarly Jervis, Merrill and Sprinz (1966) described a chronic enteritis in guinea pigs due to cryptosporidia, and Vetterling et al., 1971) considered this another new species, C. wrairi, as transmission experiments to mice, chickens, turkeys and rabbits were unsuccessful. These authors questioned earlier reports of the occurrence of cryptosporidia in reptiles and carnivores since these were based on observations of oocysts in the faeces and not on confirmation of endogenous stages. Several authors cited by Vetterling et al. postulated that these observed "oocysts" could have been free sporocysts of members of the genus Isospora (Vetterling et al., 1971).

Interest in the genus Cryptosporidium has increased over the last decade due to the emergence of cryptosporidia as potential enteric pathogens of domestic animals and man. Reports, mostly from America, have

implicated cryptosporidia in enteric disease in calves (Panciera, Thomassen and Garner, 1971; Meuten, Van Kruiningen and Lein, 1974; Schmitz and Smith, 1975; Morin, Larivière and Lallier, 1976; Powell et al., 1976; Moon et al., 1978; Pohlenz et al., 1978; Nagy, Antal and Ratz, 1979; Henrikson and Krogh, 1980), lambs (Barker and Carbonell, 1974; Berg, Peterson and Freeman, 1978), immunodeficient foals (Snyder, England and McChesney, 1978) and humans (Meisel et al., 1976; Nime et al., 1976; Lasser, Lewin and Ryning, 1979; Bird and Smith, 1980; Stemmerman et al., 1980; Tzipori et al., 1980b; Sloper et al., 1982.

In addition there have been sporadic world-wide reports of the occurrence of cryptosporidia in other host species, not always associated with disease e.g. in guinea-pigs (Jervis, Merrill and Sprinz, 1966), in monkeys (Kovatch and White, 1972; Cockrell, Valerio and Garner, 1974), in geese (Procter and Kemp, 1974), in chickens (Fletcher, Munnell and Page, 1975), in pigs (Bergeland, 1977; Kennedy, Kreitner and Strafuss, 1977), in snakes (Brownstein et al., 1977), in parrots (Doster, Mahaffey and McClearen, 1979), in rabbits (Inman and Takeuchi, 1979; Rehg, Lawton and Pakes, 1979) and in cats (Iseki, 1979), suggesting that the parasites have a wide host-range as well as an extensive geographical distribution.

Not all the reports have been of cryptosporidial parasitism of the alimentary tract although most of the sites infected with cryptosporidia in the above host species are gut or gut-associated organs. Many types of epithelial cells have been observed to be parasitised by cryptosporidia and reflecting this diversity are the sites from which the parasites have been reported e.g. gallbladder, bile and pancreatic ducts in monkeys (Kovatch and White, 1972), Bursa of Fabricius of chickens (Fletcher, Munnell and Page, 1975), stomach of snakes (Brownstein et al., 1977), respiratory epithelium of nasal passages, trachea and bronchi of turkeys (Hoerr, Ranck and Hastings, 1978), cloacal coprodeum of parrots (Doster, Mahaffey and McClearen, 1979), tonsils, pharynx, oesophagus and trachea of a human (Booth et al., 1980), conjunctiva, nasal sinuses and trachea of peacocks (Mason and Hartley, 1980). The reports of respiratory infection in turkeys and peacocks were in young birds with clinical signs of respiratory disease, in some cases fatal.

To summarise, cryptosporidia infect mainly gut epithelial cells, although a variety of gut-associated cells may be involved, and in a minority of cases non-gut cells may be infected.

CRYPTOSPORIDIA IN THE UNITED KINGDOM.

In the United Kingdom the first report of cryptosporidial enteritis was from a diarrhoeic episode of

young turkey poults in Scotland (Slavin, 1955). Much later Pearson and Logan (1978) described cryptosporidial infection in the small intestine of a colostrum-deprived experimental calf in Northern Ireland. Recently in Scotland Snodgrass et al., (1980) reported an outbreak of diarrhoea in sucking calves in which rotavirus, E. coli and cryptosporidia were demonstrated, while a separate outbreak of calf diarrhoea in another Scottish herd was attributed to cryptosporidia alone. No other enteropathogens were demonstrated (Tzipori et al., 1980c).

Subsequently these workers have described an adult human case of enteric cryptosporidiosis, not apparently associated with immunodeficiency (Tzipori et al., 1980b), and as a result of field studies of diarrhoea in lambs, calves and deer followed by experimental transmission of field isolates from calves, lambs and humans to seven host species have postulated that the genus Cryptosporidium is in fact a single-species genus, the members of which have a wide host-range (Tzipori et al., 1980a).

LIFE-CYCLE OF CRYPTOSPORIDIA.

Like other coccidia members of the genus Cryptosporidium form oocysts, containing the infective form of the parasite, the sporozoites (Levine, 1973). The cryptosporidial oocyst, containing four naked sporozoites, is passed in the faeces and infection of

the next host is by ingestion of oocysts (Iseki, 1979; Tzipori et al., 1980a; Moon and Bemrick, 1981).

Concerning the endogenous cycle of the parasite there is some controversy, firstly regarding the exact site of parasitism and secondly regarding the number of asexual generations and the number of merozoites in each generation.

Levine (1973) describes the site of parasitism as "development on the surface of the host cell or within its striated border and not in the cell proper". All authors agree that cryptosporidia parasitise the surface of the host cell, unlike most other pathogenic coccidia whose endogenous stages often have a deep intracellular site. However, there is dispute as to whether the membrane forming the external parasitophorous vacuole of the endogenous forms is of host or parasite origin and hence whether the parasite is technically an intracellular parasite or an extracellular parasite. Hence Tyzzer (1907), Pohlenz et al. (1978), and Tzipori et al. (1980a) consider the parasite to be extracellular, while Hampton and Rosario (1966), Vetterling, Takeuchi and Madden (1971), Kovatch and White (1972), Barker and Carbonell (1974), Kennedy, Kreitner and Strafuss (1977), Inman and Takeuchi (1979), Lasser, Lewin and Ryning (1979) and Bird and Smith (1980) consider the parasite to be intracellular. Iseki (1979) describes the site of parasitism as

"intramicrovillar".

As to the number and nature of the endogenous forms, most authors agree that sporozoites escaping from oocysts are likely to be the infective stage and that these initiate an asexual generation of schizonts containing eight merozoites (Tyzzer, 1907; Vetterling et al., 1971; Kovatch and White, 1972; Barker and Carbonell, 1974; Pohlenz et al., 1978; Inman and Takeuchi, 1979; Iseki, 1979; Bird and Smith, 1980).

The schizonts rupture when mature and release merozoites which parasitise other host epithelial cells and either form second generation schizonts containing only four merozoites (Vetterling et al., 1971; Pohlenz et al., 1978; Stemmermann et al., 1980), or continue to form schizonts with eight merozoites for an unknown number of generations (Iseki, 1979). After the asexual phases the gametes form the uninucleate macrogametocyte and microgametocytes containing up to sixteen microgametes (Tyzzer, 1907; Vetterling et al., 1971; Iseki, 1979). The microgametocytes are released and fertilise macrogametocytes (Bird and Smith, 1980) and oocysts, each containing four free sporozoites are formed (Barker and Carbonell, 1974; Pohlenz et al., 1978; Iseki, 1979). Oocysts are passed in the faeces ready to infect the next host although sporozoites have been observed escaping from oocysts still attached to epithelial cells which would result in autoinfection of

the same host (Tyzzer, 1907; Iseki, 1979).

CRYPTOSPORIDIA AS POSSIBLE PREDISPOSING AGENTS.

Interest in cryptosporidia as possible predisposing agents to the colonisation of epithelial cells by bacteria was stimulated by work in progress at Moredun Research Institute during 1980. Dr. Saul Tzipori and other workers there had produced cryptosporidial enteritis in experimental calves, lambs and piglets using an inoculum derived from a field outbreak of cryptosporidial enteritis in calves.

In experimental transmission of the disease to SPF lambs there was widespread destruction of the microvilli of enterocytes due to extensive attachment of cryptosporidia and this effacement of the microvilli allowed enteric bacteria to adhere apparently non-specifically to the surface membrane of host epithelial cells. Figure 7.1 is an electron micrograph kindly supplied by Dr. Tzipori, of an SPF lamb five days after infection with cryptosporidia. The area shown includes three stages in the life-cycle of the protozoon and several adherent bacteria, possibly enterococci.

It seemed possible that a similar effect could be produced by dual infection of piglets with cryptosporidia and mucosalis. Enhanced attachment of mucosalis to host cell membranes seems logically the first step in penetration of the cell, intracellular multiplication, and the development of adenomatous change.

Unfortunately there was little published information at the time on the effects of cryptosporidial infection in piglets. The experiments carried out at Moredun (vide supra) had involved very few piglets.

Accordingly several experiments were carried out, in conjunction with Dr. Tzipori and Iris Campbell from Moredun, to determine the following:

(1) Whether cryptosporidia can consistently produce enteritis or heavy infections of the surface of piglet gut.

(2) Under what conditions the maximum infection with cryptosporidia is produced.

(3) Whether dual infection with cryptosporidia and mucosalis encourages the establishment of mucosalis in the gut of piglets.

(4) Whether such enhancement, if it occurs, leads to intracellular infection with mucosalis and the development of detectable adenomatous change.

MATERIALS AND METHODS.

(i) Management of Experimental Animals.

Piglets from 5 litters, farrowed by sows from 3 farms, were used:

Litter 1 - ABRO Skedsbush sow.

Litter 2 - Easter Bush sow.

Litter 3 - ABRO Mountmarle sow.

Litter 4 - ABRO Mountmarle sow.

Litter 5 - ABRO Mountmarle sow.

(see Chapter 2 for details of farms of origin of sows).

Sows were farrowed naturally in individual isolation. In all litters the piglets were allowed to suck colostrum. The sows remained healthy throughout.

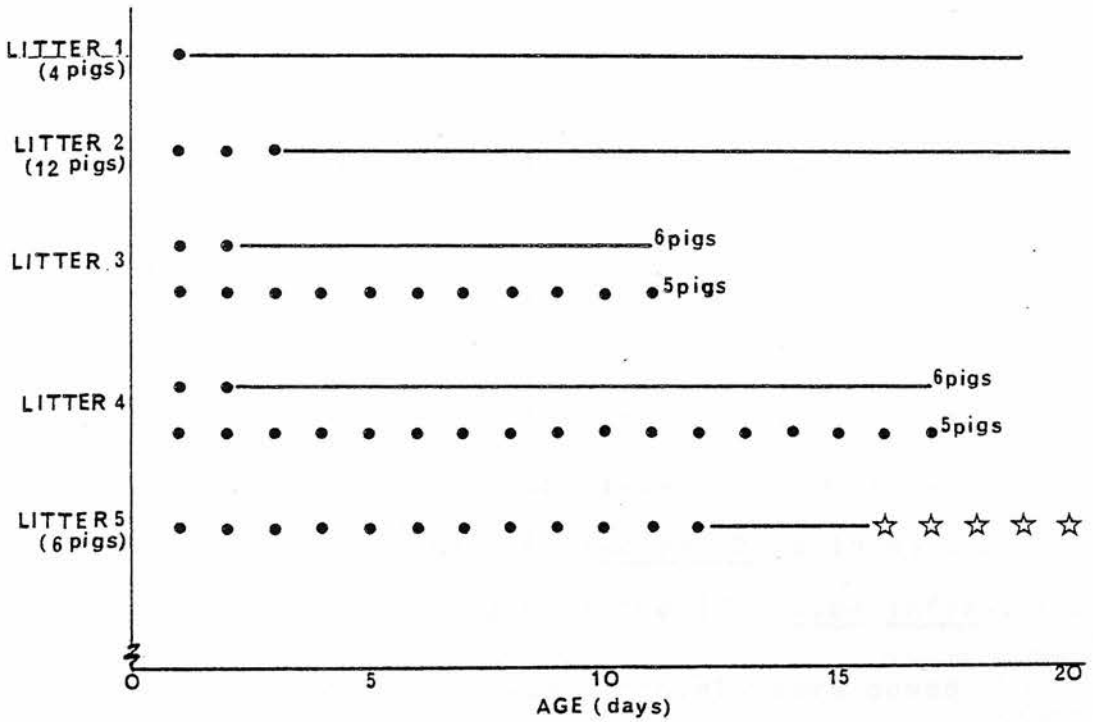
Litter 1: Four piglets were removed from the sow after 36 hours, housed individually in cages and fed the artificial milk diet described below.

Litter 2: Twelve piglets were removed after 72 hours, housed individually in cages and fed the artificial milk diet.

Litters 3 and 4: In each case 5 piglets were allowed to suck the sow throughout and the remaining 6 were removed after 48 hours, housed individually in cages and fed the artificial milk diet.

Litter 5: Six piglets were allowed to suck the sow for 12 days. Creep feed ("Finisher creep", without antibiotic, Seafield Mill, Easter Bush, Roslin) was offered from 2 days of age. The piglets were removed from the sow at 12 days of age and the artificial milk diet offered until 15 days, after which only creep feed and water were available ad libitum (Figure 7.2).

Figure 7.2: Management of Litters 1 - 5.



• = days sucking the sow

——— = artificial milk diet offered

☆ = creep feed and water ad libitum

(ii) Artificial Milk Diet.

This was fed 3 times daily and consisted of a 1:1 mixture of evaporated cows' milk (Carnation Foods Ltd., London) and mineral mixture (Appendix 5.2). Per feed each piglet was offered 200ml during the first 3 days of age, 300ml from 4 to 8 days of age and 400ml thereafter.

(iii) Design of Experiments (Figure 7.3).

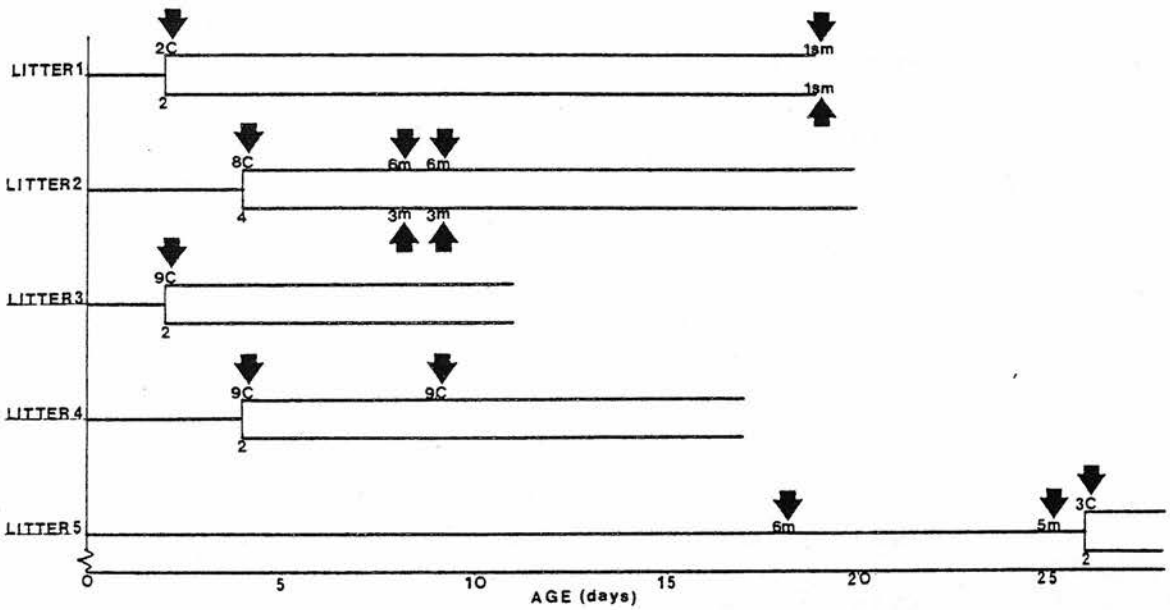
Litter 1: At 48 hours of age 2 of the 4 piglets were dosed orally with cryptosporidia (Inoculum A, vide infra) and the other 2 maintained separately as controls. One control and 1 exposed piglet were killed at 5 days of age (3 days post-cryptosporidia). The remaining 2 piglets were infected with mucosalis at 19 days of age by surgical inoculation of the TS1 (vide infra).

Litter 2: At 4 days of age 8 piglets were dosed orally with cryptosporidia (Inoculum B) and 4 piglets were maintained separately as controls. At 8 and 9 days of age 9 surviving piglets (3 controls, 6 exposed to cryptosporidia) were dosed orally with mucosalis.

Litter 3: The 5 sucking and 4 of the 6 cage-reared piglets were dosed orally at 48 hours of age with cryptosporidia (Inoculum C). The remaining 2 cage-reared piglets were maintained separately as controls.

Litter 4: Five sucking and 4 cage-reared piglets were dosed orally with cryptosporidia, at 4 days of age

Figure 7.3: Experimental Exposures of Litters 1 - 5 to Cryptosporidia and Mucosalis*



c = oral exposure to cryptosporidia

m = oral exposure to mucosalis

sm = surgical inoculation of mucosalis

* Numerals on figure denote the number of remaining piglets on each occasion

(Inoculum B) and 9 days of age (Inoculum C). The remaining 2 cage-reared piglets were maintained separately as controls.

No piglets from litters 3 and 4 were dosed with mucosalis.

Litter 5: Six piglets were orally dosed with mucosalis at 18 days of age and 5 of these remaining at 25 days of age were again dosed with mucosalis. At 26 days of age 3 of these were dosed orally with cryptosporidia (Inoculum B). The other 2 piglets were maintained separately as controls not exposed to cryptosporidia.

(iv) Experimental Inocula.

Cryptosporidia: The inocula used in these experiments were kindly provided by Dr. Tzipori, Moredun Institute, and were originally derived from a field outbreak of diarrhoea in calves attributed to cryptosporidia (Tzipori et al., 1980c). Faecal material from a scouring calf in this outbreak had been surgically inoculated into the duodenum of a conventional experimental calf (Tzipori, personal communication, 1981). Ileal scrapings from the latter calf were then homogenised 20% v/v in PBS (pH 7.2, 0.01M) and designated as Inoculum A. Inoculum A was propagated by oral dosing of first SPF rats and then SPF lambs (Tzipori et al., 1981b).

Ileal scrapings from the SPF lambs were prepared as described for Inoculum A, designated Inoculum B and passaged 5 times in SPF mice, by oral dosing. Ileal scrapings from the SPF mice, prepared as described above were designated as Inoculum C.

Inocula were maintained at 4°C until required. Aliquots of 2ml from Inocula A, B or C were used to dose each piglet. As the experiments were conducted over a 5 month period, storage times varied and were as follows:

Litter 1, Inoculum A, stored for 64 days.
Litter 2, Inoculum B, stored for 14 days.
Litter 3, Inoculum C, stored for 131 days.
Litter 4, Inoculum B, stored for 136 days.
Litter 4, Inoculum C, stored for 117 days.
Litter 5, Inoculum B, stored for 40 days.

The inocula were judged by culture to be free of enterotoxigenic *E. coli* (ETEC) and no enteric viruses were detected by electron microscopy (Tzipori, personal communication, 1980).

Mucosalis: The strain used was 209/80 L1-4 (see Chapter 2 for details). The inocula were prepared as described in Chapter 2 and the numbers of *mucosalis* in individual doses were as follows:

Litter 1, 10.18 log 10/piglet (19 days of age).
Litter 2, 10.51 log 10/piglet (8 days of age).
9.65 log 10/piglet (9 days of age).

Litter 5, 10.84 log 10/piglet (18 days of age).

10.65 log 10/piglet (25 days of age).

Piglets from litters 2 and 5 were dosed orally as described in Chapter 2. The 2 piglets from litter 1 were first anaesthetised by intracardiac barbiturate (see Chapter 2). The TSl was exteriorised, a loop 5cm long ligated and the inoculum injected using a sterile 5ml syringe and a fine needle. The piglets were maintained under anaesthesia for 3 hours and then necropsied.

(v) Clinical Observations.

Piglets were observed at least twice daily for signs of illness. The milk intake of the cage-reared piglets was recorded.

(vi) Other Procedures.

Each piglet was bled and weighed prior to the initial dosing procedure and again at necropsy.

Oral swabs were taken from piglets of litters 2 and 5 prior to and after exposure to mucosalis. These were cultured for mucosalis as described in Chapter 2.

Daily faecal samples were collected by rectal swabbing and examined for:

(a) ETEC by the method described in Chapter 6.

(b) Cryptosporidial oocysts by the examination of Giemsa-stained faecal smears (vide infra).

*c) Rotavirus by ELISA (Litters 1 - 4 only).

*d) Enteric viruses by direct electron microscopy (Litters 1 - 4 only).

(vii) Detection of Cryptosporidial Oocysts in Faeces.

Faecal swabs were moistened in 0.85% sterile saline and a thin smear made on a clean glass slide. The smears were air-dried, fixed in cold Methanol for 5 minutes and stained as follows:

** Giemsa solution	-	60 minutes
Distilled water	-	10 seconds
Absolute alcohol	-	5 seconds

The stained smears were cleared in xylol, mounted in D.P.X. and examined by light microscopy.

(viii) Indirect Immunofluorescent Test for Detection of Antibodies to Cryptosporidia.

Pre-exposure sera from piglets of litters 1 - 4 were examined using the method described by Tzipori and Campbell (1981). Briefly, cryostat sections of SPF lamb gut heavily infected with cryptosporidia were incubated with the piglet serum under test, diluted 1 in 10 in PBS (pH 7.2, 0.01M). The sections were counterstained with FITC-conjugated anti-swine IgG (Nordic Immunological Laboratories), and examined immediately for fluorescence in the area of the brush border using a Leitz-Ortholux Microscope and incident

* These procedures were kindly carried out by Dr. Tzipori, Moredun Institute.

** Giemsa 1ml, Methanol, 1.25ml, Na CO_3 1.4%

blue light. Cryostat sections from uninfected SPF lambs, stained as above, were used as negative controls. This test was performed at Moredun Institute by Iris Campbell, and interpreted by Dr. Tzipori, Iris Campbell and the present author.

(ix) Necropsy Procedures and Sites Sampled.

Piglets were necropsied under terminal anaesthesia as described in Chapter 2, and the sites sampled were as detailed in Chapter 2, with the exception of the stomach which was not sampled.

(x) Bacteriological Examinations at Necropsy.

(a) The MSl contents of all piglets were cultured for ETEC as described in Chapter 6.

(b) In litter 1, only the ligated TSl and the uninoculated USl of the piglets surgically inoculated with mucosalis were cultured for mucosalis.

(c) In litter 2, the USl, MSl, TSl, Caec and LB of the piglets dosed with mucosalis were cultured for mucosalis. Three piglets in this litter were killed or died prior to exposure to mucosalis, and in these only the MSl, TSl and LB were cultured for mucosalis.

(d) Litters 3 and 4, which were not exposed to mucosalis, were not cultured at necropsy for mucosalis.

(e) In litter 5 the USl, MSl, TSl, Caec and LB were cultured for mucosalis.

RESULTS

Exposure of some of the piglets to mucosalis did not appear to affect the results of the cryptosporidial exposures, and so for convenience this section will be presented as follows:

A. Results of Exposure to Cryptosporidia.

B. Results of Dual Exposure to Mucosalis and Cryptosporidia.

A. Results of Exposure to Cryptosporidia.

(i) Litters 1 and 2.

Clinical observations: Nine of the 10 piglets exposed to cryptosporidia in these 2 litters developed clinical signs of enteritis 1-3 days after exposure. The enteritis lasted 3-7 days and was characterised initially by vomiting (of 1-2 days duration) and then by diarrhoea (of 3-5 days duration). Two piglets (M1 and 184/80) were killed at the onset of clinical signs and another (185/80) died 3 days post-exposure. Cryptosporidial oocysts appeared in the faeces 1-3 days after the onset of clinical illness and were shed for 5-9 days (Table 7.1). Littermates not dosed with cryptosporidia remained healthy throughout.

Findings at Necropsy and Light and Electron Microscopic Observations: The findings at necropsy are correlated with the extent of cryptosporidial

TABLE 7.1.

THE CLINICAL RESPONSE OF LITTERS 1 AND 2 DOSED ORALLY AT 2 OR 4 DAYS OF AGE WITH CRYPTOSPORIDIA DERIVED FROM A CALF.

Piglet Number	DAYS OF AGE 2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
M1*	A		VSI	K															
30/80*	A		VI	VSI	SIO	SO	SO	SO	O	O	O	O						K	
184/80**			B		SIK														
185/80**			B	I	SI	D													
195/80**			B		VIS	VISO	ISO	SO	SOK										
198/80**			B		VI	IS	IS	ISO	ISO	ISO	O	OK							

Table continued overleaf:

TABLE 7.1
(CONTINUED)

Piglet Number	DAYS OF AGE																		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
200/80**			B			I S	I S O	I S O	I O	I O	O			K					
207/80**			B		I S	I S	I S O	I O	I O	I O	O	O	O			K			
208/80**			B			V I S	V I S	I S O	I S O	O	O	O	O	O	O				K
211/80**			B					O	O	O	O	O	O	O	O	O			K

Littermates not dosed with cryptosporidia remained healthy throughout.

A = Inoculum A
B = Inoculum B
V = Piglet vomiting
I = Piglet inappetent
S = Piglet diarrhoeic
O = Oocysts detected in faeces
D = Piglet found dead
K = Piglet killed
* = Piglets from litter 1
** = Piglets from litter 2

infection and the degree of mucosal damage in Table 7.2. Piglets M3 and 29/80 which were the piglets from litter 1 not exposed to cryptosporidia had no evidence of cryptosporidial infection and no mucosal abnormalities at necropsy. Piglets 189/80, 194/80, 197/80 and 206/80 which were the piglets of litter 2 not exposed to cryptosporidia likewise had no evidence of cryptosporidial infection and no mucosal abnormalities at necropsy.

In piglets exposed to cryptosporidia infection varied between sites and indeed between adjacent villi. Often a number of different forms of the life-cycle of the parasite could be seen on the same villus embedded in the brush border (Figures 7.4 - 7.7). In some heavily infected areas the cryptosporidia were seen attached deep within crypt glands (Figure 7.8). In the more severely affected piglets the large bowel was also infected, both in surface areas and deeper in the crypts (Figure 7.9).

Piglets M1, 185/80, 195/80 and 198/80, killed at the height of the clinical illness, exhibited similar intestinal lesions. These included villous stunting with fusion between adjacent villi (Figures 7.10 - 7.12), and oedema with inflammatory cell infiltration of the lamina propria. Loss of healthy vacuolated villar cells and their replacement by immature enterocytes was evident. These changes increased in severity

TABLE 7.2.

CORRELATION BETWEEN FINDINGS AT NECROPSY, DEGREE OF INFECTION WITH CRYPTOSPORIDIA AND EXTENT OF MUCOSAL DAMAGE IN THE SMALL INTESTINE, CAECUM AND SPIRAL COLON (LITTERS 1 AND 2).

Piglet Number	NECROPSY Days post-exposure	NECROPSY Intestinal Contents	DEGREE OF INFECTION* / DEGREE OF MUCOSAL DAMAGE** at Intestinal Sites Sampled: USl	Site 2	MSl	Site 4	TSl	Caec	LB
184/80	2	Fluid	++ / +	+ / +	++ / ++	+ / +	+ / +	- / -	- / -
M1	3	Fluid	+ / -	+ / -	++ / +	++ / ++	+++ / +++	+++ / +++	+++ / +++
185/80	3***	Fluid	+ / +	+ / +	+ / +	++ / ++	+++ / +++	+++ / +	++ / +
195/80	6	Fluid	+ / +	+ / -	+++ / +++	++ / +++	+++ / +++	+++ / -	+ / -
198/80	9	Fluid	+ / -	- / -	- / ++	+ / ++	++ / +++	- / -	- / -
200/80	11	Normal	- / -	- / -	- / -	++ / -	++ / -	- / -	- / -
207/80	13	Normal	- / -	- / -	- / -	- / +	- / +	- / -	- / -
208/80	16	Normal	- / -	- / -	- / -	- / -	- / -	- / -	- / -
211/80	16	Normal	- / -	- / -	- / -	- / -	- / -	- / -	- / -
30/80	17	Normal	- / -	- / -	- / -	- / -	- / -	- / -	- / -

* Degree of cryptosporidial infection: +++ = large numbers. ++ = moderate numbers. + = few. - = no cryptosporidia detected.

** Degree of mucosal damage: +++ = stunting and fusion of villi; replacement of enterocytes by immature cells; increased cellularity of lamina propria. ++ = only the first 2 of above. + = only the first 1 of above. - = no mucosal changes.

*** = piglet necropsied 4-8 hours after death.

towards the TSl where in piglet 185/80 sloughing of villous tips was apparent accompanied by an out-pouring of inflammatory cells (Figures 7.13 - 7.14). A few patchy areas of bacterial adherence to the damaged mucosal surface were observed in some areas of the TSl of piglets 195/80 and 198/80 (Figures 7.15 and 7.16). Caecal surface cells exhibited rounded luminal borders and were cuboidal rather than columnar in piglets Ml, 185/80 and 195/80.

In piglet 184/80, killed shortly after the onset of clinical illness, the histological lesions were mild and largely confined to the MSl. Cryptosporidial infection appeared more extensive in the USl and MSl with no evidence of infection of the Caec or LB. Piglets 200/80, 207/80, 208/80, 211/80 and 30/80, killed between 11 and 17 days after exposure when clinical recovery appeared complete, showed neither significant histological lesions nor, except for piglet 200/80, any evidence of cryptosporidial infection.

Aerobic Bacteriology: Bacteriological examination of daily faecal swabs and of mid-small intestinal contents at necropsy yielded a mixed growth of α-haemolytic streptococci and non-haemolytic *E. coli*. A few of the *E. coli* colonies possessed the adhesion pili 987P (987P+) or K99 (K99+). Of the 10 piglets exposed, 3 shed 987P+ *E. coli* on 1 occasion (piglets 185/80, 200/80 and 208/80) and from 3 piglets (184/80,

185/80 and 211/80) isolation was made from the MSl at necropsy. K99+ E. coli was isolated from the MSl at necropsy of piglet 200/80, 4 days after clinical recovery. However the proportion of pilus-bearing E. coli colonies was small, and there was no histological evidence of coliform adherence to the mucosa of any of these piglets at necropsy. None of the 6 piglets not exposed to cryptosporidia shed E. coli bearing K88, 987P or K99 pili.

Detection of Enteric Viruses: No enteric viruses were detected in the faeces of litters 1 and 2 at any time.

(ii) Litters 3 and 4.

Clinical Observations: The incubation period was longer (2-7 days), clinical signs were milder and of shorter duration in litter 3 after exposure to cryptosporidia. The 5 sucking piglets were more severely affected than the 4 artificially-fed piglets and there was good correlation between the clinical response, the degree of infection with cryptosporidia and the extent of mucosal damage (Table 7.3). The sucking piglets were not observed to vomit. Cryptosporidial oocysts were detected in the faeces of 5 of the exposed piglets (Table 7.3).

Litter 4 remained healthy throughout the experiment despite 2 successive exposures at 4 and 9 days of age respectively. In this litter there was also good correlation between the absence of clinical signs and

TABLE 7.3.

SUMMARY OF CLINICAL SIGNS, DEGREE OF CRYPTOSPORIDIAL INFECTION AND EXTENT OF HISTOLOGICAL CHANGES OBSERVED IN LITTER 3 DOSED WITH CRYPTOSPORIDIA (INOCULUM C) AT 2 DAYS OF AGE.

Group Management	Piglet Number	Incubation (days)	Clinical Manifestations at days of age. *** V	I	S	O	Age (days) when killed	Histological Observations (MS1, Site 4 and TS1) Cryptosporidial Infection*	Mucosal** Damage
Exposed piglets, sucking the sow	294/80	-	-	-	-	-	5	++	+
	297/80	6	-	-	8	8	8	++	+++
	299/80	7	-	-	9	9	9	++	+++
	300/80	6	-	-	8,9	10	10	++	+
	304/80	3	-	-	5,9	11	11	+	++
Exposed piglets, reared in cages	296/80	5	7	-	-	-	8	+	+
	298/80	4	6	9	-	-	9	+	+
	301/80	2	4	4,9	-	10	10	++	+++
	303/80	-	-	-	-	-	11	-	-
Not exposed, reared in cages	295/80	-	-	-	-	-	8	-	+++
	302/80	-	-	-	-	-	11	-	-

* Degree of cryptosporidial infection: ++ = moderate numbers. + = few. - = no cryptosporidia detected.

** Degree of mucosal damage: +++ = stunting and fusion of villi; replacement of enterocytes by immature cells; increased cellularity of lamina propria.
++ = only the first 2 of above. + = only the first 1 of above. - = no mucosal damage.

*** Clinical Manifestations: V = vomiting. I = inappetence. S = diarrhoea. O = Oocysts detected in faeces.

insignificant histological observations (Table 7.4). Cryptosporidial oocysts were detected in the faeces of 4 of the exposed piglets (Table 7.4).

Necropsy Findings and Histological Observations: There were no gross abnormalities apart from fluid large intestinal contents in 3 exposed piglets of litter 3 (piglets 297/80, 300/80 and 298/80). Eight of the 9 exposed piglets in litter 3 had evidence of light to moderate cryptosporidial infection in the small intestine but not in the large bowel. Mucosal changes varied from slight to moderate and were restricted to the mid and lower small intestine (Table 7.3). One exposed piglet (295/80) had a moderate degree of mucosal damage in the mid and lower ileum, including stunting and fusion of villi accompanied by increased cellularity of the lamina propria and replacement of enterocytes by immature cells. These changes were similar to those found in the exposed piglets of litter 3 but no cryptosporidia were detected, nor were other recognised enteropathogens detected.

In litter 4 mucosal damage, which was confined to the ileum, was slight. In 4 of the 9 exposed piglets infection was not detected, either by histological examination or by searching Giemsa-stained faecal smears for oocysts.

TABLE 7.4.

SUMMARY OF HISTOLOGICAL FINDINGS IN LITTER 4 WHICH DID NOT SHOW CLINICAL ILLNESS AFTER 2 CHALLENGES WITH CRYPTOSPORIDIA AT 4 DAYS (INOCULUM B) AND 9 DAYS (INOCULUM C) OF AGE.

Group Management	Piglet Number	Oocysts in Faeces at days of age	Age(days) when killed	Histological Observations in the small intestine. Degree of: Cryptosporidial Infection*	Mucosal Damage**
Exposed piglets, sucking the sow	273/80	11	12	++	+
	275/80	-	15	+	+
	277/80	-	16	-	+
	278/80	-	16	-	+
	282/80	-	17	-	+
Exposed piglets, reared in cages	272/80	-	12	-	+
	276/80	11,14	15	-	+
	279/80	11	16	+	+
	283/80	11	17	-	+
Not exposed, reared in cages	274/80	-	15	-	-
	281/80	-	17	-	-

* Degree of cryptosporidial infection: ++ = moderate numbers. + = few. - = no cryptosporidia detected.

** Degree of mucosal damage: + = minor changes of villous stunting and increased cellularity of the lamina propria.
- = no mucosal damage.

Aerobic Bacteriology: Culture of faecal swabs and of mid-small intestinal contents did not result in isolation of E. coli possessing K88, K99 or 987P pili from litters 3 and 4.

Detection of Enteric Viruses: No enteric viruses were detected in the faeces of litters 3 and 4.

(iii) Litter 5.

Clinical Observations: None of the 6 piglets of litter 5 showed any signs of illness throughout the experiment. No cryptosporidial oocysts were detected in the faeces at any time.

Necropsy Findings and Histological Observations: There were no gross abnormalities in any piglet at necropsy.

Piglet Y22/80, not exposed to cryptosporidia and killed 4 days prior to exposure of littermates, was found at necropsy to have a light cryptosporidial infection throughout the small intestine. There was no associated mucosal damage (Table 7.5). Three exposed piglets (Y28/80, Y23/80, Y25/80), killed between 3 and 13 days after exposure, and one other control piglet (Y27/80) were moderately infected with cryptosporidia at necropsy. Histological changes in these included mild stunting and fusion of villi, slightly increased cellularity of the lamina propria and mild crypt hyperplasia. The large bowel was

TABLE 7.5.

SUMMARY OF RESULTS OF CRYPTOSPORIDIAL EXPOSURE (LITTER 5).

Piglet Number	Clinical Signs	Oocysts in Faeces	Age(days) when killed	Days post-exposure	Gross findings at necropsy	Histological Observations in the small intestine Degree of:- Cryptosporidial Infection*	Mucosal Damage**
Y28/80	None	None	29	3	None	+	+
Y23/80	None	None	34	8	None	+	+
Y25/80	None	None	39	13	None	+	-
Y22/80	None	None	22	Not exposed	None	+	-
Y27/80	None	None	35	Not exposed	None	+	+
Y21/80	None	None	40	Not exposed	None	-	-

* Degree of cryptosporidial infection: + = a few cryptosporidia detected
- = no cryptosporidia detected

** Degree of mucosal damage: + = slight stunting and fusion of villi accompanied by increased cellularity of the lamina propria.
- = no mucosal damage.

unaffected. The third control piglet (Y21/80), killed at the end of the experiment, showed neither histological changes nor any evidence of cryptosporidial infection (Table 7.5).

Aerobic Bacteriology: There were no isolations of pilus-possessing E. coli from litter 5 during the course of the experiment.

(iv) Litters 1-4 Immunofluorescent Results (Cryptosporidia): All the pre-inoculation sera from the 22 piglets of litters 3 and 4 were positive in the indirect test, producing extensive fluorescence in the brush borders of infected sections. Weak reactions were produced by the pre-inoculation sera of 7 piglets in litters 1 and 2 while 9 others were negative.

B. RESULTS OF DUAL EXPOSURE TO CRYPTOSPORIDIA AND MUCOSALIS (LITTERS 1, 2 AND 5).

(i) Litter 1: Piglet 29/80 and 30/80 of litter 1 were exposed to mucosalis 3 hours prior to necropsy at 19 days of age, by surgical inoculation of the TS1 (see Materials and Methods).

Findings at Necropsy: There was no gross or histological evidence of adenomatous change in either piglet. The histological observations were as described in section A. In addition the surgically

inoculated portions of TSl had minor changes of submucosal oedema and distension of the lymphatics.

Young's Stains: In both piglets CLO's were observed in clumps in the lumen of the TSl. Occasional groups of CLO's were in close association with villar cells but these were not a prominent feature. No bacteria were seen below the level of the crypt-villus junctions nor were any found in intracellular sites. In piglet 30/80 a few CLO's were observed in the lumen of the USl, occasionally close to villar cells.

Electron Microscopy: There were no ultrastructural abnormalities and no CLO's were observed.

Immunofluorescence: Bright particulate fluorescence was observed in the lumen of both the USl and TSl of piglets 29/80 and 30/80, but appeared more extensive in the TSl.

Microaerophilic Bacteriology: High numbers of mucosalis were isolated from the surgically inoculated TSl of both piglets. Surprisingly mucosalis was also isolated, but in lower numbers, from the uninoculated portions of USl (Table 7.6).

(ii) Litter 2.

Findings at Necropsy: There was no gross or histological evidence of adenomatous change in any piglet. The histological observations were as described in section A. Piglets not exposed to

TABLE 7.6.

LITTER 1, ISOLATION OF MUCOSALIS FROM PIGLETS 29/80 AND 30/80.

Piglet 29/80*	Piglet 30/80**
US1 - 2.30 log 10/g	US1 - 4.11 log 10/g
TS1 - 7.81 log 10/g	TS1 - 7.98 log 10/g

* Piglet 29/80 - no previous exposure to cryptosporidia.

** Piglet 30/80 - exposed to cryptosporidia at 2 days of age.

cryptosporidia had little evidence of bacteria in close association with the surface of enterocytes. In piglets exposed to cryptosporidia close bacterial association with the surface of the mucosa was more common but still infrequently observed. In piglets 195/80 and 198/80 there were patchy areas in the lower small intestine where bacteria of mixed morphology were seen adhering to the surface of the damaged mucosa (Figures 7.15 - 7.20). Few of the bacteria could be identified as vibrioid and the majority were rod-shaped or coccal.

Microaerophilic Bacteriology: The numbers and sites of isolation of mucosalis are listed in Table 7.7. Relatively low numbers of mucosalis were isolated from a minority of sites.

Statistical analysis of the data in Table 7.7 (see Appendices 7.1 and 7.2) indicated that:

(a) Mucosalis did not colonise significantly different sites in the gut of piglets exposed to cryptosporidia in comparison to piglets not exposed to cryptosporidia.

(b) Mucosalis did not colonise the alimentary tract of piglets exposed to cryptosporidia more readily than piglets not exposed to cryptosporidia.

(c) The numbers of mucosalis isolated from piglets exposed to cryptosporidia were not significantly different from the numbers isolated from piglets not exposed to cryptosporidia.

TABLE 7.7.

LITTER 2, ISOLATIONS OF MUCOSALIS FROM THE ORAL CAVITY AND FROM THE GUT AT NECROPSY.

Piglets exposed to cryptosporidia and mucosalis			Piglets exposed to mucosalis only		
Piglet Number	Site	log 10/g mucosa	Piglet Number	Site	log 10/g mucosa
195/80	Mouth	-	194/80	Mouth	-
	USl	0		USl	0
	MSl	2.78		MSl	0
	TSl	2.90		TSl	2.60
	Caec	0		Caec	0
	LB	0		LB	0
198/80	Mouth	+	197/80	Mouth	-
	USl	2.78		USl	0
	MSl	2.30		MSl	0
	TSl	0		TSl	0
	Caec	0		Caec	0
	LB	0		LB	0
200/80	Mouth	+	206/80	Mouth	+
	USl	2.30		USl	0
	MSl	3.00		MSl	2.78
	TSl	0		TSl	0
	Caec	2.78		Caec	0
	LB	0		LB	0

Table continued overleaf:-

TABLE 7.7.

(CONTINUED)

Piglets exposed to cryptosporidia and mucosalis			Piglets exposed to mucosalis only		
Piglet Number	Site	log 10/g mucosa	Piglet Number	Site	log 10/g mucosa
207/80	Mouth	+			
	USl	0			
	MSl	0			
	TSl	0			
	Caec	0			
	LB	0			
208/80	Mouth	+			
	USl	2.60			
	MSl	0			
	TSl	0			
	Caec	0			
	LB	0			
211/80	Mouth	+			
	USl	0			
	MSl	0			
	TSl	3.00			
	Caec	0			
	LB	0			

\+ = isolation of mucosalis but not quantified.

0 } = mucosalis not isolated.

TABLE 7.8.

LITTER 5, ISOLATIONS OF CAMPYLOBACTERS FROM THE ORAL CAVITIES.

Piglet Number	AGE (DAYS) WHEN:					
	Pre-exposure sample 12	Exposed to *Mucosalis*	Post-exposure samples			
			20	25	35	Killed
Y22/80	NAC's	18	M	na	na	22
Y28/80	-	18,25	-	M	na	29
Y23/80	NAC's	18,25	M	M	na	34
Y27/80	NAC's	18,25	M	M	M	35
Y25/80	-	18,25	-	M	M	39
Y21/80	-	18,25	-	-	-	40

NAC's = non-agglutinating campylobacters isolated.

M = *mucosalis* isolated.

na = not applicable.

- = no campylobacters isolated.

(iii) Litter 5.

Findings at Necropsy: There were no gross or histological lesions of adenomatosis in any piglet. The main histological findings have been described in section A.

Young's Stains: There was no evidence in any piglet of close bacterial association with either villar or crypt cells and no bacteria were observed in intracellular sites.

Microaerophilic Bacteriology: Mucosalis was not recovered from the oral cavity of any piglet prior to exposure but was recovered from 5 of the 6 piglets after exposure. Catalase-negative, non-agglutinating campylobacters (NAC's) were isolated from 3 piglets prior to exposure to mucosalis (Table 7.8).

No isolations of mucosalis were made from the gut at necropsy of any piglet.

DISCUSSION

Effects of Exposure to Cryptosporidia.

The results of these experiments demonstrate that cryptosporidia can cause enteritis, under certain conditions, in piglets exposed during the first week

of life. The clinical response varied among litters 1-4 from severe illness, characterised by anorexia, vomiting and diarrhoea, to subclinical infection only. However variations were much greater between litters than within, indicating that external factors, such as the level of maternal immunity or the nature of the inoculum, could have been responsible. The correlations between the length of the incubation period, the severity of clinical illness, the extent and degree of mucosal damage and the extent and degree of intestinal infection by cryptosporidia, suggest that these protozoa were the causative agents. Full assessment of the enterocolitis which cryptosporidia induce in piglets may have to be attempted in animals devoid of maternal protection and free of previous exposure to cryptosporidia.

It seems possible that cryptosporidial infection is prevalent. Two piglets from litter 5 (Y22/80 and Y23/80) which had not been dosed with cryptosporidia were found at necropsy to be infected. As 1 of these (Y22/80) had been killed 4 days prior to dosing of littermates with cryptosporidia, natural infection seems more likely than cross-contamination from experimentally-exposed piglets. A serological study conducted recently on a variety of species, including pigs, further supports the theory that cryptosporidial infection is widespread (Tzipori and

Campbell, 1981). The extent of infection in the national pig herd is unknown at present. Whether cryptosporidiosis occurs as a primary disease, a sub-clinical infection or as a predisposing agent to other enteric pathogens also remains to be determined.

Cryptosporidiosis in other animals is now recognised as a cause of diarrhoea. It has been suggested as a potential zoonosis (Tzipori et al., 1980a; Tzipori and Campbell, 1981), and the oocysts are extremely resistant to the action of many common disinfectants (Campbell, personal communication, 1981), factors which should be considered in relation to the management of effluent disposal.

Examination of pre-exposure sera by the indirect immunofluorescent test demonstrated that piglets from litters 1 and 2, the 2 most severely affected litters, received little or no specific maternal antibodies against cryptosporidia. Differences in levels of antibodies may have accounted for the clinical variations observed between litters. The serological reaction between pig sera and lamb ileum infected with calf-derived cryptosporidia further supports the view that both calf and pig cryptosporidia share common antigens or may even be members of the same species (Tzipori et al., 1980a; Tzipori and Campbell, 1981).

The experiments were conducted over a 5 month period and methods thus far evaluated for long term

storage of the organism in the laboratory or quantification of viable oocysts have not been successful (Tzipori, personal communication, 1980). Although the 3 inocula were derived from the same original source, an outbreak of cryptosporidiosis in calves, they were passaged in different species of animals, stored for various time periods, and probably contained different numbers of viable oocysts. These variables pertaining to the inocula may in part explain the observed differences in the response of dosed piglets. However the passage of cryptosporidia in rodents does not appear to affect the pathogenicity of the protozoa for SPF lambs (Tzipori et al., 1981b).

The isolation of 987P+ and K99+ E. coli from 5 piglets of litter 2 (piglets 184/80, 185/80, 200/80, 208/80 and 211/80) may have been a factor in precipitating a more acute disease in this litter compared with litters 3 and 4. The limited excretion of these pilus-possessing E. coli over the experimental period and the lack of bacterial adherence to the mucosa of these piglets at necropsy suggest, however, that their role at best was minor. It could be argued that the mucosal damage and digestive disturbance in piglets infected with cryptosporidia are changes which favour luminal proliferation of coliforms. No pilus-possessing E. coli were

isolated from the faeces of 4 littermates not exposed to cryptosporidia.

The histological changes observed in one control piglet (295/80) of litter 3 cannot be explained. No known enteric pathogens were observed in association with the lesions or demonstrated by the techniques described, and neither were cryptosporidia observed attached to the mucosa. Litter 4 remained healthy throughout the observation period despite 2 successive exposures at 4 and 9 days of age respectively. However in comparison to litters 1, 2 and 3, the longer interval between initial exposure and necropsy could account for the failure to detect infection and the lack of mucosal damage at necropsy.

It was unexpected that the sucking piglets of litter 3 were more severely affected than artificially-reared littermates (Table 7.3). In contrast rotavirus induces severe diarrhoea and mortality in artificially-reared, colostrum-fed piglets but very little in sucking littermates (Tzipori and Williams, 1978).

The demonstration of oocysts in the faeces as a method for the diagnosis of cryptosporidiosis in piglets is unsatisfactory. Even in acute cases of diarrhoea, shedding of oocysts commenced 1-3 days after the onset of illness. In less acute cases detection of oocysts in the faeces was extremely

difficult. Often prolonged searching of the faecal smears was required before an oocyst was found.

The pathogenesis and clinical manifestations of cryptosporidiosis in piglets, apart from vomiting which appears characteristic of man and piglets (Tzipori et al., 1980b), are similar to those observed in lambs (Tzipori et al., 1981b), calves (Tzipori et al., 1980c) and deer (Tzipori et al., 1981a).

Dual Infection of Cryptosporidia and Mucosalis.

Surgical inoculation of mucosalis into the TSl of 2 piglets (29/80 and 30/80) of litter 1 resulted in recovery of high numbers of mucosalis at necropsy 3 hours later (Table 7.6). There was, however, no evidence of bacterial attachment to the mucosa or intracellular penetration by CLO's in either piglet.

It was not possible to assess accurately whether multiplication of mucosalis occurred in the TSl of these piglets. The inoculated dose of mucosalis per piglet was 1.5×10^{10} and the mean isolation /g mucosa was 8.0×10^{7} mucosalis (mean = [numbers/g 29/80 + numbers/g 30/80] ÷ 2). Thus the weight of mucosa required for the counts of mucosalis to equal the total inocating dose was 188g ($1.5 \times 10^{10} \div 8.0 \times 10^{7}$). The total weight of mucosa in each ligated loop was not known but it is unlikely to have exceeded 188g since the loops were only 5cm in length. Other

unknown factors which prevented accurate assessment of the multiplication of mucosalis within the loops were the weight of the intestinal contents, the numbers of mucosalis present in the contents at necropsy, and whether mucosalis infection was present in the loops prior to surgical exposure. Mucosalis was also isolated, but in lower numbers, from the uninoculated USl of these 2 piglets (Table 7.6).

The above findings are consistent with those of Kurtz, Soto and McAllister (1980) who performed similar surgical exposures in pigs aged 8 weeks, sacrificing at 24-72 hours post-inoculation. These workers found no evidence of penetration of the mucosa by mucosalis and a substantial fall in the total numbers of mucosalis by the time of necropsy. Occasionally they isolated mucosalis from control loops.

The maximum attachment of mucosalis to cells cultured in vitro occurs at about 8 hours post-infection (Rajasekhar, 1981) so it is possible that if a similar mechanism operates in vivo, piglets killed 8 hours post-infection may show such attachment. However other complex factors such as the immune status of the piglets, prior to exposure to mucosalis and mucosal defence mechanisms are likely to influence such in vivo experiments.

Perhaps the most interesting findings in litter 1 are the numbers of mucosalis isolated from the USl

of piglets 29/80 and 30/80. If these mucosalis were indeed a "natural" infection and unrelated to the surgical exposure of the TSl, then piglet 30/80, which had recovered from cryptosporidial enteritis, had acquired approximately 2 log 10 more mucosalis/g than its littermate 29/80 (Table 7.6). It is tempting to suggest that cryptosporidia had created more favourable conditions for or enhanced in some way the establishment of mucosalis in the mucosa of piglet 30/80. One possible mechanism whereby such enhancement could occur has been mentioned in the introduction to this chapter i.e. destruction of host microvilli by cryptosporidia could allow initial attachment of mucosalis to the host cell, followed logically by penetration and intracellular multiplication. However there was no evidence from the light microscopy that CLO's were present in intracellular situations by the time of necropsy. On the other hand disturbance of gut motility by cryptosporidial-induced enteritis could favour luminal multiplication of mucosalis, in a similar manner to the enhanced establishment of mucosalis in the gut of benzetimide-treated piglets (Roberts, 1978).

In order to more fully appreciate the effect of cryptosporidia on the establishment of mucosalis in the gut, the results of the dual exposures in litters 2 and 5 must be considered.

Heavy infection of the gut by cryptosporidia was

achieved in litter 2 and mucosalis was isolated from piglets in both groups, whether exposed to cryptosporidia or not. However statistical analysis of these results suggests that, for litter 2, cryptosporidial infection did not influence the colonisation patterns of mucosalis or promote the establishment of significantly higher numbers of mucosalis. It seems likely therefore that the difference in the numbers of mucosalis isolated from the USI of piglets 30/80 and 29/80 of litter 1 may not have been due to previous cryptosporidiosis in piglet 30/80.

Cryptosporidial infection in litter 5 was light and not confined to those piglets experimentally-exposed to cryptosporidia. It was not possible therefore to compare the colonisation patterns of mucosalis in the 2 groups since it seems likely that a "natural" exposure to cryptosporidia had occurred and resulted in the infection of 2 controls. Mucosalis was not recovered from the gut of any piglet in litter 5, results which are consistent with Roberts (1978) who found weaned pigs refractory to infection.

The results of the dual exposure experiments suggest that, overall, cryptosporidia do not promote mucosal penetration by mucosalis. Recently Isospora suis, another enteric coccidian parasite, has been implicated in outbreaks of piglet enteritis (Sangster et al., 1978; Roberts and Walker, 1980). This

parasite may be worth investigating as an alternative initiator to cryptosporidia, since the endogenous forms penetrate deep within enterocytes and may therefore allow mucosalis improved "access" to host cells.

CONCLUSIONS*

(a) It seems possible that exposure to cryptosporidia can, under certain conditions, result in heavy infections and enterocolitis in piglets.

(b) The maximum effect is produced in piglets exposed at an early age and particularly if specific maternal antibody is low or absent.

(c) Dual exposure to cryptosporidia and mucosalis does not enhance the establishment of mucosalis in the gut of piglets, whether milk-fed or creep-fed.

(d) It is unlikely that cryptosporidia are important in the pathogenesis of intestinal adenomatosis.

* Aspects of this work have been published (Tzipori et al., 1981c).

CHAPTER 8

FINAL DISCUSSION AND CONCLUSIONS.

The primary aims of the work reported in this thesis, to reproduce intestinal adenomatosis in experimental piglets and hence to study the aetiology and pathogenesis of the disease, were not achieved. Cross-suckled piglets exposed to adenomatous mucosa and cultures of *mucosalis*, in a similar experiment to a previously successful transmission (Roberts, 1978), did not develop intestinal adenomatosis. The lack of a consistently successful experimental method of reproducing PIA inevitably leaves the aetiology and pathogenesis of the disease in doubt.

There is much evidence that in the majority of field cases of PIA, *mucosalis* is the intracellular bacterium associated with adenomatous lesions, although in one study of PIA in a minimal disease herd, *mucosalis* was not isolated (Roberts *et al.*, 1979).

Hence 2 suppositions dictated the experimental designs in Chapters 4 - 7:

(i) that lesions of adenomatosis may contain all the necessary factors for transmission of the disease, whether the identity of the intracellular bacterium is *mucosalis* or another antigenically-distinct campylobacter.

(ii) since *mucosalis* is inextricably associated with the majority of cases of PIA, transmission attempts

should involve exposure of susceptible piglets to mucosalis.

In the absence of evidence to the contrary it was considered that:

(a) maternally-acquired immunity could inhibit the establishment of mucosalis in the gut lumen.

(b) the establishment of substantial numbers of mucosalis in the lumen of the gut is a necessary prerequisite before extensive parasitism of host enterocytes.

(c) extensive adherence of mucosalis to host enterocytes may be an important transitional step between extracellular commensalism and intracellular parasitism.

(d) once mucosalis succeed in extensive adherence to gut epithelial cells, penetration of cells and intracellular multiplication results.

(e) lesions of adenomatosis develop after extensive infection of epithelial cells by mucosalis, and infected cells spread throughout the depth of the mucosa, probably by continued host-cell division.

Colostrum-deprived piglets were exposed to adenomatous mucosa, in order to avoid the potentially inhibitory effects of sow colostrum and milk on the establishment of mucosalis, or other essential agents, in the gut. The results suggested that the absence of maternally-acquired protection did not particularly enhance the establishment of mucosalis. Moderate

numbers were isolated from only 1 exposed piglet, the last one necropsied. This apparent delay in the establishment of mucosalis may have been due to the crude nature of the inoculum, which probably contained antibody-coated mucosalis. There was some evidence of limited penetration of enterocytes by CLO's in the exposed group, but the isolation of large numbers of catalase-positive campylobacters prevented the assertion that the occasional intracellular CLO's were mucosalis.

The colostrum-deprived piglets did not survive for long enough to assess whether lesions of adenomatosis would have developed. Future work aiming to identify essential initiators (other than mucosalis) present in adenomatous mucosa could prove fruitful if older colostrum-deprived piglets are used. These would be more likely to survive exposures to crude inocula.

Gnotobiotic piglets exposed to mucosalis provided convincing evidence that limited uptake of mucosalis by enterocytes occurs in the early period post-exposure, but that substantial intracellular multiplication does not. These results are in accordance with studies in conventional piglets (Duncan, 1974; Roberts, 1978). There was some evidence from the gnotobiotic work that the presence of a large number of contaminating bacterial species severely

depress the numbers of *mucosalis* *in vivo*. Interestingly there was strong evidence, again from the gnotobiotic experiments, that neither crypt epithelial cells nor villar enterocytes are *per se* very susceptible to invasion by *mucosalis*, and that *mucosalis* "in isolation" is not able to penetrate the mucosa or establish intracellularly to any great extent. However the gnotobiotic piglet proved itself to be the most useful experimental system to date in which to study factors enabling *mucosalis* to forsake the lumen of the gut and invade host cells, a challenge for future investigators.

Pilot studies in weaned pigs did not implicate either rotavirus or 987P+ ETEC as possible initiators of intracellular *mucosalis* infection. Weaned pigs were extremely refractory to the establishment of *mucosalis* infection, results consistent with those of Roberts (1978).

Cryptosporidia were examined as possible precursors of intestinal adenomatosis for 3 reasons:
(i) there was evidence that these protozoa encouraged bacterial adherence to the enteric mucosa of lambs.
(ii) it was considered that if cryptosporidia could enhance the adherence of *mucosalis* to pig enteric mucosa, there could result extensive invasion of enterocytes.
(iii) the gnotobiotic results suggested that some

other agent would be necessary to promote penetration of the mucosa by mucosalis.

However there was little difference between the behaviour of mucosalis in piglets exposed to cryptosporidia and its behavior in controls, and cryptosporidia did not appear to enhance greatly bacterial adherence to the enteric mucosa of exposed piglets.

Future work of this kind is not an encouraging prospect as the investigator perforce has to adopt a "trial and error" approach in the search for initiating agents or factors. However should further in vivo studies be undertaken, the gnotobiotic pig seems the most promising subject. The advantages offered are:

(a) Mucosalis establishes and persists in substantial numbers in the lumen of the alimentary tract of this type of animal.

(b) Important and unknown variables such as the level of maternally-acquired immunity, prior exposure to mucosalis, and the contaminating microflora can, to some extent, be eliminated or controlled.

Improvement in the techniques for rearing gnotobiotic piglets would of course facilitate future studies. For example it is unclear whether the presence of specific agglutinating antibody to mucosalis in the sera of older weaned pigs reflects a prior episode of mucosalis infection, and in particular if

serum antibody is produced as a result of infection of the oral cavity, the lumen of the gut, or only after multiplication of *mucosalis* within host enterocytes. To investigate this problem would require the maintenance of gnotobiotic pigs for a much longer period than was possible in the work reported here, since it is rare to find specific circulating antibody versus *mucosalis* in pigs less than 70 days of age (Lawson *et al.*, 1982).

Although the expense of such long-term gnotobiotic experiments tends to discourage "trial and error" approaches, promising results could perhaps be studied further in the *in vitro* systems developed by Rajasekhar (1981). Such *in vitro* studies may be of use in explaining observed biological facts, thus enabling economy in the use of experimental gnotobiotic animals.

BIBLIOGRAPHY

ABELSETH, M.K. (1962). Canadian Veterinary Journal. 3, 48.

ADSERSEN, V. (1929). Medlemsblad for den danske Dyrlaegeforening. 12, 149.

ADSERSEN, V. (1930). Medlemsblad for den danske Dyrlaegeforening. 13, 183.

ADSERSEN, V. (1931). Maanedsskrift for Dyrlaeger. 43, 145.

ADSERSEN, V. (1932). Maanedsskrift for Dyrlaeger. 44, 465.

AHERNE, F., HAYS, V.W., EWAN, R.C. and SPEER, V.C. (1969). Journal of Animal Science. 29, 444.

ALEXANDER, T.J.L. (1960). "The Establishment of Isolation Units for Newborn Pigs and Studies on an Encephalomyelitis of Nursing Pigs". Masters Thesis. Ontario Veterinary College, Canada.

ALEXANDER, T.J.L. (1981). in "Diseases of Swine.", 5th Edition, Edited by Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., Iowa State University Press, Ames, Iowa, U.S.A., p 696.

ALEXANDER, T.J.L. and ROE, C.K. (1962). Canadian Veterinary Journal. 3, 299.

ALEXANDER, V.A.W. (1969). "Studies on the Nutrition of the Neonatal Pig". PhD Thesis. University of Edinburgh.

ALLEN, M.M. and SAUNDERS, R.W. (1976). Proceedings of the International Pig Veterinary Society Congress. Ames, Iowa, U.S.A., pNS.

ALLEN, W.D., SMITH, C.G. and PORTER, P. (1973). Immunology. 25, 55.

ALLEN, W.D., SMITH, C.G. and PORTER, P. (1976). Immunology. 30, 449.

AL-MASHAT, R.R. and TAYLOR, D.J. (1980). Veterinary Record. 107, 31.

AMEND, N.K., LOEFFLER, D.G., WARD, B.C. and VAN HOOSER, G.L. (1976). Laboratory Animal Science. 26, 566.

AMTOWER, W.C. and CALHOUN, J.R. (1964). Proceedings of the Animal Care Panel. 14, 382.

ASKAA, J. and BLOCH, B. (1981). Acta Veterinaria Scandinavica. 22, 32.

AUMAITRE, A. and SEVE, B. (1978). Annales de Recherches Vétérinaires. 9, 181.

BAILEY, N.T.J. (1969). "Statistical Methods in Biology". 5th Impression, The English Universities Press Ltd., London.

BALO, J. and KORPASSY, B. (1935). Allatorvosi Lapok. 58, 219.

BALO, J. and KORPASSY, B. (1939). Allatorvosi Lapok. 62, 215.

BARKER, I.K. and CARBONELL, P.L. (1974). Zeitschrift für Parasitenkunde. 44, 289.

BARRICK, E.R., MATRONE, G. and OSBORNE, J.C. (1954). Proceedings of the Society for Experimental Biology and Medicine. 87, 92.

BARTHOLD, S.W., COLEMAN, G.L., BHATT, B.N., OSBALDISTON, G.W. and JONAS, A.M. (1976). Laboratory Animal Science. 26, 889.

BARTHOLD, S.W., COLEMAN, G.L., JACOBY, R.O., LIVSTONE, E.M. and JONAS, A.M. (1978). Veterinary Pathology. 15, 223.

BARTZ, C.R., CONKLIN, R.H. and STEELE, J.H. (1979). Proceedings of the United States Animal Health Association. 83, 378.

BAURIEDEL, W.R., HOERLEIN, A.B., PICKEN, J.C. Jr., and UNDERKOFLER, L.A. (1954). Agricultural and Food Chemistry. 2, 468.

BELLIS, D.B. (1957). Proceedings of the Nutritional Society. 16, 98.

BERG, I.E., PETERSON, A.C., FREEMAN, T.P. (1978). Journal of the American Veterinary Medical Association. 173, 1586.

BERGLAND, M.E. (1977). Proceedings of the American Association of Veterinary Laboratory Diagnosticians. 20, 151.

BERGELAND, M.E., McADARAGH, J.P., WOHLGEMUTH, K. and STOTZ, I. (1975). Proceedings of the American Association of Veterinary Laboratory Diagnosticians. 18, 31.

BERRY, T.H., JENSEN, A.H., BECKER, O.E. and WOODS, G.T. (1962). Journal of the American Veterinary Medical Association. 141, 689.

BETTS, A.O. (1960). Research in Veterinary Science. 1, 57.

BETTS, A.O. (1961). Veterinary Record. 73, 1349.

BETTS, A.O. and JENNINGS, A.R. (1960). Research in Veterinary Science. 1, 160.

BETTS, A.O., JENNINGS, A.R., LAMONT, P.H. and PAGE, Z. (1962). Nature. 193, 45.

BETTS, A.O., LAMONT, P.H. and LITTLEWORT, M.C.G. (1960). Veterinary Record. 72, 461.

BEYNON, V.H. (1978). in "The Pig Veterinary Society Proceedings". 2, 83. The Burlington Press, Foxton, Royston, Herts.

BIESTER, H.E. and SCHWARTE, L.H. (1931). American Journal of Pathology. 7, 175.

BIESTER, H.E., SCHWARTE, L.H. and EVELETH, D.F. (1939). American Journal of Pathology. 15, 385.

BINNS, R.M. (1968). "The Ontogeny of Immune Responses in the Pig". Ph.D. Thesis. University of Cambridge.

BIRD, R.G. and SMITH, M.D. (1980). Journal of Pathology. 132, 217.

BLASER, M., CRAVENS, J., POWERS, B.W. and WANG, W.L.L. (1978). Lancet. 2, 979.

BOHL, E.H. (1981). in "Diseases of Swine", 5th Edition, Edited by Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B. Iowa State University Press, Ames, Iowa, USA. p. 195.

BOHL, E.H., KOHLER, E.M., SAIF, L.J., CROSS, R.F., AGNES, A.G. and THEIL, K.W. (1978). Journal of the American Veterinary Medical Association. 172, 458.

BOLITHO, W.S. (1978). in "The Pig Veterinary Society Proceedings". 2, 93. The Burlington Press, Foxton, Royston, Herts.

BOOTH, C.C., SLAVIN, G., DOURMASHKIN, R.R., DONIACH, I., WEBSTER, D., BIRD, R.G., BRYCESON, A., ASHERSON, G.L., BLACK, D., LAMBERT, H. and VALMAN, B. (1980). British Medical Journal. 281, 1123.

BOOTH, A.O. and CHEVILLE, N.F. (1967). Pathologia Veterinaria. 4, 31.

BOURNE, F.J. (1973). Proceedings of the Nutrition Society. 32, 205.

BRAMBELL, F.W.R. (1958). Biological Reviews. 33, 488.

BRAMBELL, F.W.R. (1970). in "Frontiers of Biology". North Holland Publishing Company, p18.

BRAMBELL, F.W.R., HEMMING, W.A. and HENDERSON, M. (1951). in "Antibodies and Embryos". Athlone Press, London.

BRIDGER, J.C. (1980). Veterinary Annual. 20, 172.

BRIDGER, J.C., WOODE, G.N., JONES, J.M., FLEWETT, T.H., BRYDEN, A.S. and DAVIES, H. (1975). Journal of Medical Microbiology. 8, 565.

BROWNSTEIN, D.G., STRANDBERG, D., MONTALI, R.J., BUSH, M. and FORTNER, J. (1977). Veterinary Pathology. 14, 606.

BUSTAD, L.K., HAM, W.E. and CUNHA, T.J. (1948). Archives of Biochemistry. 17, 249.

BUTZLER, J.P., DEKEYSER, P., DETRAIN, M. and DEHAEN, F. (1973). Journal of Paediatrics. 82, 493.

BYWATER, R.J. and WOODE, G.M. (1980). Veterinary Record. 106, 75.

CALVERLEY, J.L. (1963). Proceedings of the Animal Care Panel. 13, 678.

CAMPBELL, R.M., BROUGH, H., and FELL, B.F. (1971). Journal of Agricultural Science. 76, 531.

CATRON, D.V., NELSON, L.F., ASHTON, G.C. and MADDOCK, H.M. (1953). Journal of Animal Science. 12, 62.

CHANG, K., KURTZ, H.J., WARD, G.E. and GEBHART, C.J. (1981). Abstracts of the 62nd Annual Meeting Conference of Research Workers in Animal Disease. Abstract No. 124, p.22.

CHEVILLE, N.F. (1976). "Cell Pathology", The Iowa State University Press, Ames, Iowa, USA.

CHIDLOW, J.W. and PORTER, P. (1978). Veterinary Record. 104, 494.

CHRISTIE, B.R. (1963). Australian Veterinary Journal. 39, 148.

CHRISTOFINIS, G.J., EDINGTON, N., BETTS, A.O., PRIDIE, J. and PIRIE, G. (1972). American Jounral of Veterinary Research. 33, 1915.

COALSON, J.A., MAXWELL, C.V. and HILLIER, J.C. (1971). Journal of Animal Science. 32, 375.

COATES, M.E. (1968). "The Germ-Free Animal in Research." Academic Press. London and New York.

COCKRELL, B.Y., VALERIO, M.G. and GARNER, F.M. (1974). Laboratory Animal Science. 24, 881.

COMBS, B.O. (1960). The Allied Veterinarian. 31, 108.

COOK, M.G. and TURNBULL, G.J. (1975). Virchows Archiv A: Pathological Anatomy and Histology. 365, 327.

COOPER, J.E. (1975). Laboratory Animals. 9, 329.

CORLEY, L.D., STALEY, T.E. and JONES, E.W. (1973). Research in Veterinary Science, 14 69.

CORTHIER, G., COHEN, J. and SCHERRER, R. (1980). Annales de Recherches Vétérinaires. 11, 45.

COWAN, S.T. and STEEL, K.J. (1965). "Manual for the Identification of Medical Bacteria". Cambridge University Press, Cambridge.

COWAN, S.T. and STEEL, K.J. (1974). "Manual for the Identification of Medical Bacteria". 2nd Edition. Cambridge University Press, Cambridge.

CROSS, R.F., SMITH, C.K. and PARKER, C.F. (1973). Journal of American Veterinary Medical Association. 162, 564.

CROUCH, C.F. and WOODE, G.N. (1978). Journal of Medical Microbiology. 11, 325.

CRUICKSHANK, R. (1965). "Medical Microbiology", 11th Edition, E. and S. Livingstone Ltd., Edinburgh.

CRUICKSHANK, R., DUGUID, J.P., MARMION, B.P. and SWAIN, R.H.A. (1975). "Medical Microbiology". 12th Edition. Churchill Livingston, Edinburgh, London and New York.

DAHLQVIST, A. (1964). Analytical Biochemistry, 7, 18.

DAVIDSON, G.P., GALL, D.G., PETRIC, M., BUTLER, D.G. and HAMILTON, J.R. (1977). Journal of Clinical Investigation. 60, 1402.

DAVIS, G.K. and FREEMAN, V.A. (1940). Proceedings of the 33rd Annual Meeting of the American Society for Animal Production. p. 316.

DELLMAN, H.D. and BROWN, E.M. (1976). "Textbook of Veterinary Histology". Lea and Febiger, Philadelphia.

DODD, D.C. (1968). Pathologia Veterinaria. 5, 333.

DONE, J.T. (1955). Veterinary Record. 67, 623.

DOSTER, A.R., MAHAFFEY, E.A. and McCLEAREN, J.R. (1979). Avian Diseases. 23, 654.

DOYLE, L.P. (1948). American Journal of Veterinary Research. 9, 50.

DOZSA, L. (1965). Journal of the American Veterinary Medical Association. 147, 620.

DUFFY, M.C., BENSON, J.B. and RUBIN, S.J. (1980). American Journal of Clinical Pathology, 73, 706.

DUNCAN, A.L. (1974). "A Preliminary Study of the Fate of an Oral Dose of Campylobacter Sputorum subspecies Mucosalis in the Neonatal Pig". M.Sc. Dissertation, University of Edinburgh.

EDINGTON, N., CHRISTOFINIS, G.J. and BETTS, A.O. (1972). Journal of Comparative Pathology. 82, 393.

EDINGTON, N., WATT, R.G., PLOWRIGHT, W., WRATHALL, A.E. and DONE, J.T. (1977). Journal of Hygiene. 78, 243.

EDWARDS, R.R. and EWING, W.H. (1972). "Identification of Enterobacteriaceae". Burgess Publishing Co., Minneapolis, USA.

ELLENS, D.J. and DE LEEUW, P.W. (1977). Journal of Clinical Microbiology. 6, 530.

ELLIOT, J.I., OWEN, B.D. and McCALLUM, I.M. (1975). Journal of Animal Science. 41, 266.

EMSBO, P. (1951). Nordisk Veterinaermedizin. 3, 1.

FARRINGTON, D.O. (1981). in "Diseases of Swine". 5th Edition. Edited by Leman A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., Iowa State University Press, Ames, Iowa, USA. p. 378.

FIELD, H.I., BUNTAIN, D. and JENNINGS, A.R. (1953). Journal of Comparative Pathology and Therapeutics. 63, 153.

FIREHAMMER, B.D. (1979). in "CRC Handbook Series in Zoonoses". Edited by Steele, J.H., CRC Press, Inc., Boca Raton, Florida. Section A, Volume I, p 295.

FIREHAMMER, B.D. and DAVIES, J. (1981). in "Campylobacter: Epidemiology, Pathogenesis and Biochemistry"., Edited by D.G. Newell, MTP Press Limited, Lancaster-Boston - The Hague, p 87.

FLETCHER, O.J., MUNNELL, J.F. and PAGE, R.K. (1975). Avian Diseases. 19, 630.

FLEWETT, T.H. and WOODE, G.H. (1978). Archives of Virology. 57, 1.

FLORENT, A. (1953). Compte Rendu de la Societé de Biologie. 147, 2066.

FLORENT, A. (1959). Mededelingen der Veeartsenijschool van de Rijksuniversite it te Gent. 3, 1.

FRETER, R. (1974). in "The Immunoglobulin A System". Edited by Mestecky, J. and Lawton, A.R., Plenum Press, New York and London. p. 350.

FRIEDMAN, M.H. (1965). Laboratory Animal Digest. 1, 18.

FRISK, C.S. (1976). "The Etiology and Pathogenesis of Hamster Enteritis". Ph.D. Thesis, University of Missouri, Columbia, U.S.A.

FRISK, C.S. and WAGNER, J.E. (1977a). American Journal of Veterinary Research. 38, 1861.

FRISK, C.S. and WAGNER, J.E. (1977b). Laboratory Animals. 11, 79.

GEBHART, C.J., KURTZ, H.J. and WARD, G.E. (1981). Abstracts of the 62nd Annual Meeting Conference of Research Workers in Animal Disease. Abstract No. 125, p. 22.

GEISSINGER, H.D., MINIATS, O.P., QUINN, P.J. (1969). Zentralblatt für Veterinärmedizin. 16B, 689.

GHADIALLY, F.N. (1977). "Ultrastructural Pathology of the Cell". Butterworths, London and Boston.

GHADIALLY, F.N. (1982). "Ultrastructural Pathology of the Cell and Matrix". 2nd Edition, Butterworths, London and Boston.

GIRARD, A. and MITCHELL, D. (1962). Canadian Journal of Comparative Medicine. 26, 279.

GOIS, M., FRANZ, J., KUSKA, F., POKORNY, J. and CERNY, M. (1972). Zentralblatt für Veterinärmedizin. 19B, 379.

GOLDMAN, P.M., ANDREW, E.J. and LANG, C.M. (1972). Laboratory Animal Science, 22, 721.

GOMEZ-GARCIA, G.G. and MATRONE, G. (1967). Journal of Nutrition. 92, 237.

GOODWIN, R.F.W. (1962). Journal of Comparative Pathology. 72, 214.

GOODWIN, R.F.W. (1965). Veterinary Record. 77, 1070.

GOODWIN, R.F.W. and JENNINGS, A.R. (1959). Journal of Comparative Pathology. 69, 313.

GORDON, H.A. and PESTI, L. (1971). Bacteriological Reviews. 35, 390.

GOVIER, R.J. (1978). in "The Pig Veterinary Society Proceedings". 2, 100. The Burlington Press, Foxton, Royston, Herts.

GRADY, G.F. and KEUSCH, G.T. (1971). New England Journal of Medicine. 285, 891.

GRUFFYDD-JONES, T.J., MARSTON, M. and WHITE, E. (1980). Lancet. 2, 366.

GUINÉE, P.A.M., VELDKAMP, J. and JANSEN, W.H. (1977). Infection and Immunity. 15, 676.

HAELTERMAN, E.O. (1956). American Journal of Veterinary Research, 17, 129.

HAELTERMAN, E.O. (1965). Journal of the American Veterinary Medical Association. 147, 1661.

HALL, G.A., BRIDGER, J.C., CHANDLER, R.L. and WOODE, G.N. (1976). Veterinary Pathology. 13, 197.

HAMPTON, J.C. and ROSARIO, B. (1966). Journal of Parasitology. 52, 939.

HARRIS, D.L. and GLOCK, R.D. (1981). in "Diseases of Swine". 5th Edition, Edited by Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., Iowa State University Press, Ames, Iowa, U.S.A. p. 432.

HEARD, T.W. and JOLLANS, J.L. (1970). Veterinary Record. 86, 418.

HENRIKSON, S.A. and KROGH, H.V. (1980). Nordisk Veterinaermedicin. 32, 501.

HENRY, D.P. (1965). Australian Veterinary Journal. 41, 125.

HILL, E.B. and LARSON, N.L. (1955). Journal of Animal Science. 14, 1116.

HODGES, R.T., BETTS, A.O. and JENNINGS, A.R. (1969). Veterinary Record. 84, 268.

HOERLAIN, A.B., ADAMS, C.H. and MEADE, R.J. (1956). Journal of the American Veterinary Medical Association. 128, 127.

HOERR, F.J., RANCK, F.M. and HASTINGS, T.F. (1978). Journal of the American Veterinary Medical Association. 173, 1591.

HOORENS, J. (1962). "Regional Enteritis in the Pig". Thesis. Ghent.

HOORENS, J., OYAERT, W., MEYVISCH, C., VANDENBERGHE, J. and DERIJCKE, J. (1977). Vlaams Diergeneeskundig Tijdschrift. 43, 10.

HORSFALL, F.L. Jr. and BAUER, J.H. (1940). Journal of Bacteriology. 40, 569.

HUNGERFORD, T.G. (1975). "Diseases of Livestock". 8th Edition. McGraw-Hill Book Co., Sydney, Australia.

INMAN, L.R. and TAKEUCHI, A. (1979). Veterinary Pathology. 16, 89.

INTERNATIONAL COMMITTEE ON LABORATORY ANIMALS (1964). "Terms and Definitions.", ICLA Bulletin No. 14, Annex I, London.

ISAACSON, R.E. (1980). Proceedings of the 3rd International Symposium on Neonatal Diarrhoea. Published by Veterinary Infectious Disease Organisation, University of Saskatchewan, Saskatchewan, Canada. p 213.

ISAACSON, R.E., FUSCO, P.C., BRINTON, C.C. and MOON, H.W. (1978). Infection and Immunity. 21, 392.

ISAACSON, R.E., NAGY, B. and MOON, H.W. (1977). Journal of Infectious Diseases. 135, 531.

ISEKI, M. (1979). Japanese Journal of Parasitology. 28, 285.

ISOMÄKI, A.M. (1973). Acta Pathologica et Microbiologica Scandinavica. 240, 1.

IZAWA, H., HOWARTH, J.A. and BANKOWSKI, R.A. (1962). American Journal of Veterinary Research. 23, 1142.

JACKSON, G.H. (1980). Proceedings of the International Pig Veterinary Society Congress, Copenhagen, Denmark. p 261.

JACKSON, S.T. and WAGNER, J.E. (1970). Laboratory Animal Digest. 6, 12.

JACOBY, R.O. (1978). American Journal of Pathology. 91, 433.

JACOBY, R.O. and JOHNSON, E.O. (1981). Advances in Experimental Medicine and Biology. 134, 267.

JACOBY, R.O., ONDERDONK, A.B. and JONAS, A.M. (1978). 29th Annual Session of the American Association of Laboratory Animal Science. Abstract No. 99.

JACOBY, R.O., OSBALDISTON, G.W. and JONAS, A.M. (1975). Laboratory Animal Science. 25, 465.

JERVIS, H.R., MERRILL, T.G. and SPRINZ, H. (1966). American Journal of Veterinary Research. 27, 408.

JOHNSON, E. and BARTHOLD, S.W. (1979). American Journal of Pathology. 97, 291.

JONAS, A.M. (1965). Journal of the American Veterinary Medical Association. 147, 1102.

JONAS, A.M., TOMITA, Y. and WYAND, D.S. (1965). Journal of the American Veterinary Medical Association. 147, 1102.

JONES, F.S., ORCUTT, M. and LITTLE, R.B. (1931). Journal of Experimental Medicine. 58, 853.

JONES, G.W. and RUTTER, J.M. (1972). Infection and Immunity. 6, 918.

JÖNSSON, L. and MARTINSSON, K. (1976). Acta Veterinaria Scandinavica. 17, 223.

KALIMA, T.V., SALONIEMI, H. and RAHKO, T. (1976). Scandinavian Journal of Gastroenterology. 11, 353.

KASZA, L., HODGES, R.T., BETTS, A.O. and TREXLER, P.C. (1969). Veterinary Record. 84, 262.

KEKWITH, R.A. (1959). Advances in Protein Chemistry. 14, 231.

KELLY, W.R. and CAMERON, R.D.A. (1976). Proceedings of the 53rd Annual Conference of the Australian Veterinary Association. p 146.

KENNEDY, G.A., KREITNER, G.L. and STRAFUSS, A.C. (1977). Journal of the American Veterinary Medical Association. 170, 348.

KENWORTHY, R. (1976). Research in Veterinary Science. 21, 69.

KENWORTHY, R., STUBBS, J.M. and SYME, G. (1967). Journal of Pathology and Bacteriology. 93, 493.

KERR, P.A. (1938). American Journal of Clinical Pathology. 2, 63.

KNOP, J., BREU, H., WERNET, P. and ROWLEY, D. (1971). Australian Journal of Experimental Biology and Medical Science. 49, 405.

KNOP, J. and ROWLEY, D. (1975). Australian Journal of Experimental Biology and Medical Science. 53, 137.

KOHLER, E.M. and BOHL, E.H. (1966). Canadian Journal of Comparative Medicine. 30, 199.

KOHLER, E.M. and CROSS, R.F. (1969). Canadian Journal of Comparative Medicine. 33, 173.

KORPASSY, B. and TIBOLDI, T. (1957). Oncologia. 10, 330.

KOSUNEN, T.U., DANIELSSON, D. and KJELLANDER, J. (1980). Acta Pathologica et Microbiologica Scandinavica. 88, 207.

KOVATCH, R.M. and WHITE, J.D. (1972). Veterinary Pathology. 9, 426.

KUBO, M., OKUDA, K., SUGIYAMA, T. and KONNO, S. (1979). National Institute of Animal Health Quarterly. 19, 134.

KURTZ, H.J. (1973). Abstracts of the 54th Annual Meeting Conference of Research Workers in Animal Disease. Abstract No. 130, p 21.

KURTZ, H.J., MIROCHA, C. and MEADE, R. (1974). Proceedings of the 17th Annual North Central Conference of Veterinary Diagnosticians. Urbana, Illinois, U.S.A. Abstract.

KURTZ, H.J., MIROCHA, C. and MEADE, R. (1976). Proceedings of the International Pig Veterinary Society Congress., Ames, Iowa, U.S.A., p N6.

KURTZ, H.J., SOTO, J. and McALLISTER, J.S. (1980). Proceedings of the International Pig Veterinary Society Congress., Copenhagen, Denmark., p 262.

LA REGINA, M.S. (1979). "Studies on Proliferative Ileitis of Hamsters". M.Sc. Thesis. University of Missouri - Columbia, U.S.A.

LA REGINA, M.S., HALES, W.F. and WAGNER, J.E. (1980). Laboratory Animal Science. 30, 38.

LASSER, K.H., LEWIN, K.J. and RYNING, F.W. (1979). Human Pathology. 10, 234.

LAWSON, G.H.K., LEAVER, J.L., PETTIGREW, G.W. and ROWLAND, A.C. (1981). International Journal of Systematic Bacteriology. 31, 385.

LAWSON, G.H.K., ROBERTS, L., McCARTNEY, E., ROWLAND, A.C. and LUCKINS, A.G. (1982). Research in Veterinary Science. 32, 89.

LAWSON, G.H.K., ROBERTS, L. and ROWLAND, A.C. (1980). Proceedings of the International Pig Veterinary Society Congress, Copenhagen, Denmark. p 260.

LAWSON, G.H.K. and ROWLAND, A.C. (1974). Research in Veterinary Science. 17, 331.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1975). Veterinary Record. 97, 308.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1976). Journal of Medical Microbiology. 9, 163.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1977a). Research in Veterinary Science. 23, 378.

LAWSON, G.H.K., ROWLAND, A.C. and ROBERTS, L. (1977b). Veterinary Record. 100, 144.

LAWSON, G.H.K., ROWLAND, A.C., ROBERTS, L., FRASER, G. and McCARTNEY, E. (1979). Research in Veterinary Science. 27, 46.

LAWSON, G.H.K., ROWLAND, A.C., SMITH, W.J., ROBERTS, L. and LUNNEY, D. (1980). Veterinary Record. 107, 424.

LAWSON, G.H.K., ROWLAND, A.C. and WOODING, P. (1975). Research in Veterinary Science. 18, 121.

LECCE, J.G. (1960). Annals of the New York Academy of Science. 79, 670.

LECCE, J.G. (1966). Biology of the Neonate. 9, 50.

LECCE, J.G. and COALSON, J.A. (1976). Journal of Animal Science. 42, 622.

LECCE, J.G. and KING, M.W. (1978). Journal of Clinical Microbiology. 8, 454.

LECCE, J.G. and KING, M.W. (1980). Proceedings of the 3rd International Symposium on Neonatal Diarrhea. Published by Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatchewan, Canada. p 21.

LECCE, J.G., KING, M.W. and MOCK, R. (1976). Infection and Immunity. 14, 816.

LECCE, J.G. and MATRONE, G. (1960). Journal of Nutrition. 70, 13.

LECCE, J.G. and REEP, B.R. (1962). Journal of Experimental Medicine. 115, 491.

LEE, A. and GEMMELL, E. (1972). Infection and Immunity. 5, 1.

LEE, F.D. and TONER, P.G. (1980). in "Biopsy Pathology of the Small Intestine"., Chapman and Hall, London., p 27.

LEVADITI, C. and MANOUELIAN, Y. (1906). as quoted by Drury, R.A.B. and Wallington, E.A. (1967) "Carleton's Histological Techniques". 4th Edition, Oxford University Press, London. p 346.

LEVINE, N.D. (1973). "Protozoan Parasites of Domestic Animals and of Man." 2nd Edition. Burgess Publishing Company, Minneapolis, Minnesota.

LOESCHE, W.J., GIBBONS, R.J. and SOCRANSKY, S.S. (1965). Journal of Bacteriology. 89, 1109.

LOVE, D.N. and LOVE, R.J. (1979). Veterinary Pathology. 16, 41.

LOVE, R.J. and LOVE, D.N. (1977). Veterinary Record. 100, 473.

LOVE, R.J., LOVE, D.N. and EDWARDS, M.J. (1977). Veterinary Record. 100, 65.

LUCKEY, T.D. (1963). "Germfree Life and Gnotobiology." Academic Press, New York and London.

LUDVIGSEN, G. (1958). Proceedings of the 8th Nordic Veterinary Congress, Helsinki. p 415.

McADARAGH, J.P., BERGELAND, M.E., MEYER, R.C., JOHNSHOY, M.W., STOTZ, I.J., BENFIELD, D.A. and HAMMER, R. (1980). American Journal of Veterinary Research. 41, 1572.

McCALLUM, I.M., ELLIOTT, J.I. and OWEN, B.D. (1977). Canadian Journal of Animal Science. 57, 151.

McNULTY, M.S. (1978). Journal of General Virology. 40, 1.

McNULTY, M.S., PEARSON, G.R., McFERRAN, J.B., COLLINS, D.S. and ALLAN, G.M. (1976). Veterinary Microbiology. 1, 55.

McROBERTS, V.F. and HOGAN, A.G. (1944). Journal of Nutrition. 28, 165.

MAKIN, T.J. and TZIPORI, S. (1980). Australian Veterinary Journal. 56, 353.

MANDEL, L., LANE, A. and SYRINEK, F. (1960). Ved-Zwocisna Sbornik Ceskoslovenske Akademic Zene-delskych Vyroba. 5, 37.

MARSBOOM, R., TEMMERMAN, R. and SYMOENS, J. (1973). Veterinary Record. 93, 382.

MARTINSSON, K. and EKMAN, L. (1974). Svensk Veterinärtidning. 26, 824.

MARTINSSON, K., EKMAN, L. and JÖNSSON, L. (1976). Acta Veterinaria Scandinavica. 17, 233.

MARTINSSON, K., HOLMGRON, N., JÖNSSON, L. and NORDSTRÖM, G. (1974). Svensk Veterinärtidning. 26, 347.

MASON, R.W. and HARTLEY, W.J. (1980). Avian Diseases. 24, 771.

MATTHEWS, N., MAYBERRY, J.F., RHODES, J., NEALE, L., MUNRO, J., WENSINCK, F., LAWSON, G.H.K., ROWLAND, A.C., BERKHOFF, G.A. and BARTHOLD, S.W. (1980). Gut. 21, 376.

MAYER, P. (1903). Zeitschrift für Wissenschaftliche Mikroskopie und für Mikroskopiche Technick. 20, 409.

MEISEL, J.L., PERERA, D.R., MELIGRO, C., RUBIN, C.E. (1976). Gastroenterology. 70, 1156.

METZGER, J.J., MILEN, A. and BORDIEU, C. (1978). Annales de Recherches Vétérinaires. 9, 301.

MEUTEN, D.J., VAN KRUININGEN, H.J. and LEIN, D.H. (1974). Journal of the American Veterinary Medical Association. 165, 914.

MEYER, R.C., BOHL, E.H. and KOHLER, E.M. (1964). Applied Microbiology. 12, 295.

MEYER, R.C. and SIMON, J. (1972). Veterinary Pathology. 9, 360.

MICKELSON, M.J. and KLIPSTEIN, F.A. (1975). Infection and Immunity. 12, 1121.

MILES, A.A. and MISRA, S.S. (1938). Journal of Hygiene. 38, 732.

MINIATS, O.P. and JOL, D. (1978). Canadian Journal of Comparative Medicine. 42, 428.

MINIATS, O.P., MITCHELL, L. and BARNUM, D.A. (1970). Canadian Journal of Comparative Medicine. 34, 269.

MINISTRY OF AGRICULTURE. (1965). Advisory Leaflet No. 514, HMSO, London.

MOON, H.W. (1972). Veterinary Pathology. 9, 3.

MOON, H.W. (1974). Advances in Veterinary Science and Comparative Medicine. 18, 179.

MOON, H.W. and BEMRICK, W.J. (1981). Veterinary Pathology. 18, 248.

MOON, H.W., NAGY, B., ISAACSON, R.E. and ØRSKOV, I. (1977). Infection and Immunity. 15, 614.

MOON, H.W., FUNG, P.Y., WHIPP, S.C. and ISAACSON, R.E. (1978). Infection and Immunity. 20, 36.

MORGAN, J.T. and LEWIS, D. (1962). "Nutrition of Pigs and Poultry"., Butterworths, London.

MORIN, M., LARIVIÈRE, S. and LALLIER, R. (1976). Canadian Journal of Comparative Medicine. 40, 228.

MORSON, B.C. and DAWSON, I.M.P. (1972). "Gastrointestinal Pathology", Blackwell Scientific Publications, Oxford and Edinburgh.

MOSTRATOS, A. and BESWICK, T.S.L. (1969). Journal of Pathology. 98, 17.

MOUNT, L.E. (1959). Journal of Physiology. 147, 333.

MOUNT, L.E. (1963). Nature. 199, 1212.

MOYNIHAN, I.W. and GWATKIN, R. (1941). Canadian Journal of Comparative Medicine and Veterinary Science. 5, 167.

MUIRHEAD, M.R. (1978). in "The Pig Veterinary Society Proceedings". 2, 113. The Burlington Press, Foxton, Royston, Herts.

NABEYAMA, A. and LEBLOND, C.P. (1974). American Journal of Anatomy. 140, 147.

NAGY, B., ANTAL, A. and RATZ, F. (1979). Magyar Allatorvosok Lapja. 34, 585.

NAGY, B., MOON, H.W. and ISAACSON, R.E. (1976). Infection and Immunity. 13, 1214.

NAGY, B., MOON, H.W. and ISAACSON, R.E. (1977). Infection and Immunity. 16, 344.

NAIRN, R.C. (1964). in "Fluorescent Protein Tracing". 2nd Edition, E. and S. Livingstone Ltd., Edinburgh. p 19.

NECOECHA, R.R., MELGAR, H.C. and GUZMAN DE LAS CASAS, M. (1969). Technica Pecuaria en Mexico., Enero-Julio, 12-13, p 55.

NEWLAND, H.W., McMILLEN, W.N. and REINEKE, E.P. (1952). Journal of Animal Science. 11, 118.

NIELSEN, S.V. (1955). Journal of the American Veterinary Medical Association. 127, 437.

NIME, F.A., BUREK, J.D., PAGE, D.L., HOLSCHER, M.A. and YARDLEY, J.H. (1976). Gastroenterology. 70, 592.

O'HARA, P.J. (1972). Veterinary Record. 91, 517.

OLSEN, D.P., WAXLER, G.L. and ROBERTS, A.W. (1973). American Journal of Veterinary Research. 34, 1239.

O'NEILL, P.A. (1970). Veterinary Record. 87, 742.

OWEN, B.D., BELL, J.M., WILLIAMS, C.M. and OAKES, R.G. (1961). Canadian Journal of Animal Science. 41, 236.

PANCIERA, R.J., THOMASSEN, R.W. and GARNER, F.M. (1971). Veterinary Pathology. 8, 479.

PASTEUR, L. (1885). Compte Rendu Hebdomadaire des Séances de l' Académie des Sciences, Paris. 100, 68.

PEARSON, G.R. and LOGAN, E.F. (1978). Veterinary Record. 103, 212.

PENNY, R.H.C. and MUIRHEAD, M.R. (1981). in "Diseases of Swine". 5th Edition., Edited by Leman, A.D., Glock, E.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., Iowa State University Press, Ames, Iowa, USA. p 90.

PENSAERT, M.B. and DE BOUCK, P. (1978). Archives of Virology. 58, 243.

PERRIN, D.R. (1954). Journal of Dairy Research. 21, 55.

PILL, A.H. (1971). Veterinary Record. 88, 27.

PINK, I.J. (1970). in "Modern Trends in Gastro-enterology - 4"., Butterworths, London. pl.

POHLENZ, J., BEMRICK, W.J., MOON, H.W. and CHEVILLE, N.F. (1978). Veterinary Pathology. 15, 417.

POLAND, J. (1973). "Studies on Mycoplasma Hyorhinis and its Pathogenicity for Pigs". PhD. Thesis, University of London.

POLLARD, M. (1967). Advances in Veterinary Science. 11, 139.

POND, W.G., ROBERTS, S.J., DUNN, J.A., KING, J.M., MANER, J.H., LOWREY, R.S. and OLAFSON, P. (1961). Journal of Animal Science. 20, 88.

PORTER, P. (1969). Biochemica et Biophysica Acta. 181, 381.

PORTER, P. and ALLEN, W.D. (1972). Journal of the American Veterinary Medical Association. 160, 511.

PORTER, P. and HILL, I.R. (1970). Immunology. 18, 565.

PORTER, P. and KENWORTHY, R. (1969). Research in Veterinary Science. 10, 440.

POWELL, H.S., HOLSCHER, M.A., HEALTH, J.E. and BEASLEY, F.F. (1976). Veterinary Medicine and Small Animal Clinician. 71, 205.

PRESCOTT, J.F. and BRUIN-MOSCH, C.W. (1981). American Journal of Veterinary Research. 42, 164.

PRÉVOT, A.R. (1940). Annales de L' Institut Pasteur Paris, 64, 117.

PROCTOR, S.J. and KEMP, R.L. (1974). Journal of Protozoology. 21, 664.

PULLAR, E.M. (1958). Australian Veterinary Journal. 34, 305.

RAHKO, T. and SALONIEMI, H. (1972). Nordisk Veterinaermedicin. 24, 132.

RAHKO, T., SALONIEMI, H. and KALIMA, V. (1973). Journal of Gastroenterology. 20, 36.

RAJAN, A., NAIR, M.K. and MARYAMMA, K.I. (1975). Kerala Journal of Veterinary Science. 6, 128.

RAJASEKHAR, M. (1981). "Growth Characteristics of Campylobacter Sputorum subspecies Mucosalis in Cell Cultures." Ph.D. Thesis, University of Edinburgh.

RANLOV, P., NIELSEN, M.H. and WANSTRUP, J. (1972). Scandinavian Journal of Gastroenterology. 7, 471.

REDMAN CHU, R.M. and HONG, C.B. (1973). Veterinary Record. 93, 562.

REHG, J.E., LAWTON, G.W. and PAKES, S.P. (1979). Laboratory Animal Science. 29, 656.

REYNOLDS, E.S. (1963). Journal of Cell Biology. 17, 208.

RHODIN, J.A.G. (1963). "An Atlas of Ultrastructure.", W.G. Saunders Co., Philadelphia and London.

ROBERTS, L. (1978). "A Study of Porcine Intestinal Adenomatosis." Ph.D. Thesis, University of Edinburgh.

ROBERTS, L. (1981). Veterinary Record. 109, 17.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1977). Research in Veterinary Science. 23, 257.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1980a). Research in Veterinary Science. 28, 145.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1980b). Research in Veterinary Science. 28, 148.

ROBERTS, L., LAWSON, G.H.K. and ROWLAND, A.C. (1980c). Veterinary Microbiology. 5, 249.

ROBERTS, L., LAWSON, G.H.K., ROWLAND, A.C. and LAING, A.H. (1979). Veterinary Record. 104, 366.

ROBERTS, L., ROWLAND, A.C. and LAWSON, G.H.K. (1977). Veterinary Record. 100, 12.

ROBERTS, L., ROWLAND, A.C. and LAWSON, G.H.K. (1980a). Gut. 21, 1035.

ROBERTS, L., ROWLAND, A.C. and LAWSON, G.H.K. (1980b). Proceedings of the International Pig Veterinary Society Congress., Copenhagen, Denmark. p 259.

ROBERTS, L. and WALKER, E.J. (1980). Veterinary Record. 108, 62.

ROBERTSON, V.A.W., JONES, A.S., FULLER, M.E. and ELSLEY, F.W.H. (1971). Research in Veterinary Science. 12, 59.

RODGER, S.M., CRAVEN, J.A. and WILLIAMS, I. (1975). Australian Veterinary Journal. 51, 536.

ROE, C.K. and ALEXANDER, T.J.L. (1961). Canadian Veterinary Journal. 2, 139.

ROWAN, T.G. and LAWRENCE, T.L.J. (1982). Veterinary Record. 110, 306.

ROWLAND, A.C. and HUTCHINGS, D.A. (1978). Veterinary Record. 103, 338.

ROWLAND, A.C. and LAWSON, G.H.K. (1974). Research in Veterinary Science. 17, 323.

ROWLAND, A.C. and LAWSON, G.H.K. (1975a). Research in Veterinary Science. 18, 263.

ROWLAND, A.C. and LAWSON, G.H.K. (1975b). Veterinary Record. 97, 178.

ROWLAND, A.C. and LAWSON, G.H.K. (1981). in "Diseases of Swine". 5th Edition., Edited by Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., The Iowa State University Press, Ames, Iowa, USA. p 517.

ROWLAND, A.C., LAWSON, G.H.K. and MAXWELL, A. (1973). Nature. 243, 417.

ROWLAND, A.C., LAWSON, G.H.K. and ROBERTS, L. (1978). Research in Veterinary Science. 24, 191.

ROWLAND, A.C., ROBERTS, L., LAWSON, G.H.K. and McCARTNEY, E. (1980). Proceedings of the International Pig Veterinary Society Congress., Copenhagen, Denmark. p 258.

ROWLAND, A.C. and ROWNTREE, P.G.M. (1972). Veterinary Record. 91, 235.

RUNNELLS, R.A., MONLUX, W.S. and MONLUX, A.W. (1965). "Principles of Veterinary Pathology". 7th Edition. Iowa State University Press, Ames, Iowa, USA.

SALONIEMI, H., RAHKO, T. and KALIMA, T.V. (1974). Proceedings of the 12th Nordic Veterinary Congress, Reykjavik. p 297.

SANDBORN, E.B. (1970). in "Cells and Tissues by Light and Electron Microscopy". Volume 2, Academic Press, New York. p 48.

SANGSTER, L.T., STUART, B.P., WILLIAMS, D.J. and BEDELL, D.M. (1978). Veterinary Medicine. 73, 1317.

SCHMITZ, J. and SMITH, D.H. (1975). Journal of the American Veterinary Medical Association. 167, 731.

SCHNEIDER, D.L. and SARETT, H.P. (1966). Journal of Nutrition. 89, 43.

SCOOT, A., OWEN, B.D. and AGAR, J.L. (1972). Journal of Animal Science. 35, 1201.

SEBALD, M. and VÉRON, M. (1963). Annales de L' Institut Pasteur Paris. 105, 897.

SELLWOOD, R. (1979). Veterinary Record. 10, 228.

SELLWOOD, R., GIBBONS, R.A., JONES, G.W. and RUTTER, J.M. (1975). Journal of Medical Microbiology. 8, 405.

SENFT, B. and KLOBASA, F. (1971a). Züchtungskundeband. 43, 371.

SENFT, B. and KLOBASA, F. (1971b). Züchtungskundeband. 43, 437.

SENFT, B. and KLOBASA, F. (1971c). Züchtungskundeband. 43, 451.

SHADDUCK, J.A., KOESTNER, A. and KASZA, L. (1967). Pathologia Veterinaria. 4, 537.

SHARPE, H.B.A. (1965). Research in Veterinary Science. 6, 490.

SHAW, B.T. (1953). Report of the Administrator of Agricultural Research, U.S. Dept. Agriculture. p 129.

SHEFFY, B.E., GALLEGOS, C.A, GRUMMER, R.H., PHILLIPS, P.H. and BOHSTEDT, G. (1951). Journal of Animal Science. 10, 867.

SIBALIN, M. and LANNEK, N. (1960). Archiv für die Gesamte Virusforschung. 10, 31.

SKERMAN, V.B.O., McGOWAN, V. and SNEATH, P.H.A. (1980). International Journal of Systematic Bacteriology. 30, 225.

SKIRROW, M.B. (1977). British Medical Journal. 2, 9.

SKIRROW, M.B. (1980). Society for General Microbiology Quarterly. 8, 18.

SKIRROW, M.B. and BENJAMIN, J. (1980a). Journal of Clinical Pathology. 33, 1122.

SKIRROW, M.B. and BENJAMIN, J. (1980b). Journal of Hygiene, Cambridge. 85, 427.

SLAVIN, D. (1955). Journal of Comparative Pathology. 65, 262.

SLOPER, K.S., DOURMASHKIN, R.R., BIRD, R.B., SLAVIN, G. and WEBSTER, A.D.B. (1982). Gut. 23, 80.

SMIBERT, R.M. (1974). in "Bergey's Manual of Determinative Bacteriology.", Edited by Buchanan, R.E. and Gibbons, N.E., The Williams and Williams Company, Baltimore, U.S.A. p 207.

SMIBERT, R.M. (1978). Annual Review of Microbiology. 32, 673.

SMITH, H.W. and JONES, J.E.T. (1963). Journal of Pathology and Bacteriology. 86, 387.

SMITH, H.W. and LINGGOOD, M.A. (1971). Journal of Medical Microbiology. 4, 467.

SMITH, I.M. (1971). "Studies on the Role of Some Microorganisms in Respiratory Infections of the Pig with Special Reference to the Involvement of Pasteurella Septica.", Ph.D. Thesis, University of London.

SMITH, I.M. and HAYWARD, A.H.S. (1969). Journal of Comparative Pathology. 79, 237.

SMITH, I.M., BETTS, A.O., WATT, R.G. and HAYWARD, A.H.S. (1973a). Journal of Comparative Pathology. 83, 1.

SMITH, I.M., HODGES, R.T., BETTS, A.O. and HAYWARD, A.H.S. (1973b). Journal of Comparative Pathology. 83, 307.

SMITH, T. and TAYLOR, M.S. (1919). Journal of Experimental Medicine. 30, 313.

SNODGRASS, D.R., ANGUS, K.W., GRAY, E.W., KEIR, W.A. and CLERIHEW, L.W. (1980). Veterinary Record. 106, 458.

SNODGRASS, D.R., FERGUSON, A., ALLAN, F., ANGUS, K.W. and MITCHELL, B. (1979). Gastroenterology. 76, 477.

SNODGRASS, D.R. and WELLS, P.W. (1976). Archives of Virology. 52, 201.

SNYDER, S.P., ENGLAND, J.J. and McCHESNEY, A.E. (1978). Veterinary Pathology. 15, 12.

SOJKA, W.J. (1965). "Escherichia Coli in Domestic Animals and Poultry." Commonwealth Agricultural Bureaux, Farnham Royal, Bucks., England.

SOJKA, W.J. (1971). Veterinary Bulletin. 41, 509.

SOKAL, R.R. and ROHLF, F.J. (1981). "Biometry: The Principles and Practices of Statistics in Biological Research.", 2nd Edition, W.H. Freeman and Co., San Francisco, U.S.A.

STEMMERMANN, G.N., HAYASHI, T., GLOBER, G.A., OISHI, N. and FRANKEL, R.I. (1980). The American Journal of Medicine. 69, 637.

STERZL, J., REJNEK, J. and TRAVNICEK, J. (1966). Folia Microbiologica. 11, 7.

TANNOCK, G.W. and SAVAGE, D.C. (1974). Infection and Immunity. 9, 591.

TAYLOR, D.J. (1981). in "Pig Diseases". 2nd Edition, The Burlington Press (Cambridge) Ltd., Foxton, Cambridge, p 1.

TAYLOR, D.J. and ALEXANDER, T.J.L. (1971). British Veterinary Journal. 127, 58.

TAYLOR, D.J. and OLUBUNMI, P.A. (1981). Veterinary Record. 109, 112.

TAYLOR, D.J., SIMMONS, J.R. and LAIRD, H.M. (1980). Veterinary Record. 106, 326.

THEIL, K.W., BOHL, E.H., CROSS, R.E., KOHLER, E.M. and AGNES, A.G. (1978). American Journal of Veterinary Research. 39, 213.

THOMPSON, C.M., WHITEHAIR, C.K., MacVICAR, R.W. and HILLIER, J.C. (1952). Oklahoma Agricultural Experimental Station M.P. 27, 74.

THREADGOLD, L.T. (1976). "The Ultrastructure of the Animal Cell". 2nd Edition. Pergamon Press, Oxford.

TIME-LIFE BOOKS (1981). "The Good Cook-Pork"., Time-Life Books Amsterdam. 3rd European English Language Printing., European Editor: Kit Van Tullekan.

TONER, P.G., CARR, K.E. and WYBURN, G.M. (1971). "The Digestive System - An Ultrastructural Atlas and Review"., Butterworth and Co. Ltd., London.

TORRES-MEDINA, A. and UNDERDAHL, N.R. (1980). Canadian Journal of Comparative Medicine. 44, 403.

TORRES-MEDINA, A., WYATT, R.G., MEBUS, C.A., UNDERDAHL, N.R. and KAPIKIAN, A.Z. (1976a). Intervirology. 7, 250.

TORRES-MEDINA, A., WYATT, R.G., MEBUS, C.A., UNDERDAHL, N.R. and KAPIKIAN, A.Z. (1976b). Journal of Infectious Diseases. 133, 22.

TREXLER, P.C. (1971). Veterinary Record. 88, 15.

TREXLER, P.C. and BETTS, A.O. (1975). Veterinary Annual. 15, 405.

TREXLER, P.C. and REYNOLDS, L.I. (1957). Applied Microbiology. 5, 406.

TRIBE, G.W., MACKENZIE, P.S. and FLEMING, M.P. (1979). Veterinary Record. 105, 333.

TRIER, J.S. (1963). Journal of Cell Biology. 18, 599.

TRIER, J.S. and RUBIN, C.E. (1965). Gastroenterology. 49, 574.

TYZZER, E.E. (1907). Proceedings of the Society for Experimental Biology and Medicine. 5, 12.

TYZZER, E.E. (1910). Journal of Medical Research. 23, 487.

TYZZER, E.E. (1912). Archiv für Protistenkunde. 26, 394.

TYZZER, E.E. (1929). American Journal of Hygiene. 10, 269.

TZIPORI, S., ANGUS, K.W., CAMPBELL, I. and GRAY, E.W. (1980a). Infection and Immunity, 30, 884.

TZIPORI, S., ANGUS, K.W., CAMPBELL, I. and SHERWOOD, D. (1981a). Journal of Infectious Diseases. 144, 170.

TZIPORI, S., ANGUS, K.W., GRAY, E.W. and CAMPBELL, I. (1980b). New England Journal of Medicine. 303, 818.

TZIPORI, S., ANGUS, K.W., GRAY, E.W., CAMPBELL, I. and ALLAN, F. (1981b). American Journal of Veterinary Research. 42, 1400.

TZIPORI, S. and CAMPBELL, I. (1981). Journal of Clinical Microbiology. 14, 455.

TZIPORI, S., CAMPBELL, I., SHERWOOD, D., SNODGRASS, D.R. and WHITELAW, A. (1980c). The Veterinary Record. 107, 579.

TZIPORI, S., CHANDLER, D., MAKIN, T. and SMITH, M. (1980d). Australian Veterinary Journal. 56, 279.

TZIPORI, S., McCARTNEY, E., LAWSON, G.H.K., ROWLAND, A.C. and CAMPBELL, I. (1981c). Research in Veterinary Science. 31, 358.

TZIPORI, S. and WILLIAMS, I.H. (1978). Australian Veterinary Journal. 54, 150.

ULLREY, D.E., LONG, C.H. and MILLER, E.R. (1966). in "Swine in Biomedical Research.", Edited by Bustad, L.K., McClellan, R.O. and Burns, M.P., Frayn Printing Co., Seattle, U.S.A. p 249.

UNDERDAHL, N.R. and YOUNG, G.A. (1959). Journal of the American Veterinary Medical Association. 131, 222.

UNDERDAHL, N.R., MEBUS, C.A., STAIR, E.L. and TWIEHAUS, M.J. (1972). Canadian Veterinary Journal. 13, 9.

VANDENBERGHE, J. and HOORENS, J. (1980). Research in Veterinary Science. 29, 390.

VAN ULSEN, F.W. (1971). Veterinary Record. 88, 433.

VÉRON, M. and CHATELAIN, R. (1973). International Journal of Systematic Bacteriology. 23, 122.

VETTERLING, J.M., JERVIS, H.R., MERRILL, T.G. and SPRINZ, H. (1971). Journal of Protozoology. 18, 243.

VETTERLING, J.M., TAKEUCHI, A. and MADDEN, P.A. (1971). Journal of Protozoology. 18, 248.

WAGNER, J.E., OWENS, D.R. and TROUTT, H.F. (1973). American Journal of Veterinary Research. 34, 249.

WAXLER, G.L. and BRITT, A.L. (1972). Canadian Journal of Comparative Medicine. 36, 226.

WAXLER, G.L., SCHMIDT, D.A. and WHITEHAIR, C.K. (1966). American Journal of Veterinary Research. 27, 300.

WEISER, M.M. (1973). Journal of Biological Chemistry. 248, 2536.

WENSVOORT, P. (1962). Tijdschrift voor Diergeneeskunde. 87, 841.

WESTENDORP, J.F. (1965). "Terminale-Jejuno-Ileitis Bij Het Varken.", Thesis, Utrecht.

WHITEHAIR, C.K. and THOMPSON, C.M. (1956). Journal of the American Veterinary Medical Association. 128, 94.

WHITEHAIR, C.K. and WAXLER, G.L. (1963). Laboratory Animal Care. 13, 655.

WILLIAMS, G.V. and DEACON, G.J. (1980). Medical Journal of Australia. 2, 268.

WOODE, G.N. and BOHL, E.H. (1981). in "Diseases of Swine.", 5th Edition, Edited by Leman, A.D., Glock, R.D., Mengeling, W.L., Penny, R.H.C., Scholl, E. and Straw, B., Iowa State University Press, Ames, Iowa, U.S.A. p 310.

WOODE, G.N. and BRIDGER, J.C. (1975). Veterinary Record. 96, 85.

WOODE, G.N., BRIDGER, J.C., HALL, G.A., JONES, T.M. and JACKSON, G. (1976). Journal of Medical Microbiology. 9, 203.

WOODE, G.N. and CROUCH, C.F. (1978). Journal of the American Veterinary Medical Association. 173, 522.

WORLD HEALTH ORGANISATION SCIENTIFIC WORKING GROUP (1980). Bulletin of the World Health Organisation. 58, 519.

YANG, T.S., HOWARD, B. and MacFARLANE, W.V. (1980). Animal Production. 31, 201.

YATES, W.D.G., CLARK, E.G., OSBORNE, A.D., ENWEANI, C.C., RADOSTITS, O.M. and THEEDE, A. (1979). Canadian Veterinary Journal. 20, 261.

YOUNG, B.J. (1969). Journal of Medical Laboratory Technology. 26, 248.

YOUNG, G.A. (1959a). Journal of the American Veterinary Medical Association. 134, 491.

YOUNG, G.A. (1959b). Veterinary Medicine. 54, 339.

YOUNG, G.A. (1964). Advances in Veterinary Science. 9, 61.

YOUNG, G.A. and UNDERDAHL, N.R. (1951). Archives of Biochemistry and Biophysics. 32, 449.

YOUNG, G.A. and UNDERDAHL, N.R. (1953). American Journal of Veterinary Research. 14, 571.

YOUNG, G.A., UNDERDAHL, N.R. and CARPENTER, L.E. (1949). Proceedings of the Society for Experimental Biology and Medicine. 72, 695.

YOUNG, G.A., UNDERDAHL, N.R. and HINZ, R.W. (1955). American Journal of Veterinary Research. 16, 123.

APPENDICES.

APPENDIX 5.1.

COMPTON FEEDING SCHEDULE FOR GNOTOBIOTIC PIGLETS.

Age (days)	Volume (ml) per feed per piglet	
	Evaporated cows' milk	Water/ mineral mixture
0 - 7	75	75
8 - 14	100	75
15 - 21	150	100
22 - 28	200	100
29 - 35	250	150

APPENDIX 5.2.

COMPTON MINERAL SUPPLEMENT - STOCK SOLUTION.

2 large flasks (6000ml) both containing 2000ml distilled water.

1 measuring cylinder containing 1000ml distilled water.

Add 12.5ml HCl conc. into one flask and mark with a letter A.

Dissolve each of the following ingredients separately in the below order, into the other flask making sure that each has dissolved completely before adding the next:-

Potassium Iodide	K1	1.3g
Manganous Chloride	$MnCl_2$ $4H_2O$	18.0g
Cupric Sulphate	$CuSO_4$ $5H_2O$	19.5g
Ferrous Sulphate	$FeSO_4$ $7H_2O$	249.0g

Slowly add this solution to flask A mixing occasionally, then rinse out the empty flask with the 1000ml water in the measuring cylinder and add this to flask A and stir well.

To make up water/mineral solution for piglets add 3ml stock solution to 1000ml water.

APPENDIX 5.3.

(Experiment 2, Chapter 5).

AVERAGE WEIGHTS (kg) OF PIGLETS IN ISOLATOR MC* AND ISOLATORS RMA** AND RMB**.

Isolator	AGE IN DAYS															
	1	4	5	8	12	14	15	17	18	19	20	21	22	26	33	40
MC	1.1	1.1	1.1	1.3	1.3	1.4	1.4	1.7	1.7	2.1	2.1	2.1	2.0	3.4	4.8	6.6
RMA and RMB	1.2	1.2	1.2	1.6	1.6	1.7	1.4	1.3	1.5	1.7	1.8	1.7	1.7	1.8	2.6	3.6

* Isolator MC = piglets exposed to mucosalis only.

** Isolators RMA and RMB = piglets exposed to mucosalis and rotavirus.

APPENDIX 5.4.

(EXPERIMENT 2, CHAPTER 5) AVERAGE VOLUNTARY FOOD INTAKE (ml/feed) OF PIGLETS IN ISOLATOR MC* AND ISOLATORS RMA** AND RMB**.

	DAYS OF AGE																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Isolator MC	38	42	48	62	64	50	60	102	99	100	100	83	86	72	84	105	118	108	125	126	143	150	160	143	177
Isolators RMA and RMB	42	39	47	71	62	50	62	97	94	102	90	94	90	100	32	44	70	62	67	76	78	71	80	61	90

	DAYS OF AGE																				
	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
Isolator MC	220	209	206	233	250	288	267	237	365	425	375	600	500	450	377	410	393	625	550	630	600
Isolators RMA and RMB	86	89	99	111	115	132	127	125	162	192	170	250	214	241	170	210	200	316	332	450	310

* Isolator MC = piglets exposed to mucosalis only.

** Isolators RMA and RMB = piglets exposed to mucosalis and rotavirus.

APPENDIX 5.5.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM MUCOSAL SITES AT NECROPSY OF UNCONTAMINATED PIGLETS* (EXPERIMENT 2, CHAPTER 5).

CBA count	NBGT count	Difference (d)
5.20	4.90	0.30
6.20	6.20	0
6.68	6.68	0
7.35	6.98	0.37
6.50	5.38	1.12
6.50	6.28	0.22
6.50	6.50	0
6.20	6.11	0.09
5.50	4.90	0.60
6.68	6.20	0.48
3.90	3.26	0.64
7.11	6.81	0.30
		$\bar{d}$ = 0.34

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$$= \frac{0.33}{3.46}$$

$$= 0.10$$

t = 3.4 (11 degrees of freedom).

t is significant ($p < 0.1$)

i.e. in uncontaminated piglets CBA plates are significantly better than NBGT plates at isolating mucosalis.

* Data from Table 5.9.

APPENDIX 5.6.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN VITRO (EXPERIMENT 2, CHAPTER 5).

DATA FROM DEPARTMENT VETERINARY PATHOLOGY FILES.

CBA Count	NBGT Count	Difference (d)
7.28	7.24	0.04
7.28	7.16	0.12
7.28	7.20	0.08
7.30	6.98	0.32
7.30	7.02	0.28
7.30	6.90	0.40
7.30	6.93	0.37
7.27	6.88	0.39
7.27	6.60	0.67
7.27	6.84	0.43
7.15	6.18	0.97
7.15	6.65	0.50
6.98	6.60	0.38
6.98	7.00	-0.02
6.98	6.70	0.28
6.98	6.60	0.38
7.28	7.27	0.01
	$\bar{d}$ =	0.33

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}} = \frac{0.25}{4.12} = 0.06$$

$t = 5.5$ (16 degrees of freedom)

t is significant ($p < 0.001$)

i.e. CBA plates are significantly better than NBGT plates at isolating *mucosalis in vitro*.

APPENDIX 5.7.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM MUCOSAL SITES AT NECROPSY OF CONTAMINATED PIGLETS (EXCLUDING PIGLET 3).

DATA FROM TABLE 5.9*(EXPERIMENT 2, CHAPTER 5).

$$t = \frac{\bar{d}}{s.e.}$$

$\bar{d} = -1.29$ $\quad t = -2.93$ (32 degrees of freedom)

$$s.e. = \frac{\sigma n - 1}{\sqrt{n}}$$

$$= \frac{2.50}{5.74}$$

$$= 0.44$$

t is significant ($p < 0.01$)

i.e. in contaminated piglets NBGT plates are significantly better than CBA plates at isolating mucosalis.

* Data listed overleaf.

APPENDIX 5.7 (CONTINUED)

CBA Count	NBGT Count	Difference (d)
0	4.08	-4.08
0	4.68	-4.68
0	0	0
0	6.81	-6.81
0	0	0
0	3.60	-3.60
0	3.00	-3.00
0	5.68	-5.68
0	2.90	-2.90
0	2.30	-2.30
0	2.30	-2.30
7.50	3.20	4.30
6.81	2.78	4.03
4.90	4.90	0
4.81	5.20	-0.39
4.30	6.50	-2.20
0	3.90	-3.90
0	4.08	-4.08
6.20	4.90	1.30
6.20	6.50	-0.30
0	3.08	-3.08
4.90	3.90	1.00
0	0	0
6.32	6.33	0.01
6.68	6.20	0.48
0	0	0
0	2.30	-2.30
4.90	3.90	1.00
5.68	6.20	-0.52
0	2.60	-2.60
0	0	0
0	0	0
0	0	0
	$\bar{d}$ =	-1.29

APPENDIX 5.8.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM FAECAL SWABS OF UN-CONTAMINATED PIGLETS (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

CBA Count	NBGT Count	Difference (d)
5.48	1.70	3.78
5.00	4.70	0.30
6.40	4.00	2.40
4.00	0	4.00
6.57	4.70	1.87
6.40	0	6.40
5.81	0	5.81
6.31	0	6.31
4.70	4.70	0
0	0	0
		$\bar{d}$ = 3.09

$$t = \frac{\bar{d}}{s.e.}$$

t = 3.81 (9 degrees of freedom)

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$$= \frac{2.56}{3.16}$$

$$= 0.81$$

t is significant ($p < 0.01$)

i.e. in uncontaminated piglets CBA plates are significantly better than NBGT plates at isolating mucosalis.

APPENDIX 5.9.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATION OF MUCOSALIS FROM FAECAL SWABS OF CONTAMINATED PIGLETS (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

CBA Count	NBGT Count	Difference (d)
6.18	7.00	-0.82
0	4.70	-4.70
0	2.40	-2.40
0	0	0
0	0	0
0	0	0
0	4.70	-4.70
0	0	0
0	2.70	-2.70
0	4.78	-4.78
4.70	3.70	1.00
4.70	3.81	0.89
6.40	4.70	1.70
4.70	3.60	1.10
0	6.78	-6.78
0	6.06	-6.06
6.00	6.02	-0.02
6.00	5.95	0.05
6.00	5.28	0.72
6.00	6.40	-0.40
4.70	3.30	1.40
4.70	6.13	-1.43
4.70	0	4.70
0	5.70	-5.70
6.00	6.70	-0.70
5.90	0	5.90
7.00	5.70	1.30
		$\bar{d}$ = -0.83

APPENDIX 5.9 (CONTINUED).

$$t = \frac{\bar{d}}{s.e.}$$

$\bar{d} = -0.83$ $\quad t = -1.41$ (26 degrees of freedom).

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$$= \frac{3.08}{5.20}$$

$$= 0.59$$

t is not significant ($p > 0.10$)

i.e. in contaminated piglets there is no significant difference between CBA and NBGT plates in their ability to isolate mucosalis from faecal swabs.

APPENDIX 5.10.

PAIRED t-test COMPARING THE EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM MUCOSAL SITES AT NECROPSY OF PIGLETS CONTAMINATED WITH STREPTOCOCCI* (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.9.

CBA Count	NBGT Count	Difference (d)
0	4.08	-4.08
0	4.68	-4.68
0	0	0
0	6.81	-6.81
0	0	0
0	3.60	-3.60
0	3.00	-3.00
0	5.68	-5.68
0	2.90	-2.90
0	2.30	-2.30
0	2.30	-2.30
7.50	3.20	4.30
		$\bar{d}$ = -2.59

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}} \qquad t = -3.01 \text{ (11 degrees of freedom)}$$

$$= \frac{2.96}{3.46}$$

$$= 0.86$$

t is significant ($p < 0.02$)

i.e. in piglets contaminated with streptococci NBGT plates are significantly better than CBA plates at isolating mucosalis.

* excluding Piglet 3.

APPENDIX 5.11.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM FAECAL SWABS OF PIGLETS CONTAMINATED WITH STREPTOCOCCI (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

CBA Count	NBGT Count	Difference (d)
6.18	7.00	-0.82
0	0	0
0	0	0
0	4.70	-4.70
0	2.40	-2.40
0	0	0
0	4.70	-4.70
0	0	0
0	2.70	-2.70
0	4.78	-4.78
4.70	3.70	1.00
		$\bar{d}$ = -1.74

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}} \qquad t = -2.64 \quad \text{(10 degrees of freedom).}$$

$$= \frac{2.20}{3.32}$$

$$= 0.66$$

t is significant ($p < 0.05$)

i.e. in piglets contaminated with streptococci NBGT plates are significantly better than CBA plates at isolating mucosalis.

APPENDIX 5.12.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATING MUCOSALIS FROM MUCOSAL SITES AT NECROPSY OF PIGLETS CONTAMINATED WITH A YEAST. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.9.

CBA Count	NBGT Count	Difference (d)
0	3.90	-3.90
0	4.08	-4.08
6.20	4.90	1.30
6.20	6.50	-0.30
0	0	0
0	2.30	-2.30
4.90	3.90	1.00
5.68	6.20	-0.52
		$\bar{d}$ = -1.10

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}} \qquad t = -1.49 \text{ (7 degrees of freedom).}$$

$$= \frac{2.09}{2.83}$$

$$= 0.74$$

t is not significant ($p > 0.10$)

i.e. in piglets contaminated with a yeast there is no significant difference between the ability of CBA and NBGT plates in isolating mucosalis.

APPENDIX 5.13.

PAIRED t-test COMPARING THE EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATION OF MUCOSALIS FROM FAECAL SWABS OF PIGLETS CONTAMINATED WITH A YEAST. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

CBA Count	NBGT Count	Difference (d)
6.00	6.02	-0.02
6.00	5.28	0.72
4.70	3.30	1.40
4.70	0	4.70
5.90	0	5.90

$t = \frac{\bar{d}}{s.e.}$ $\quad \bar{d} = 2.54$

$s.e. = \frac{\sigma n-1}{\sqrt{n}}$ $\quad t = 2.19$ (4 degrees of freedom).

$= \frac{2.60}{2.24}$

$= 1.16$

t is not significant ($p > 0.10$)

i.e. in piglets contaminated with a yeast there is no significant difference between the ability of CBA and NBGT plates in isolating mucosalis.

APPENDIX 5.14.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATION OF MUCOSALIS FROM MUCOSAL SITES AT NECROPSY OF PIGLETS CONTAMINATED WITH BACILLI. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.9.

CBA Count	NBGT Count	Difference (d)
6.81	2.78	4.03
4.90	4.90	0
4.81	5.20	-0.39
4.30	6.50	-2.20
0	3.08	-3.08
4.90	3.90	1.00
0	0	0
6.32	6.33	-0.01
6.68	6.20	0.48

$\bar{d} = -0.02$

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$$= \frac{2.00}{3}$$

$$= 0.67$$

$t = -0.03$ (8 degrees of freedom).

t is not significant ($p > 0.10$)

i.e. in piglets contaminated with bacilli there is no significant difference between the ability of CBA and NBGT plates in isolating mucosalis.

APPENDIX 5.15.

PAIRED t-test COMPARING EFFICIENCY OF CBA AND NBGT PLATES IN ISOLATION OF MUCOSALIS FROM FAECAL SWABS OF PIGLETS CONTAMINATED WITH BACILLI. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

CBA Count	NBGT Count	Difference (d)
4.70	3.81	0.89
6.40	4.70	1.70
4.70	3.60	1.10
0	6.78	-6.78
0	6.06	-6.06
6.00	5.95	0.05
6.00	6.40	-0.40
4.70	6.13	-1.43
0	5.70	-5.70
6.00	6.70	-0.70
7.00	5.70	1.30

$\bar{d} = -1.46$

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$t = -1.52$ (10 degrees of freedom).

$$= \frac{3.18}{3.32}$$

$$= 0.96$$

t is not significant ($p > 0.10$).

i.e. in piglets contaminated with bacilli there is no significant difference between CBA and NBGT plates in isolating mucosalis.

APPENDIX 5.16.

STUDENT'S t-test COMPARING THE NUMBERS OF MUCOSALIS ISOLATED FROM UNCONTAMINATED PIGLET NOT EXPOSED TO ROTAVIRUS AND UNCONTAMINATED PIGLETS EXPOSED TO ROTAVIRUS. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.9 (CBA Counts)

Piglet 11	Piglets 2 and 6
5.20	6.50
6.20	6.50
6.68	6.50
7.35	6.20
	5.50
	6.68
	3.90
	7.11
$n_1 = 4$	$n_2 = 8$
$\bar{x}_1 = 6.36$	$\bar{x}_2 = 6.11$
$\sigma n_1 - 1 = 0.90$	$\sigma n_2 - 1 = 1.00$

$t = 0.42$ (10 degrees of freedom)

which is not significant ($p > 0.10$)

i.e. there is no significant difference between the numbers of mucosalis isolated from piglets exposed to mucosalis only and those exposed to mucosalis and rotavirus.

APPENDIX 5.17.

TWO BY TWO CONTINGENCY TABLES COMPARING THE NUMBER OF SUCCESSFUL ISOLATIONS OF MUCOSALIS AT NECROPSY OF PIGLETS EXPOSED TO MUCOSALIS ONLY AND THOSE EXPOSED TO MUCOSALIS AND ROTAVIRUS. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.9 (EXCLUDING PIGLET 3).

(i) General 2 x 2 Contingency Table:

Site at necropsy	Absence of M*	Presence of M	Totals
P(R+M)**	a	b	a+b
P(M)***	c	d	c+d
Totals	a+c	b+d	n

* M = mucosalis **P(R+M) = piglets exposed to mucosalis and rotavirus.

***P(M) = piglets exposed to mucosalis only.

$$p = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{n!\ a!\ b!\ c!\ d!}$$

p must be < 0.05 to be significant.

(ii)

US1	Absence of M	Presence of M	Totals
P(R+M)	1	6	7
P(M)	1	3	4
Totals	2	9	11

APPENDIX 5.17 (CONTINUED)

$$p = \frac{7!\ 4!\ 2!\ 9!}{11!\ 1!\ 6'\ 1!\ 3!}$$

$$= 0.51$$

$p > 0.05$ ∴ not significant

(iii)

MS1	Absence of M	Presence of M	Totals
P(R+M)	1	6	7
P(M)	0	4	4
Totals	1	10	11

$$p = \frac{7!\ 4!\ 1!\ 10!}{11!\ 1!\ 6!\ 0!\ 4!}$$

$$= 0.64$$

$p > 0.05$ ∴ not significant

(iv)

TS1	Absence of M	Presence of M	Totals
P(R+M)	2	5	7
P(M)	1	3	4
Totals	3	8	11

$$p = \frac{7!\ 4!\ 3!\ 8!}{11!\ 2!\ 5!\ 1!\ 3!}$$

APPENDIX 5.17 (CONTINUED)

$= 0.07$

$p > 0.05$ ∴ not significant

(v)

Caecum and LB	Absence of M	Presence of M	Totals
P(R+M)	1	7	8
P(M)	0	4	4
Totals	1	11	12

$$p = \frac{8!\ 4!\ 1!\ 11!}{12!\ 1!\ 7!\ 0!\ 4!}$$

$= 0.67$

$p > 0.05$ ∴ not significant

(vi)

All Sites	Absence of M	Presence of M	Totals
P(R+M)	5	24	29
P(M)	2	14	16
Totals	7	38	45

$$p = \frac{29!\ 16!\ 7!\ 38!}{45!\ 5!\ 24!\ 2!\ 14!}$$

$= 0.31$

$p > 0.05$ ∴ not significant

APPENDIX 5.17 (CONTINUED)

i.e. there is no significant difference between the number of successful isolations at necropsy of piglets exposed to mucosalis and those exposed to mucosalis and rotavirus.

APPENDIX 5.18.

TWO BY TWO CONTINGENCY TABLES COMPARING THE NUMBER OF SUCCESSFUL ISOLATIONS OF MUCOSALIS FROM FAECAL SWABS OF PIGLETS EXPOSED TO MUCOSALIS ONLY AND THOSE EXPOSED TO MUCOSALIS AND ROTAVIRUS. (EXPERIMENT 2, CHAPTER 5).

DATA FROM TABLE 5.8.

(i) General 2 x 2 Contingency Table:

Days of Age	Absence of M*	Presence of M	Totals
P(R+M)**	a	b	a+b
P(M)***	c	d	c+d
Totals	a+c	b+d	n

* M = mucosalis. **P(R+M) = piglets exposed to mucosalis and rotavirus.
***P(M) = piglets exposed to mucosalis only.

$$p = \frac{(a+b)!\,(c+d)!\,(a+c)!\,(b+d)!}{n!\,a!\,b!\,c!\,d!}$$

p must be < 0.05 to be significant.

(ii) Pre-exposure to Rotavirus:

11	Absence of M	Presence of M	Totals
P(R+M)	0	7	7
P(M)	0	4	4
Totals	0	11	11

$$p = \frac{7!\,4!\,0!\,11!}{11!\,0!\,7!\,0!\,4!}$$

$$= 1$$

p > 0.05 ∴ not significant.

APPENDIX 5.18 (CONTINUED)

(iii) 7-8 days post-exposure to Rotavirus:

21-22	Absence of M	Presence of M	Totals
P(R+M)	0	3	3
P(M)	2	1	3
Totals	2	4	6

$$p = \frac{3!\ 3!\ 2!\ 4!}{6!\ 0!\ 3!\ 2!\ 1!}$$

$$= 0.20$$

$p > 0.05$ ∴ not significant.

(iv) 12 days post-exposure to Rotavirus:

26	Absence of M	Presence of M	Totals
P(R+M)	0	4	4
P(M)	1	2	3
Totals	1	6	7

$$p = \frac{4!\ 3!\ 1!\ 6!}{7!\ 0!\ 4!\ 1!\ 2!}$$

$$= 0.43$$

$p > 0.05$ ∴ not significant.

(v) 19 days post-exposure to Rotavirus:

33	Absence of M	Presence of M	Totals
P(R+M)	0	4	4
P(M)	1	2	3
Totals	1	6	7

APPENDIX 5.18 (CONTINUED)

$$p = \frac{4!\ 3!\ 1!\ 6!}{7!\ 0!\ 4!\ 1!\ 2!}$$

$$= 0.43$$

$p > 0.05$ ∴ not significant.

(vi) 26 days post-exposure to Rotavirus:

40	Absence of M	Presence of M	Totals
P(R+M)	0	3	3
P(M)	0	2	2
Totals	0	5	5

$$p = \frac{3!\ 2!\ 0!\ 5!}{5!\ 0!\ 3!\ 0!\ 2!}$$

$$= 1$$

$p > 0.05$ ∴ not significant.

(vii) All Faecal Swabs Post-exposure to Rotavirus:

All faecal swabs post-rotavirus	Absence of M	Presence of M	Totals
P(R+M)	1	14	15
P(M)	4	7	11
Totals	5	21	26

$$p = \frac{15!\ 11!\ 5!\ 21!}{26!\ 1!\ 14!\ 4!\ 7!}$$

$$= 0.08$$

$p > 0.05$ ∴ not significant.

APPENDIX 5.18 (CONTINUED)

i.e. there is no significant difference between the number of successful isolations of *mucosalis* from faecal swabs of piglets exposed to *mucosalis* only and those exposed to *mucosalis* and rotavirus.

APPENDIX 5.19

TWO BY TWO CONTINGENCY TABLES COMPARING THE NUMBER OF SUCCESSFUL ISOLATIONS OF MUCOSALIS AT NECROPSY OF PIGLETS FROM TWO LITTERS, EXPOSED TO MUCOSALIS ONLY (EXPERIMENTS 1 and 2, CHAPTER 5).

DATA FROM TABLE 5.8 AND 5.9 (EXCLUDING PIGLET 3 OF EXPERIMENT 2).

(i) General 2 x 2 Contingency Table:

Site at necropsy	Absence of M*	Presence of M	Totals
P(M1-M4)**	a	b	a+b
P(11,1,7,10)***	c	d	c+d
Totals	a+c	b+d	n

*M = mucosalis

**P(M1-M4) = piglets from Experiment 1 exposed to mucosalis.

***P(11,1,7,10) = piglets from Experiment 2 exposed to mucosalis only.

$$p = \frac{(a+b)!\ (c+d)!\ (a+c)!\ (b+d)!}{n!\ a!\ b!\ c!\ d!}$$

p must be > 0.05 to be significant.

APPENDIX 5.19 (CONTINUED)

(ii)

USl	Absence of M	Presence of M	Totals
P(M1-M4)	1	3	4
P(11,1,7,10)	1	3	4
Totals	2	6	8

$$p = \frac{4!\ 4!\ 2!\ 6!}{8!\ 1!\ 3!\ 1!\ 3!}$$

$$= 0.57$$

$p > 0.05$ ∴ not significant.

(iii)

MSl	Absence of M	Presence of M	Totals
P(M1-M4)	1	3	4
P(11,1,7,10)	0	4	4
Totals	1	7	8

$$p = \frac{4!\ 4!\ 1!7!}{8!\ 1!\ 3!\ 0!\ 4!}$$

$$= 0.50$$

$p > 0.05$ ∴ not significant.

APPENDIX 5.19 (CONTINUED)

(iv)

TS1	Absence of M	Presence of M	Totals
P(M1-M4)	0	4	4
P(11,1,7,10)	1	3	4
Totals	1	7	8

$$p = \frac{4!\ 4!\ 1!\ 7!}{8!\ 0!\ 4!\ 1!\ 3!}$$

$$= 0.50$$

$p > 0.05$ ∴ not significant.

(v)

CAEC and LB	Absence of M	Presence of M	Totals
P(M1-M4)	0	4	4
P(11,1,7,10)	0	4	4
Totals	0	8	8

$$p = \frac{4!\ 4!\ 0!\ 8!}{8!\ 0!\ 4!\ 0!\ 4!}$$

$$= 1.00$$

$p > 0.05$ ∴ not significant.

APPENDIX 5.19 (CONTINUED)

(vi) All sites (Tables 5.3 and 5.9):

All Sites	Absence of M	Presence of M	Totals
P(M1-M4)	3	21	24
P(11,1,7,10)	2	14	16
Totals	5	35	40

$$p = \frac{24!\ 16!\ 5!\ 35!}{40!\ 3!\ 21!\ 2!\ 14!}$$

$$= 0.37$$

$p > 0.05$.·. not significant

i.e. there is no significant difference between the number of successful isolations of mucosalis at necropsy from piglets of two litters, exposed to mucosalis only.

APPENDIX 5.20

TWO BY TWO CONTINGENCY TABLES COMPARING THE NUMBER OF SUCCESSFUL ISOLATIONS OF MUCOSALIS FROM FAECAL SWABS OF PIGLETS FROM TWO LITTERS, EXPOSED TO MUCOSALIS ONLY (EXPERIMENTS 1 AND 2, CHAPTER 5).

DATA FROM TABLES 5.2 AND 5.8.

(i) General 2 x 2 Contingency Table:

Days post-exposure to M*	Absence of M	Presence of M	Totals
P(M1-M4)**	a	b	a+b
P(3,1,7,10)***	c	d	c+d
Totals	a+c	b+d	n

*M = mucosalis

**P(M1-M4) = piglets from Experiment 1 exposed to mucosalis.

***P(3,1,7,10) = piglets from Experiment 2 exposed to mucosalis only.

$$p = \frac{(a+b)!\ (c+d)!\ (a+c)!\ (b+d)!}{n!\ \ a!\ \ b!\ \ c!\ \ d!}$$

p must be > 0.05 to be significant.

(ii) 1-6 days (inclusive) post-exposure to mucosalis.

1 - 6	Absence of M	Presence of M	Totals
P(M1-M4)	4	8	12
P(3,1,7,10)	0	4	4
Totals	4	12	16

APPENDIX 5.20 (CONTINUED)

$$p = \frac{12!\ 4!\ 12!\ 4!}{16!\ 4!\ 8!\ 0!\ 4!}$$

$$= 0.27$$

$p > 0.05$ ∴ not significant.

(iii) 7-15 days (inclusive) post-exposure to mucosalis.

7-15	Absence of M	Presence of M	Totals
P(M1-M4)	3	8	11
P(3,1,7,10)	2	1	3
Totals	5	9	14

$$p = \frac{11!\ 3!\ 5!\ 9!}{14!\ 3!\ 8!\ 2!\ 1!}$$

$$= 0.25$$

$p > 0.05$ ∴ not significant.

(iv) 16-33 days (inclusive post-exposure to mucosalis.

16-33	Absence of M	Presence of M	Totals
P(M1-M4)	5	2	7
P(3,1,7,10)	2	6	8
Totals	7	8	15

$$p = \frac{7!\ 8!\ 7!\ 8!}{15!\ 5!\ 2!\ 2!\ 6!}$$

$$= 0.09$$

$p > 0.05$ ∴ not significant.

APPENDIX 5.20 (CONTINUED)

(v) All Faecal Swabs:

1-33	Absence of M	Presence of M	Totals
P(M1-M4)	12	18	30
P(3,1,7,10)	4	11	15
Totals	16	29	45

$$p = \frac{30!\ 15!\ 16!\ 29!}{45!\ 12!\ 18!\ 4!\ 11!}$$

$$= 0.18$$

$p > 0.05$ $\therefore$ not significant.

i.e. there is no significant difference between the number of successful isolations of mucosalis from faecal swabs of piglets from two litters, exposed to mucosalis only.

APPENDIX 6.1: PILOT STUDY 1. BODY WEIGHTS (KG) OF PIGLETS Y11 — Y18

PIGLET NUMBER	AGE (DAYS)																									
	9	16	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Y11* (BA 315/79)	3.0	4.5	5.0	—	5.9																					
Y17* (BA 333/79)	3.0	4.5	5.0	—	5.5	5.9	5.9	6.8	7.3	7.7	8.2	8.6														
Y13* (BA 345/79)	3.2	5.2	5.2	—	5.9	6.4	6.8	7.3	7.3	7.7	8.2	8.6	9.1	9.5	10.0	10.4	10.9	11.8	11.8							
Y15* (BA 360/79)	2.8	4.0	4.1	—	5.0	5.9	5.9	5.9	6.8	6.8	7.7	7.7	8.6	9.1	9.5	10.0	10.5	10.9	11.4	11.8	12.3	—	—	14.1	14.5	15.0
Y16** (BA 316/79)	2.2	4.0	4.0	4.0	3.9																					
Y14** (BA 334/79)	2.8	4.5	4.2	4.5	4.5	4.2	4.5	4.5	5.0	5.0	5.5	5.8														
Y12** (BA 346/79)	3.2	5.0	5.5	5.0	5.2	5.2	5.2	5.2	5.5	5.8	6.0	6.2	6.2	6.2	6.5	7.0	7.2	8.0	8.1							
Y18** (BA 361/79)	3.0	4.5	5.0	5.0	5.0	5.0	5.0	5.0	5.5	5.8	6.0	6.2	6.7	7.2	7.2	8.0	8.0	9.0	9.8	9.8	10.4	11.0	11.4	11.9	12.4	12.9

* Piglets exposed to mucosalis only

** Piglets exposed to mucosalis and rotavirus

— Piglet not weighed

APPENDIX 6.2.

PILOT STUDY 1, PAIRED T-TESTS COMPARING BODY-WEIGHT GAINS OF PIGLETS EXPOSED TO MUCOSALIS ONLY AND THOSE EXPOSED TO MUCOSALIS AND ROTAVIRUS.

DATA DERIVED FROM APPENDIX 6.1.

(i) Body-weight gains (kg) prior to rotavirus infection (9-19 days).

P (M only) *	P (M + R) **	Difference (d)
2.0	1.8	0.2
2.0	1.4	0.6
2.0	2.3	- 0.3
1.3	2.0	- 0.7
		$\bar{d}$ = - 0.05

* P (M only) = piglets exposed to mucosalis only.

** P (M + R) = piglets exposed to mucosalis and rotavirus.

$$t = \frac{\bar{d}}{s.e.}$$

t = - 0.21 (3 degrees of freedom)

$$s.e. = \frac{\sigma n - 1}{\sqrt{n}}$$

t is not significant ($p > 0.10$)

$$s.e. = \frac{0.49}{2}$$

$$= 0.24$$

APPENDIX 6.2.
(Continued)

(ii) Body-weight gains (kg) post-rotavirus infection (19-21 days).

P(M only)	P (M+R)	Difference (d)
0.9	- 0.1	1.0
0.5	0.3	0.2
0.7	- 0.3	1.0
0.9	0	0.9
		$\bar{d}$ = 0.78

$t = \frac{\bar{d}}{s.e.}$

$s.e. = \frac{\sigma n-1}{\sqrt{n}}$

$= \frac{0.33}{2}$

$= 0.17$

$t = 4.59$ (3 degrees of freedom)

t is significant ($P < 0.02$)

(iii) Body-weight gains (kg) 22 - 27 days

P(M only)	P (M+R)	Difference (d)
2.3	1.3	1.0
1.8	0.8	1.0
1.8	1.0	0.8
		$\bar{d}$ = 0.93

$t = \frac{\bar{d}}{s.e.}$

$s.e. = \frac{\sigma n-1}{\sqrt{n}}$

$= \frac{0.09}{1.73}$

$= 0.05$

$t = 18.6$ (2 degrees of freedom)

t is significant ($P < 0.01$)

APPENDIX 6.2.
(Continued)

(iv) Body-weight gains (kg) 27 - 28 days.

P (M only)	P(M&R)	Difference (d)
0.4	0.3	0.1
0.4	0.2	0.2
0	0.2	- 0.2
		$\bar{d}$ = 0.03

$$t = \frac{\bar{d}}{s.e.}$$

$$s.e. = \frac{\sigma n-1}{\sqrt{n}}$$

$$= \frac{0.17}{1.73}$$

$$= 0.10$$

t = 0.30 (2 degrees of freedom)

t is not significant (P > 0.10)

Conclusions

(i) There was no significant difference in weight gains between the two groups of piglets prior to rotavirus exposure.

(ii) After exposure to rotavirus the control group showed significantly better weight gains than the rotavirus-exposed group until approximately 8 days post-exposure.

APPENDIX 6.3.

PILOT STUDY 1. STUDENT'S T-TESTS COMPARING HISTOLOGICAL MEASUREMENTS IN THE SMALL INTESTINES OF PIGLETS 11 and Y16.

DATA FROM TABLE 6.4.

(i) USl Villus heights

Y11	Y16	Y11	Y16
355	200	$n_1 = 10$	$n_2 = 10$
537	273	$\bar{x}_1 = 408$	$\bar{x}_2 = 186$
400	155	$\sigma n_1 - 1 = 70$	$\sigma n_2 - 1 = 49$
473	155		
419	191		
364	164		
419	100		
282	209		
445	246		
382	170		

t = 8.2 (18 degrees of freedom)

t is significant ($p < 0.001$)

APPENDIX 6.3.
(Continued)

(ii) USl Crypt depths

Y11	Y16	Y11	Y16
218	243	$n_1 = 10$	$n_2 = 10$
161	286	$\bar{x}_1 = 174$	$\bar{x}_2 = 251$
179	286	$\sigma n_1 - 1 = 22$	$\sigma n_2 - 1 = 41$
143	303		
164	278		
173	179		
200	196		
179	232		
161	232		
159	271		

t = - 5.2 (18 degrees of freedom)

t is significant ($P < 0.001$)

(iii) MSl Villus heights.

Y11	Y16	Y11	Y16
546	510	$n_1 = 10$	$n_2 = 10$
510	464	$\bar{x}_1 = 486$	$\bar{x}_2 = 419$
501	355	$\sigma n_1 - 1 = 43$	$\sigma n_2 - 1 = 70$
537	410		
491	300		
437	408		
464	500		
446	474		
419	345		
510	420		

t = 2.6 (18 degrees of freedom)

t is significant ($P < 0.02$)

APPENDIX 6.3.
(Continued)

(iv) MS1 Crypt depths

Y11	Y16	Y11	Y16
125	179	$n_1 = 10$	$n_2 = 10$
164	179	$\bar{x}_1 = 190$	$\bar{x}_2 = 180$
161	161	$\sigma n_1 - 1 = 44$	$\sigma n_2 - 1 = 23$
268	161		
250	139		
179	186		
179	221		
214	196		
157	203		
203	180		

t = 0.6 (18 degrees of freedom)

t is not significant ($P > 0.10$)

(v) TS1 Villus heights

Y11	Y16	Y11	Y16
355	346	$n_1 = 10$	$n_2 = 10$
328	237	$\bar{x}_1 = 290$	$\bar{x}_2 = 315$
273	209	$\sigma n_1 - 1 = 36$	$\sigma n_2 - 1 = 57$
273	355		
282	328		
282	382		
228	300		
319	300		
300	382		
264	315		

t = -1.2 (18 degrees of freedom)

t is not significant ($P > 0.10$)

APPENDIX 6.3.
(Continued)

(vi) TSl Crypt depths

Yll	Y16	Yll	Y16
136	168	$n_1 = 10$	$n_2 = 10$
118	168	$\bar{x}_1 = 127$	$\bar{x}_2 = 150$
139	86	$\sigma n_1 - 1 = 8$	$\sigma n_2 - 1 = 28$
114	171		
125	139		
125	161		
126	129		
120	146		
134	150		
130	186		

$t = -2.5$ (18 degrees of freedom)

t is significant ($P < 0.05$)

Conclusions

(i) The USl villi of Piglet Yll were significantly longer than those of Piglet Y16.

(ii) The USl crypts of Piglet Yll were significantly shorter than those of Piglet Y16.

(iii) The MSl villi of Piglet Yll were significantly longer than those of Piglet Y16.

(iv) There was no significant difference between the MSl crypt depths of Piglets Yll and Y16.

(v) There was no significant difference between the TSl villi heights of Piglets Yll and Y16.

(vi) The TSL crypts of Piglet Yll were significantly shorter than those of Piglet Y16.

APPENDIX 7.1.

LITTER 2. TWO BY TWO CONTINGENCY TABLES COMPARING THE COLONISATION PATTERNS OF MUCOSALIS IN PIGLETS EXPOSED TO CRYPTOSPORIDIA AND MUCOSALIS, AND PIGLETS EXPOSED TO MUCOSALIS ONLY.

DATA FROM TABLE 7.7.

(i) General 2 x 2 Contingency Table:

Site	Absence of M*	Presence of M	Totals
P (C + M)**	a	b	a + b
P (M) ***	c	d	c + d
Totals	a + c	b + d	n

* M = mucosalis **P(C+M) = piglets exposed to cryptosporidia and mucosalis.

*** P(M) = piglets exposed to mucosalis only.

$$P = \frac{(a+b)!\ (c+d)!\ (a+c)!\ (b+d)!}{n!\ a!\ b!\ c!\ d!}$$

P must be < 0.05 to be significant.

(ii)

USl	Absence of M	Presence of M	Totals
P (C + M)	3	3	6
P (M)	3	0	3
Totals	6	3	9

$$P = \frac{6!\ 3!\ 6!\ 3!}{9!\ 3!\ 3!\ 3!\ 0!}$$

$$= 0.24$$

APPENDIX 7.1.
(Continued)

$p > 0.05$.˙. not significant

(iii)

MS1	Absence of M	Presence of M	Totals
P(C&M)	3	3	6
P(M)	2	1	3
Totals	5	4	9

$$p = \frac{6!\ 3!\ 5!\ 4!}{9!\ 3!\ 3!\ 2!\ 1!}$$

$= 0.48$

$p > 0.05$.˙. not significant

(iv)

TS1	Absence of M	Presence of M	Totals
P(C&M)	4	2	6
P(M)	2	1	3
Totals	6	3	9

$$p = \frac{6!\ 3!\ 6!\ 3!}{9!\ 4!\ 2!\ 2!\ 1!}$$

$= 0.54$

$p > 0.05$.˙. not significant

APPENDIX 7.1.
(Continued)

(v)

Caec	Absence of M	Presence of M	Totals
P(C&M)	5	1	6
P(M)	3	0	3
Totals	8	1	9

$$P = \frac{6!\ 3!\ 8!\ 1!}{9!\ 5!\ 1!\ 3!\ 0!}$$

$$= 0.67$$

$P > 0.05$ ∴ not significant

(vi)

LB	Absence of M	Presence of M	Totals
P(C&M)	6	0	6
P(M)	3	0	3
Totals	9	0	9

$$P = \frac{6!\ 3!\ 9!\ 0!}{9!\ 6!\ 0!\ 3!\ 0!}$$

$$= 1.0$$

$P => 0.05$ ∴ not significant

(vii)

All Enteric Sites	Absence of M	Presence of M	Totals
P(C&M)	21	9	30
P(M)	13	2	15
Totals	34	11	45

$$p = \frac{30!\ 15!\ 34!\ 11!}{45!\ 21!\ 9!\ 13!\ 2!}$$

APPENDIX 7.1.
(continued)

$= 0.48$

$p > 0.05$ $\therefore$ not significant

i.e. there is no significant difference between the colonisation patterns of mucosalis in piglets exposed to cryptosporidium and mucosalis and those exposed to mucosalis only.

APPENDIX 7.2.

WILCOXON TWO-SAMPLE TEST COMPARING THE NUMBERS OF MUCOSALIS ISOLATED FROM PIGLETS EXPOSED TO CRYPTOSPORIDIA AND MUCOSALIS WITH THE NUMBERS ISOLATED FROM PIGLETS EXPOSED TO MUCOSALIS ONLY.

DATA DERIVED FROM TABLE 7.7.

Numbers of Mucosalis in P(C&M) (Total/piglet)	Rank(R)	Numbers of Mucosalis in P(M) (Total/piglet)	Rank(R)
0	1.5	0	1.5
4×10^2	3.5	4×10^2	3.5
8×10^2	6	6×10^2	5
10×10^2	7		
14×10^2	8		
18×10^2	9		
$n_1 = 6$	$\leq R = 35$	$n_2 = 3$	$\leq R = 10$

a) $C = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - \leq R(n_2)$

$= 14$

b) $n_1 n_2 - C = \underline{4}$

The Wilcoxon Statistic (U) is the largest of a) or b).

$\therefore$ U = 14 which is not significant

i.e. there is no significant difference between the numbers of mucosalis isolated from piglets exposed to cryptosporidia and mucosalis, and the numbers isolated from piglets exposed to mucosalis only.