A SURVEY OF THE PREVALENCE OF EQUID HERPESVIRUS 2 INFECTIONS

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A SURVEY OF THE PREVALENCE OF EQUID HERPESVIRUS 2 INFECTIONS

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SUMMARY

Slowly cytopathogenic viruses were isolated from the leucocytes of 17 out of 19 (89 per cent) apparently normal horses, one yearling and one of two foals under two months of age.

The viruses replicated in primary rabbit kidney cells, the RK 13 rabbit kidney cell line and the VERO monkey kidney cell line but not in the BHK 21 hamster kidney cell line.

Typical Cowdry Type A intranuclear inclusions were produced in culture cells and these were shown to contain DNA by acridine orange fluorescence.

Electron microscopy of negatively stained preparations demonstrated characteristic herpesvirus morphology.

The complement fixation reaction demonstrated an antigenic relationship between the viruses isolated and equid herpesvirus 2. Conventional serum neutralisation tests failed to demonstrate neutralising antibody in pooled normal horse serum, pooled normal rabbit serum and reference antisera to equid herpesvirus 1 and 2. Double immuno-diffusion gave no reaction.
INTRODUCTION

Herpesviruses are large viruses which have a DNA core, a capsid consisting of 162 capsomeres arranged in the form of an icosahedron and a lipid-glycoprotein envelope.

The equine herpesviruses, isolated by many workers throughout the world, appear to fall into three taxonomic groups. The diseases produced by two of these groups, namely equine rhinopneumonitis and equine coital exanthema, are well documented but little data are available on the epidemiology of slowly growing equine herpesviruses in Britain.

Leucocytes have been shown to be a rich source of these viruses in the U.S.A. and a similar situation was anticipated in Scotland.

The opportunity was taken, during routine haematological examination of horses submitted to the Royal (Dick) School of Veterinary Studies Field Station for a variety of surgical and orthopaedic conditions, to survey the prevalence of these viruses.

This dissertation presents the results of the survey and studies, *ex vivo*, on the viruses isolated with reference to growth characteristics, virion morphology and antigenic relationships.
HISTORICAL BACKGROUND

The earliest indication of herpesvirus infection in horses was in 1961 when Tajima, Shimizu and Ishizaki suggested that the aetiology of equine viral rhinopneumonitis and abortion was a herpesvirus. Their proposal was based on observations of a characteristic growth pattern and cytopathic effects in cell culture and thin section electron microscopy. However, it was not until 1963 that confirmation, based on negatively stained electron micrographs, was obtained by Plummer and Waterson. These authors recommended that the virus be designated Equine Herpesvirus 1 (EHV1) and created a new category, Equine Herpesvirus 2 (EHV2), to describe their "IK isolate" which had been obtained, by nasal swab, from a case of upper respiratory tract infection in Britain.

Since then many isolations of other herpesviruses have been reported, most of which seem to be related to EHV2 by morphology, growth character and antigenicity, where these have been studied. Many workers, in widely separate countries, have reported accidental isolation of viruses from a variety of equine tissues. Spontaneous degeneration has been observed often in equine kidney cell cultures (Kono and Kobayishi, 1963a; Karpas, 1966; Erasmus, 1970; Steck, 1970; Burrows and Goodridge, 1970; Burki, 1970;

The venereal virus of equine coital exanthema is now recognised as taxonomically distinct and the term Equine Herpesvirus 3 (EHV3), first suggested by Plummer, Goodheart and Studdert in 1973, is generally accepted. Isolation of the virus was first reported from Germany (Petzoldt, 1967) but has since been reported from the U.S.A. (Bryans, 1968), Canada (Girard, Greig and Mitchell, 1968), Australia (Pascoe, Spadbrow and Bagust, 1968), Norway (Krogsrud and Onstad, 1971) and Britain (Gibbs and Roberts, 1972).

For many years the terminology was confused, each isolate was given either a new descriptive name e.g. 'Cytopathicogenic Equine Orphan Virus' (Kono et al, 1963a) and 'Cytomegalo-like Virus' (Hsiung et al, 1969) or designated
as a separate taxon e.g. Equine Herpesvirus 3 (Karpas, 1966) and Equine Herpesvirus 4 (Hsiung et al, 1969). Detailed examination of nucleic acid content and antigenic relationships led Plummer et al (1973) to conclude that all the slowly growing herpesviruses of the horse should be grouped together as EHV2. A recent international meeting, under the chairmanship of Roizman (1973), recommended that the terminology should be Equid Herpesvirus 1, etc. The term cytomegalovirus is avoided because of the vague and ambiguous criteria on which this designation is based (Plummer et al, 1973; Plummer, 1973).

The main features of the three types will be discussed in terms of this classification.

GROWTH CHARACTERISTICS

The cytopathic effects were essentially the same in each group with foci of rounded, refractile cells becoming visible and spreading centrifugally until the whole cell sheet was involved. However, variation, both in time to first appearance of cytopathic effect and in its rate of progress, was marked. EHV1 usually produced a cytopathic effect in two to five days compared to a figure frequently in excess of ten days for EHV2 on initial isolation, blind passages often being required (Kemeney et al, 1970; Studdert, 1974). Many authors have found that passage of EHV2 reduced the time to inception of cytopathic effects to three days. EHV3 occupied an intermediate position with
the Australian isolate being comparable to EHV1 which produced cytopathic effects in less than two days after passage (Burki, Pichler and Sibalin, 1973; Pascoe et al, 1968).

The demarcation was not clear-cut but growth curves indicated that the viruses which formed small plaques (notably EHV2) replicated more slowly and had longer eclipse periods than did those which formed larger plaques (Plummer et al, 1973). Tube culture and plaque methods have been used widely to study the growth kinetics of these viruses (Plummer et al, 1973; Kemeney et al, 1971; Studdert, 1974). None were completely cell associated but EHV1 released virus into the culture fluid faster and to a higher titre than EHV2 (Plummer, Bowling and Goodheart, 1969; Plummer et al, 1973; Studdert, 1974). EHV3 was liberated to high titre into the culture fluid (Burki et al, 1973; Studdert, 1974). There is evidence that individual isolates of EHV2 differ in their tendency to remain cell associated (Turner, Studdert and Peterson, 1970).

Variation was also seen in the spectrum of cell types which will support viral replication. EHV1 grew in a great variety of primary cultures and cell lines (Bagust, 1971), EHV2 was reported to be restricted to a narrower range of primary cultures (Plummer et al, 1963; Karpas, 1966) whilst EHV3 was restricted to tissues of equine origin (Bryans, Rogers, McCollum and Ludwig, 1971). Growth of EHV2 in a
rabbit kidney cell line was mentioned by Plummer et al., (1969) and Russell and Crawford (1964), and in a rabbit kidney cell line and VERO monkey kidney cell line by Ratulà et al. (1972) and Erasmus (1970). However, Moraillon et al. (1971) failed to establish growth in the VERO cell line.

Light microscopy of cell culture preparations, stained with haematoxylin and eosin, showed that all three types produced eosinophilic intranuclear inclusions which were typical Cowdry Type A inclusions, characterised by margination of chromatin and surrounding halo. They were particularly prominent in EHV2 where many adjacent cells showed striking inclusions without any macroscopic evidence of cytopathic effect (Studdert, 1974). Syncytia were seen commonly in EHV2, especially when grown in a rabbit epithelial cell line (Plummer et al., 1969) and EHV3 regularly produces large syncytia (Studdert, 1974). Intracytoplasmic inclusions have been described by few authors for EHV2 and were faint and less marked than in human cytomegalovirus infection (Plummer et al., 1969; Erasmus, 1970). Juxtanuclear cytoplasmic vacuoles were mentioned by Moraillon et al., (1971).

Staining with acridine orange caused the intranuclear inclusion to fluoresce a characteristic yellow-green with ultraviolet light and the Feulgen staining technique gave the red colour associated with DNA (Karpas, 1966; Kemeney et al., 1970).
MORPHOLOGY

The three types of virus have been described as essentially similar in structure to each other and to other herpes viruses (Plummer et al., 1963; Karpas, 1966; Girard et al., 1968; Pascoe et al., 1968; Studdert, 1974).

In negatively stained electron micrographs the complete virion consisted of an envelope, approximately 200 nm in diameter, surrounding an apparently circular or hexagonal nucleocapsid, approximately 100 nm in diameter. The icosahedral capsid consisted of 162 hollow capsomeres (Karpas, 1966; Bagust, 1971; Studdert, 1974) and the nucleoid was reported to measure 25 to 30 nm (Studdert, 1974), or 60 nm (Okaniwa et al., 1969) in diameter. A distinctive electron-lucent cross within the nucleocapsid has been reported for EHV1 (Reczko, Bohm and Straub, 1965).

Virions lacking envelopes have been seen frequently and, in EHV1, they have been shown to be less infective (Abodeely, Lanson and Randall, 1970). Similarly nucleocapsids without nucleoids have been described for EHV1 and EHV2 (Okaniwa, Fukunaga and Kono, 1969; Erasmus, 1970; Studdert, 1974).

REPLICATION

In electron micrographs of thin sections of infected cells, masses of fine granular material in the nucleus, thought to represent the inclusion body observed by light microscopy, were shown to contain single membrane-bound
virions. Double membrane-bound virions were demonstrated external to the nuclear membrane and virions with triple membranes occurred in cytoplasmic vacuoles (Okaniwa et al., 1969). These findings support the statement of Andrewes and Pereira (1972) that development commenced in the nucleus and different elements were added to the virion in different compartments of the nucleus and cytoplasm. They also stated that release of virus from cells was gradual and might not involve cell destruction. However, Roizman and Spear (1973) considered that lysis was the eventual fate of infected cells. Infection of adjacent cells has been postulated and the mechanism of entry was shown to be pinocytosis (Abodeely et al., 1970; Andrewes and Pereira, 1972).

Eclipse periods were similar for EHV1 and EHV3 at about six hours compared to 18 hours for EHV2. Maximum levels of cell-associated virus were reached by 24 hours with EHV1 and EHV3 and maximum extracellular virus about two hours later. EHV2 showed a marked tendency to remain cell-associated and maximum levels of cell-associated and extracellular virus were not attained until after 96 hours. The titre of extracellular virus exceeded $10^6$ plaque forming units (pfu) per ml in EHV1, reached between $10^5$ and $10^6$ pfu per ml in EHV3 but rarely exceeded $10^5$ pfu per ml in EHV2 (Karpas, 1967; Plummer, Bowling and Goodheart, 1969; Studdert, 1974).

DNA COMPOSITION

In one study, density gradient analysis of DNA extracted
from ten isolates of EHV2 showed a remarkable similarity in the DNA density, all being in the range 1.716 to 1.717 g per ml which also was similar to EHV1 (Plummer et al., 1969). Studdert (1974) obtained figures of 1.714 and 1.715 g per ml for EHV1 and EHV2 respectively. EHV3 was shown to differ markedly with 1.725 g per ml (Plummer et al., 1969; Ludwig et al., 1971; Studdert, 1974).

The ratio between guanine plus cytosine and total nucleotides showed a similar relationship, the figures being approximately 56 per cent for EHV1 and EHV2, and 66 per cent for EHV3 (Plummer et al., 1969; Studdert, 1974).

ACTION OF PHYSICAL AND CHEMICAL AGENTS

In common with other herpes viruses the equine representatives were inactivated by lipid solvents, a pH less than 3 and by heating at 56°C for 30 minutes (Bagust, 1971; Studdert, 1974). Trypsin sensitivity was demonstrated by Agthe and Schmidt (1971).

ANTIGENIC RELATIONSHIPS

The antigenic relationship of the three groups has been reviewed by Bagust (1971), Plummer et al (1973) and Studdert (1974).

There was a striking degree of antigenic uniformity in all strains of EHV1, whatever their origin (Studdert, 1974). However, analysis of cross-neutralisation products has distinguished at least two sub-types. Sub-type 1 was
represented by the Ky-D strain, isolated in the U.S.A.,
and sub-type 2 included the Army 183 strain and a number
of European strains (Burrows, 1970). The Japanese isolate,
H45, was shown to belong to sub-type 2 (Shimizu, Ishizaki,
Ishii, Kawakami, Kaji, Sugimura and Matuomoto, 1959).
Antigenic differences have been demonstrated between
respiratory and abortigenic strains of the virus (Burrows

Some antigenic relationship has been shown to infectious
bovine rhinotracheitis virus by complement fixation and gel
diffusion reactions but not by neutralisation tests
(Carmichael and Barnes, 1962).

Considerable antigenic heterogeneity has been observed
amongst different isolates of EHV2 using neutralisation
tests, but all were distinguishable from EHV1 (Plummer et al,
1969; Bryans, 1970; Turner et al, 1970; Plummer et al,
1973). However, Kemeney (1971) used plaque reduction and
neutralisation kinetics tests and concluded that his isolates
and three other strains were so closely related that they
formed a single serotype.

The complement fixation reaction has been used to
investigate relationships between Japanese isolates and for
serological surveys (Kono et al, 1963b). Homogeneity was
found. The test has not yet been used to compare isolates
from different countries.
Those strains of EHV3, so far tested, have been antigenically very similar but distinct from EHV1 and EHV2 (Studdert, 1974).

ASSOCIATION WITH DISEASE

Acute respiratory disease and abortion are commonly described attributes of EHV1 infection (McGee, 1969). In addition, genital lesions (Studdert, 1974) and a paralytic syndrome (Saxegaard, 1966) have been described.

Most isolations of EHV2 have been made from clinically normal horses but isolations from horses with respiratory disease have been reported (Plummer et al, 1963; Studdert et al, 1970; Turner et al, 1970; Ratulld et al, 1972; Scatotta et al, 1972). An aetiological rôle in disease has not been proven and inoculation of a mare and foal did not reproduce the respiratory disease (Moraillon et al, 1971). Inoculation of a gnotobiotic foal with the IK isolate produced no evidence of pathogenicity (Plowright, personal communication). Kono et al (1963b) demonstrated seroconversion, in the absence of clinical disease, in a group of recently purchased horses.

In contrast to EHV1, EHV2 has shown evidence of latency following initial infection and reactivation by stress has been reported (Erasmus, 1968; Studdert, 1974).

The association of EHV3 with coital exanthema (blaschenausslag) has been well documented and mild
respiratory manifestations noted (Petzoldt, 1967; Gibbs and Roberts, 1972).

PREVALENCE STUDIES

Serological surveys have indicated a high incidence of neutralising antibodies to EHV1, e.g. 92 per cent in one survey (Burrows, 1968). Latency has not been proven and isolation of EHV1 virus was only successful within 14 days of infection. However, abortion was often delayed for several months after infection of the dam. Nevertheless, virus was still isolated from the aborted foetus (Studdert, 1974).

EHV2 viruses have been isolated from infected horses for periods over a year and Studdert (1974) has demonstrated infection as early as 30 days after birth but he failed to isolate virus from neonatal foals and foetuses. The virus has been isolated from a large proportion of sampled horses, e.g. Kemeney (1970) found a prevalence of 88.7 per cent by leucocyte sampling.

Serological surveys of EHV2 have also indicated high infection rates, 66.3 per cent by complement fixation test (Kono et al, 1963) and 45 per cent by neutralisation test (Burrows, 1968).

EHV3 virus has been isolated only within 14 days of infection. Between 21 and 53 per cent of horses over two years of age were shown to have neutralising antibodies in
one survey (Bagust, Pascoe and Harden, 1972).

In short, two equine herpesviruses are recognised pathogens whereas a third, the commonest, may well be non-pathogenic.
MATERIALS AND METHODS

REAGENTS

Cell Culture Media

Growth medium was prepared to the following formula:-

- Medium 199 (10X concentration) 10 ml
- Calf serum (foetal or inactivated normal) 10 ml
- Sodium bicarbonate solution (4.4 per cent), gassed with carbon dioxide to neutral pH 2 ml and autoclaved
- Sodium benzylpenicillin solution (100,000 IU per ml) 0.1 ml
- Streptomycin solution (50,000 µg per ml) 0.1 ml
- Amphotericin solution (50 µg per ml) 0.1 ml
- Deionised distilled water ad 100 ml

Maintenance medium was of similar composition except that the calf serum content was reduced to five ml and the sodium bicarbonate content was increased to three ml.

When single strength medium 199 was used, already buffered and containing antibiotics, the serum and amphotericin were added to 100 ml quantities.

BHK21 medium was prepared in the following manner:-

- Eagles Minimal Essential Medium (Glasgow modification 10X concentration) 8 ml
- Tryptose phosphate broth 10 ml
- Foetal or normal calf serum 10 ml
Sodium bicarbonate solution buffered with carbon dioxide 5 ml
Sodium benzylpenicillin solution (100,000 IU per ml) 0.1 ml
Streptomycin solution (50,000 μg per ml) 0.1 ml
Amphotericin solution (50 μg per ml) 0.1 ml
Deionised distilled water 72 ml

**Balanced Salt Solution**
Hank's balanced salt solution was used with the addition of antibiotics and sodium bicarbonate solution in proportions similar to those employed in growth medium.

**Phosphate Buffered Saline**
Dulbecco A phosphate buffered saline (PBS) was used throughout.

**Trypsin Solution**
An 0.25 per cent solution of trypsin in PBS was supplemented with 0.2 ml volumes of sodium benzylpenicillin solution (100,000 IU per ml), streptomycin solution (50,000 μg per ml) and amphotericin solution (50 μg per ml).

**Saline Trypsin-Versene (STV)**
Five ml each of one per cent trypsin solution in PBS and one per cent versene solution in distilled water were added to 500 ml of PBS. This gave approximately 1/10,000 dilutions of the constituents.

**Reference Antisera**
Sera from gnotobiotic foals infected with the H45 strain
of EHV1 and the IK isolate of EHV2 were obtained from The Royal Veterinary College, London by courtesy of Professor W. Plowright.

CELL CULTURES

Primary Rabbit Kidney

Rabbits up to nine days old were killed with carbon dioxide and pinned to a board. The abdominal skin was wiped with methylated spirits and flamed prior to opening the abdomen with sterile scissors and forceps. The instruments were discarded and a new set used to detach the kidneys from surrounding fat and to remove them to a sterile petri dish. The capsule was removed, thin strips of cortex were cut, transferred to balanced salt solution at 37°C and finely minced with scissors. The tissue fragments were washed twice with balanced salt solution, transferred to a conical flask with 100 ml of trypsin solution prewarmed to 37°C and agitated with a magnetic stirrer at room temperature. After 15 minutes the tissue fragments were allowed to settle and the supernatant fluid was discarded. A fresh 100 ml of trypsin solution at room temperature was added and the flask placed on a magnetic stirrer* which maintained the contents at between 3 and 4°C. Trypsinisation was continued for approximately 18 hours

* Fryka - Kältetechnik KP250
before the supernatant fluid was collected and centrifuged at 150 g for ten minutes. Trypsin solution at room temperature was added to the remaining tissue fragments which were forcefully broken up by pipetting. The trypsin was allowed to act at room temperature for 20 minutes before repeating the harvesting procedure. Cell harvests obtained by centrifugation were resuspended in growth medium and stored at 4°C until harvesting of cells was complete. Harvests were then pooled, centrifuged, resuspended in growth medium in the ratio of one ml of packed cells to 100 ml of medium and counted in a haemocytometer. The cell concentration was adjusted to three million per ml for seeding into prewarmed flasks; five ml for a 25 cm² plastic flask*; 25 ml for a 75 cm² plastic flask*; one ml for Leighton tubes and test tubes and pro rata for glass medicinal flats.

Flasks with a volume greater than 30 ml were gassed with five per cent carbon dioxide. All cultures were incubated for 48 hours, undisturbed, at 37°C before decanting the medium and replacing it with an equal volume of maintenance medium. Changes of medium were made when indicated by an acid change in the pH of the medium, usually every two days. Confluent monolayers were normally obtained within three days.

Becton Dickinson & Co., California, U.S.A.
Secondary and Tertiary Rabbit Kidney

The medium was decanted from the initial culture flasks and the cell sheets were washed with PBS prewarmed to 37°C. STV solution at the same temperature was added and allowed to act, with constant agitation, until no clumps of cells remained. Dispersal of the cells was aided by pipetting the fluid vigorously. The harvested fluid was centrifuged at 150 g for ten minutes, the supernatant fluid discarded and the cells resuspended in growth medium. After counting and adjusting the concentration to one million cells per ml, flasks were seeded and tended as described for primary cultures. Confluent monolayers were normally obtained within three days.

Rabbit Kidney Cell Line (RK13)

The cell stocks of this epithelial-like cell line were grown in 500 ml glass medicinal flats containing 40 ml of culture medium. These cultures were split in the ratio of 1:2 using STV. When required to seed Leighton tubes, test tubes and 25 cm² plastic flasks, a cell suspension containing 400,000 cells per ml was prepared in growth medium and dispensed as for primary cultures. Media were changed as necessitated by an acid pH change.

Monkey Kidney Cell Line (VERO)

The cell stocks of this fibroblast-like cell line were maintained, split and used to seed culture flasks in the same manner as the RK13 cell line.
Hamster Kidney Cell Line (BHK21)

This fibroblast-like cell line was treated in the same manner as the RK13 cell line except that the specific medium was used.

TECHNIQUES

Equine Leucocyte Suspensions

Blood was collected by jugular venepuncture into 50 ml evacuated containers * and allowed to stand at room temperature for 40 minutes. The plasma was removed and centrifuged at 300 g for ten minutes to separate the leucocytes. The clear plasma was removed with a sterile pasteur pipette and stored at -20°C for use later in serology. The leucocytes were suspended in 0.3 ml of growth medium at 37°C and two tenfold dilutions in growth medium were made.

Virus Isolation

Three day cultures in 25 cm² plastic flasks were prepared by removing all medium from the cell sheet. The three dilutions were seeded, in 0.3 ml volumes onto primary rabbit kidney cell cultures, using one culture for each dilution. After a one hour adsorption period at 37°C, with agitation every ten minutes, five ml of maintenance medium was added to each flask and incubation continued. Changes of medium were made when necessitated by pH change. Cultures were observed daily for cytopathic effect.

* Becton Dickinson & Co., New Jersey, U.S.A.
Virus Passage and Preparation of Stocks

Cell sheets were scraped off the flask surface with a nichrome wire loop, flame sterilised, usually when approximately 70 per cent of cells were affected by the cytopathic effect, or after at least 14 days in culture. The resultant suspension of cells in culture medium were used in 0.3 ml volumes to inoculate monolayers in plastic flasks and in 0.1 ml volumes for Leighton tube cultures. Media were removed prior to inoculation. When samples had shown cytopathic effects the contents of the three flasks were pooled before passage to two fresh cultures. If no cytopathic effects had been observed 0.3 ml of the contents of each flask was passaged individually to one new flask.

One hour at 37°C was allowed for adsorption, with agitation every ten minutes, before adding maintenance medium. The remainder of each harvest was stored at either -180°C in the vapour above liquid nitrogen or at -20°C. Half of each stock received ten per cent (v/v) dimethylsulphoxide (DMSO) as a cryoprotectant.

Light Microscopy

Cell cultures were grown on coverslips in Leighton tubes, infected and examined for cytopathic effect in the normal way. When required, the coverslips were washed out with PBS and fixed by immersion in methanol for two minutes. Ehrlich's haematoxylin and aqueous eosin were used for staining and the coverslips were mounted on glass slides.
Fluorescence Microscopy

Leighton tube coverslip cultures, fixed with methanol, were air dried and immersed in acridine orange stain for two minutes. Excess stain was rinsed off with McIlvane's buffer and the coverslips were air dried before mounting on thin glass slides in non-fluorescent immersion oil. The preparations were viewed with ultraviolet light excitation.

Electron Microscopy

Inoculated cultures, showing approximately 70 per cent of cells affected by the cytopathic effect, were scraped from the flask surface. The suspension of cells in cell culture medium was sonicated for three minutes using a sonic probe and then centrifuged at 150 g for ten minutes. The clear fluids were then centrifuged at 35,000 g for one hour to pellet the virus contents. The pellets were resuspended in the small volume of fluid remaining after decantation and a little of each suspension was removed to suitable shallow containers. Carbon coated copper grids were floated on the suspensions for 30 seconds and excess fluid was removed by touching a filter paper to the grid edge. The grids were then floated on a solution of phosphotungstic acid (PTA) at pH 7.2 or 7.7. Excess PTA was removed with filter paper.

Immunodiffusion

Plates were prepared by pouring 15 ml of 2 per cent
immunodiffusion standard ion agar onto a 100 cm\(^2\) glass plate. A template was used to cut the wells in a pattern which consisted of six equidistant holes surrounding one central well. The well diameters were 2.5 mm and the reaction arena measured 5.0 mm. Dilutions of plasma over the range 1/2 to 1/64 were placed in the peripheral wells with antigen in the central well. Complement fixation antigen and the virus suspension used for the preparation of grids for electron microscopy were employed as antigen. The prepared plates were placed in a high humidity chamber in a refrigerator and examined daily for three days.

**Quantitative Virus Assay**

Stock virus samples, which had been stored at -20\(^\circ\)C or -180\(^\circ\)C were thawed and centrifuged at 150 g for ten minutes to remove cell debris. Dilutions of the supernatant fluid in maintenance medium were prepared using tenfold steps over the range 10\(^{-1}\) to 10\(^{-8}\). Confluent monolayer cultures in test tubes each received 0.1 ml of a dilution after removal of all culture medium. Five tubes were used per dilution and five control tubes received maintenance medium. One hour at 37\(^\circ\)C, with agitation every ten minutes, was allowed for adsorption and one ml of maintenance medium was then added to each tube. The cultures were observed daily for cytopathic effects and scored as negative or positive on the 12th day post inoculation.
The 50 per cent infective dose (TCID\textsubscript{50}) was calculated using the method of Spearman and Karber (cited by Dougherty, 1964).

**Neutralisation Test**

Sera, diluted with an equal volume of PBS, were heat inactivated at 56°C for 30 minutes, to remove non-specific thermolabile inhibitors, and allowed to cool to room temperature (23°C). Dilutions of serum in serum-free maintenance medium were prepared using twofold steps over the range 1/16 to 1/1024. A stock virus suspension containing 200 TCID\textsubscript{50} per 0.1 ml was prepared using serum-free maintenance medium as diluent and added to equal quantities of each serum dilution. The mixtures were left for one hour at room temperature. A virus sample diluted to contain 100 TCID\textsubscript{50} per 0.1 ml was treated similarly.

Confluent RK13 monolayer cultures in test tubes each received 0.1 ml of a virus/serum mixture after the removal of all culture medium. Five tubes were used per dilution and five control tubes each received 0.1 ml of the diluted virus stock. Five additional control tubes each received 0.1 ml of serum-free maintenance medium. One hour at 37°C, with agitation every ten minutes, was allowed for adsorption and one ml of maintenance medium was then added to each tube. The cultures were observed daily for cytopathic effects and scored as negative or positive on the 12th day post inoculation.
Complement Fixation Test

Diluent. Barbitone buffered saline (CFT diluent) was used.

Antigen Preparation. Primary cell cultures were grown in 25 cm² plastic flasks and inoculated with 0.5 ml of third passage virus stock. Cells and fluid were harvested when approximately 90 per cent of cells showed cytopathic effect, sonicated for three minutes and centrifuged at 150 g for ten minutes. The resultant clear supernatant fluid was dispensed in one ml aliquots in hard glass bottles and stored at -20°C.

Plasma Preparation. When required plasma samples were thawed and diluted with an equal volume of sterile CFT diluent. Complement was inactivated by heating at 56°C for 30 minutes.

Complement. Blood was collected from guinea pigs by cardiac puncture and allowed to clot. After clot retraction the extruded serum was preserved by Richardson's method and stored between 0 and 4°C. Complement was titrated before each day's tests in which two haemolytic units were used.

Haemolytic System. A stock sheep, known to have suitable erythrocytes, was bled into Alsever's solution and the cells were washed once with Alsever's solution to remove plasma before storage at 0 to 4°C at least three days prior to use. When required the cells were washed three
times with CFT diluent and a three per cent solution prepared. Equal volumes of haemolysin solution and erythrocyte suspension were mixed and placed in an incubator at 37°C for ten minutes immediately before use. Five minimal haemolytic doses of haemolysin were used to sensitisé the erythrocytes, this figure having been calculated from a complement/haemolysin optimum proportions test.

**Antigen Titration.** A checker-board titration using a homologous antigen/plasma system was performed to calculate the antigen strength. Four units of antigen were used in the actual tests.

**The Complement Fixation Test.** The standard reagent volume used throughout was 0.1 ml in WHO perspex haemagglutination plates. Plates which had received antigen, plasma and complement were placed in a refrigerator overnight and then warmed in an incubator at 37°C for 25 minutes before the addition of sensitised erythrocyte suspension. After heating at 37°C for one hour with constant agitation the plate was placed in a refrigerator for two hours before reading the result. The 50 per cent haemolysis end point was recorded.

**Test One – Comparison with Reference Antisera.** Doubling dilutions of two horse plasmas and the two reference sera were tested with the two homologous antigens.
Controls consisted of antigen without antibody, plasma dilutions without antigen, complement titration checks and sensitised cells. All sera and plasmas were tested against a negative antigen prepared from an uninoculated cell culture.

Test Two - Fixation by Different Antigens. Virus antigens were tested against one plasma which had shown a reaction comparable to the EHV2 reference antiserum. Antigen, plasma, complement and sensitised cell controls were included.

Sighting Survey. In order to assess different techniques for preparation of cell cultures and leucocyte suspensions, virus isolation and staining methods, a preliminary sighting trial was undertaken involving samples from 16 horses. This trial enabled a standardised procedure to be evolved for use in the definitive survey.

Definitive Survey. Two foals and 20 adult horses, presented for surgical and orthopaedic attention, were sampled. The survey group included thoroughbreds, hunters and various ponies of both sexes and ranged in age from one to 20 years. The foals were aged seven and five weeks.
RESULTS

SURVEYS

Sighting
Viruses were apparently isolated on so many occasions that doubt arose as to whether cross-contamination between samples had occurred. Precautions were devised to eliminate this possibility in the definitive trial. The viruses were slowly cytopathogenic and one isolate was examined by light microscopy, fluorescence microscopy and electron microscopy. The characteristics agreed with those described in the definitive survey.

Specific complement fixation was demonstrated between antigen and hemologous antibody antigen and heterologous antibody.

Definitive
The details of the horses which were sampled are shown in Table 1. Viruses were isolated from the leucocytes of 17 out of 19 adult horses (89 per cent), from the yearling and from the older of the two foals. Those which were positive ranged in age from seven weeks to 20 years and included all the breeds examined. The positive foal exhibited no sign of disease and its temperature remained normal.
<table>
<thead>
<tr>
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<tbody>
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<tr>
<td>74/268</td>
<td>Pony</td>
<td>NN</td>
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</table>

TB = Thoroughbred  M = Male  y = Years
F = Female  MN = Gelding  w = Weeks
Primary Rabbit Kidney

Cytopathic effects were visible in 16 of the 22 isolation attempts at the first passage and three more after one blind passage. The one foal isolate was obtained at the first passage. Two adult and one foal samples failed to yield virus during three passages. The dams of both foals were positive.

Spontaneous degeneration of control cultures was not observed even when observation was continued for three weeks.

The times to inception of the cytopathic effects, during the first passage, ranged from five to 14 days and, during the second passage, they ranged from three to seven days (Table 2). The samples which failed to show cytopathic effects at the first passage had a mean time and standard deviation at the second passage of $5.7 \pm 1.5$ days which was significantly longer than the rest which had a mean time and standard deviation of $3.2 \pm 0.5$ days ($t_{(17)} = 5.4^{**}$, $p < 0.001$). At the third passage all isolates showed cytopathic effects in three days.

The rate of cell destruction was significantly more rapid at the second passage than at the first. Approximately 70 per cent of cells were affected in a mean time and standard deviation of $13.3 \pm 2.5$ days at the first
<table>
<thead>
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<th>HORSE</th>
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<th>AT 2nd PASSAGE</th>
<th>AT 3rd PASSAGE</th>
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</tr>
<tr>
<td>74/290</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\[ \bar{x} \pm SD \quad 9.4 \pm 2.6 \quad 3.6 \pm 1.2 \quad 3.0 \pm 0.0 \]

- no macroscopic cytopathic effects seen
passage, compared with 5.3 ± 0.9 days at the second passage, in eight isolates studied \((t_{(14)} = 8.5^{**}, p < 0.001)\) (Table 3).

The 1/100 dilution of leucocytes yielded virus every time virus was isolated. The 1/10 dilution yielded virus 14 times out of 16 and the undiluted suspension only ten times out of 16 (Table 4). The difference in the proportions of viruses isolated were significant (Chi - square\((2) = 8.4^*, p < 0.02)\).

All isolates behaved similarly in cell culture, giving rise to similar cytopathic effects, characterised by the appearance of groups of rounded, refractile cells, cytoplasmic streaming and disruption of the cell sheet. These foci extended centrifugally and new foci developed over a period of several days until the whole cell sheet was destroyed (Figures 1 and 2). After passage, the first foci often appeared around the site of attachment of cell clumps derived from the inoculum.

Virus stocks retained infectivity during storage for four months at -180°C irrespective of whether or not DMSO had been added. Similarly, the majority of stocks, stored at -20°C for up to two and a half months, without DMSO, were still infective. However, two isolates lost all infectivity during storage at -20°C and therefore could not be studied further. Virus stocks, which had been
<table>
<thead>
<tr>
<th>VIRUS</th>
<th>AT 1st PASSAGE</th>
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<td>74/210F</td>
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<td>4</td>
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</table>

\[ \bar{x} \pm SD \]

| \bar{x} \pm 3D | 13.3 \pm 2.5 | 5.3 \pm 0.9 |
### Table 4.

DAYS TO INCEPTION OF CYTOPATHIC EFFECTS

AT EACH LEUCOYOTE DILUTION

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</tr>
<tr>
<td>74/290</td>
<td>9</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

U/D undiluted

- no macroscopic cytopathic effects seen
Figure 1.

Normal primary rabbit kidney cell culture.

Magnification X 250

Figure 2.

Primary rabbit kidney cell culture.

Typical cytopathic effects.

Magnification X 250
centrifuged at 150 g for ten minutes to remove cell debris, were infective.

**Rabbit Kidney Cell Line**

The 17 available isolates were successfully passaged to monolayers of the RK13 cell line after three passages in primary cell cultures. Slight differences were noted in the cytopathic effects. The first indication of cytopathogenicity was usually the appearance of rounded, refractile cells and morulae projecting above the surface of an apparently intact cell sheet. Visible syncytia were present at this stage in ten isolates and extensive syncytial formation, with cytoplasmic vacuolation, involving many cells, was the first indication in four (Figures 3 and 4). Macroscopic syncytia became visible on all but two occasions during the course of infection. Cells detached from the cell sheet and cytoplasmic streaming became apparent leaving holes (Figure 5). The formation of syncytia and rounded cells extended to involve the whole cell sheet which was eventually destroyed (Figure 6). Large syncytia were frequently the last cells to detach from the vessel surface. The mean times to appearance of cytopathic effects and destruction of the cell sheet were $2.1 \pm 0.3$ days and $6.9 \pm 1.5$ days respectively (Table 5).
Figure 3.

Normal rabbit kidney cell line.
Magnification X 100

Figure 4.

Rabbit kidney cell line.
Extensive syncytium formation.
Magnification X 250
Figure 3.

Figure 4.
Figure 5.

Rabbit kidney cell line.
Early foci of cytopathic effects.
Magnification X 250

Figure 6.

Rabbit kidney cell line.
Advanced destruction of cell sheet.
Magnification X 250
### TABLE 5.

DAYS TO INCEPTION OF CYTOPATHIC EFFECTS AND DESTRUCTION OF CELL SHEET IN RK13 CELL LINE

<table>
<thead>
<tr>
<th>VIRUS</th>
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<th>DESTRUCTION</th>
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<td>9</td>
</tr>
<tr>
<td>74/290</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

\[ \bar{X} \pm SD \quad 2.1 \pm 0.3 \quad 6.9 \pm 1.5 \]
Monkey Kidney Cell Line

Isolates 73/369, 74/R and 74/119 were successfully passaged to this cell line after passage in rabbit kidney, primary and cell line culture. The cytopathic effects appeared on the third to fourth days and consisted of foci of detachment of rounded, refractile cells. The foci increased in number and diameter during the six days that observations were made.

Hamster Kidney Cell Line

Passage was attempted with isolates 73/369, 74/R and 74/119 of both primary rabbit kidney cell culture and rabbit kidney cell line culture origin. No cytopathic effects were seen in the six days during which observations were made.

Virus Titres

Isolate 74/70, second passage virus, was titrated in primary rabbit kidney cell cultures after storage, without DMSO, for three months at -180°C. The titre ± standard error of the fluid was $10^{4.8 ± 0.3}$ TCID$_{50}$ per ml. Isolate 73/369, passaged once in RK13 cells, was frozen at -20°C for one day and then titrated in the RK13 cell line. The titre was $10^{6.3 ± 0.3}$ TCID$_{50}$ per ml.

LIGHT MICROSCOPY

Primary Rabbit Kidney Cell Culture

Typical eosinophilic, Cowdry Type A intranuclear
inclusions were seen in all infected preparations. In an otherwise normal cell sheet many adjacent cells often contained inclusions. Stages of development of an inclusion body were recognised. The initial appearance was of multiple, small, eosinophilic bodies with narrow, surrounding, unstained halos and this was followed by the formation of one large, polymorphic, eosinophilic inclusion, with a clear surrounding halo, occupying most of the nucleus. These two stages were seen in otherwise normal cells. The mature inclusion, seen in rounded cell detaching from the flask surface and in syncytia, was condensed and more densely staining with a wide halo separating it from the margined chromatin which consisted of discrete particles confined to the nuclear membrane (Figures 7, 8 and 9).

Syncytia were not seen on every examination but were eventually recognised for all isolates and consisted of up to 27 nuclei arranged circumferentially (Figure 10).

No definite intracytoplasmic inclusions were seen but large cytoplasmic vacuoles, stained with eosin, could be produced by staining normal and infected cultures heavily with eosin and smaller juxtanuclear eosinophilic vacuoles were occasionally seen. The intranuclear inclusions had a definite granular structure whereas stained cytoplasmic vacuoles were amorphous (Figure 11).
Figure 7.

Primary rabbit kidney cell culture.

Early appearance of intranuclear inclusions.

Magnification X 700
Figure 8.

Primary rabbit kidney cell culture.
Intermediate stage of intranuclear inclusion development.
Magnification X 700

Figure 9.

Primary rabbit kidney cell culture.
Mature intranuclear inclusions in the nuclei of a syncytium.
Magnification X 700
Figure 10.

Primary rabbit kidney cell culture.

A syncytium.

Magnification X 175

Figure 11.

Primary rabbit kidney cell culture.

Granular intranuclear inclusions and juxtanuclear cytoplasmic vacuoles.

Magnification X 1750
**Rabbit Kidney Cell Line**

Multinucleate cells, containing two to five nuclei, were occasionally seen in uninfected control cultures. Syncytia were seen more commonly than in primary cultures and were more prominent, containing from ten to 40 nuclei each with a mature intranuclear inclusion. These were present in all the preparations examined. On several occasions the syncytial response was the main cytopathic effect and the fusion of cells seemed to have produced holes in the cell sheet (Figures 12 and 13). Isolated inclusion-containing cells were seen around such foci and developmental stages of the inclusions were recognised (Figures 14 and 15).

**Monkey Kidney Cell Line**

When examined on the third day post inoculation, scattered groups of cells, containing immature intranuclear inclusions, were seen and mature inclusions were seen in rounded cells. Very few syncytia were present and those seen contained from two to six nuclei, each with immature inclusions.

**Hamster Kidney Cell Line**

No evidence of viral replication was obtained.

**FLUORESCENCE MICROSCOPY**

A characteristic pale green fluorescence of intranuclear inclusions was demonstrated in all infected primary
Figure 12.

Normal rabbit kidney cell line.

Magnification X 700

Figure 13.

Rabbit kidney cell line.

Syncytium formation producing holes in the cell sheet.

Magnification X 350
Figure 14.
Rabbit kidney cell line.
Early and intermediate stages of intranuclear inclusion development.
Magnification X 700

Figure 15.
Rabbit kidney cell line.
Mature intranuclear inclusions in nuclei of a syncytium.
Magnification X 1750
Figure 14.

Figure 15.
cultures examined (all isolates) and infected RK13 cell cultures (isolates 73/308, 73/369, 74/R, 74/119, 74/269 and 74/21OF).

**ELECTRON MICROSCOPY**

Electron micrographs of negatively stained preparations showed abundant particles in every isolate, none were seen in control preparations.

The nucleocapsid appeared circular or hexagonal in outline and measured approximately 100 nm in diameter. Detail of the capsomeric structure was seen but the electron micrographs were not of sufficient quality to allow a count or identification of the type of symmetry. The capsomeres were most clearly seen around the periphery and appeared to be elongated, hollow structures, measuring approximately 10 nm by 15 nm (Figure 16).

Many enveloped particles were seen, as well as naked virions, and the envelope was frequently irregular in outline. Where it was nearly circular, the diameter varied between 150 nm and 190 nm (Figure 17). Collections of virions within a single membrane were occasionally observed (Figure 18).

Particles varied in the degree to which they were penetrated by the PTA and some envelopes were not penetrated at all (Figure 17). On occasion, penetration inside the capsid revealed the presence of an inner body, (the nucleoid
Figure 16.

Details of capsomeric structure in naked and enveloped virions.

Bar = 100 nm

Figure 17.

Enveloped and naked virions penetrated to varying degree by P.T.A.

Bar = 100 nm
Figure 16.

Figure 17.
Figure 18.

Nucleoid structure in a group of naked virions enclosed in a limiting membrane.

Bar = 100 nm

Figure 19.

Group of naked virions showing one 'empty' capsid.

Bar = 100 nm
approximately 60 nm in diameter. 'Empty' capsids were recognised (Figure 19).

IMMUNODIFFUSION

This study was completely negative; no precipitation lines were observed between dilutions of plasma 73/369 and antigens of both types prepared from all 17 available isolates.

COMPLEMENT FIXATION TESTS

Test One - Comparison with Reference Antisera

Fixation was demonstrated between antigens 73/369 and 73/308 and homologous plasmas and the reference EHV2 antiserum. Fixation did not occur with these antigens and the reference EHV1 antiserum or with a negative antigen and the reference antisera and plasmas. Homologous antibody titres were 1/64 and 1/128 whereas the reference EHV2 antiserum gave a titre of 1/16 in both instances (Table 6). Titres less than 1/8 were considered negative.

Test Two - Fixation by Different Antigens

Every antigen fixed complement with plasma from horse 73/369 and the antibody titres were confined to the narrow range 1/64 to 1/256, the homologous titre being 1/128. With eight antigens the titres were 1/64, with six 1/128 and with three 1/256 (Table 7).
**TABLE 6.**

**COMPLEMENT FIXATION ANTIBODY TITRES**

**TEST ONE - COMPARISON WITH REFERENCE ANTISERA**

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>PLASMA 73/308</th>
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**TABLE 7.**

**COMPLEMENT FIXATION ANTIBODY TITRES**

**TEST TWO - FIXATION BY DIFFERENT ANTIGENS**

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<td>74/135</td>
<td>74/74</td>
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<td>74/119</td>
<td>73/390</td>
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<td>74/210F</td>
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<td>74/142</td>
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<td>74/70</td>
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The titres are expressed as the reciprocal of the highest dilution of plasma or serum which gave 50 per cent haemolysis.

Homologous system results are underlined.
NEUTRALISATION TESTS

The two reference antisera, pooled rabbit serum and pooled horse serum were tested for neutralisation with isolate 73/369. Complete neutralisation did not occur with any of the sera at the lowest dilution tested, i.e. 1/16.
DISCUSSION

The prevalence of EHV2 in horses has previously been estimated by serological means, e.g. Burrows (1968) in Britain, but only on one occasion by virus techniques (Kemeney et al., 1970). The finding that slowly cytopathogenic herpesviruses can be isolated from the leucocytes of apparently normal horses was confirmed by this trial and the prevalence was in close agreement with the results which Kemeney et al. (1970) obtained in the U.S.A. I would agree with Studdert (1974) that infection with this group of viruses is widespread in horse populations and would add that isolation is to be anticipated whenever horse tissues are sampled for virological investigations.

The horses sampled in our series were all free from clinical signs of infectious disease and, moreover, the foal, which proved to be viraemic, was also normal. Others have attempted to ascribe an aetiological significance to these viruses and it is possible that they may be involved in the undoubtedly complex aetiology of equine respiratory disease ('colds') and neonatal disease.

It may be that all horses are infected and that the negatives in this series represent failure to isolate virus rather than absence of infection, especially as
fluctuating levels of viraemia are to be expected by extrapolation from other persistent viral infections, e.g. malignant catarrhal fever (Plowright, 1963). It is interesting to note that the younger foal was negative, although the dam was positive, and this was interpreted to mean that the foal had not yet acquired infection; a hypothesis that agrees with the finding of Studdert (1974) that infection occurs during the first few months of life.

The results obtained, in this study, by the use of several dilutions of leucocyte suspension, emphasises the need to use more than one dilution for efficient isolation. Occasional failures to isolate virus, when the lower dilutions were employed, may be explained by the presence of neutralising antibody in the inoculum or by an auto-interference phenomenon (Fenner, 1968a).

Increasing cytopathogenicity during the first three passages was considered to be due to a combination of two factors; a process of adaptation to the cells used and an increase in the virus titre of the inoculum. A variation in the multiplicity of infection would alter the kinetic parameters of viral replication and may account for the observation that the time required before the appearance of cytopathic effects was always relatively long on initial isolation, even when blind passage had
intervened. During the course of a virus titration, cytopathic effects were seen later and progressed more slowly in higher dilutions leading to an apparent increase in the titre reading during the first eight days post inoculation. Stable readings were obtained by the twelfth day. The higher titre of the two was of the order that would be expected to be a maximum for EHV2 and compares with the results of Karpas (1967) for his isolate of kidney origin. Retention of infectivity to moderate titre by cell-free fluid indicated that cell association was not marked. However, the appearance of foci of cytopathic effects more frequently around cell debris derived from the inoculum than elsewhere suggested that cells contained much of the infectious virus.

Characteristic cytopathic effects, comparable to those described in the literature, were obtained with the viruses studied and they contrasted, markedly, with those described for EHV1 and EHV3. Variation in the degree of syncytium formation occurred possibly because the virus titre of the inoculum varied; syncytia may be formed by a physico-chemical phenomenon when high levels of virus are used (Penner, 1968b). However, the rabbit kidney cell line did seem to be more prone to syncytium formation compared with primary cultures and the monkey kidney cell line, confirming the observation of Plummer et al (1969).
The spectrum of cell types supporting growth, namely primary rabbit kidney, rabbit kidney cell line and monkey kidney cell line, conflicts with the reports of Russell et al (1964) and Plummer et al (1969) for the LK isolate but confirmed the findings of Ratuld et al (1972) for their leucocyte derived viruses. Accounts of the susceptibility of the VERO monkey kidney cell line have differed. Plummer et al (1969) and Moraillon et al (1971) recorded lack of growth of the LK isolate and other isolates obtained from leucocytes. However, Erasmus (1970) described growth of the LK isolate and Ratuld et al (1972) obtained positive results with their leucocyte derived isolates. Similarly, growth was obtained with the three isolates from this study which were tested.

Moraillon et al (1971) and Ratuld et al (1972) found that BHK cells did not support the growth of their isolates and no evidence of growth was obtained with the three isolates I tested. In contrast EHV1 has been reported to grow in the BHK cell line (Moraillon et al, 1971).

Intranuclear inclusion bodies, seen by light microscopy, were typical of herpesviruses in general and conformed to the descriptions of equine herpesviruses and especially EHV2, where many adjacent cells have been seen to contain
inclusions (Studdert, 1974). Acridine orange staining confirmed the DNA content of the inclusions. Definite evidence of intracytoplasmic inclusions, as described by Erasmus (1970), was not obtained but the small, eosinophilic, juxtanuclear vacuoles seen may represent these structures. Confusion arose in these studies with large, stained, cytoplasmic vacuoles, of unknown origin, which were seen in both infected and control cultures. Typically, the structure of the viruses isolated consisted of a capsid, approximately 100 nm in diameter, composed of individual, elongated, hollow capsomeres, each approximately 10 nm by 15 nm, containing a nucleoid, approximately 60 nm in diameter and surrounded by an envelope of variable diameter; a morphology that conforms to the general description of the herpesvirus group (Roizman et al, 1973), equine herpesviruses (Studdert, 1974) and the leucocyte derived isolates of Kemeney et al (1970). However, the size of the nucleoid is at variance with the figure quoted by Studdert (1974) although it agrees with that given by Okaniwa et al (1969).

Aggregations of naked nucleocapsids within a single limiting membrane were also observed by Turner et al (1970) and were considered to be restricted to slowly cytopathogenic viruses. They may represent accumulations of virus in cytoplasmic vacuoles and be responsible for
the reports of intracytoplasmic inclusions (Plummer et al., 1969; Erasmus, 1970) and juxtanuclear cytoplasmic vacuoles (Moraillon et al., 1971) in EHV2. Similar cytoplasmic inclusions have been described for cytomegaloviruses and varicella virus (Plummer, 1973).

Particles devoid of central cores and with incomplete envelopes were seen. Similar structures have been described for cytomegaloviruses and varicella virus (Plummer, 1973).

Immunodiffusion studies have not been reported for EHV2 and were of no value in this study.

There was close agreement between all the complement fixation titres of one plasma to the different viral antigens indicating that the isolates were closely related antigenically. A relationship with the IK isolate of EHV2 was demonstrated. Low levels of cross-reaction with other herpesviruses were anticipated (Bagust et al., 1972) and the titre of the reference EHV1 antiserum to the one isolate tested fell into this category. The level of fixation with the EHV2 antiserum was lower than with plasma from two horses which yielded virus but this relationship can not be considered to be quantitative because it may be a reflection of a lower antibody titre. Reference viruses and extensive cross-checking of reactions would be required to determine accurately the antigenic relationship between these viruses.
The serum neutralisation test was not sufficiently sensitive to detect activity of low levels of neutralising antibody. It was not possible, in the tests, to quantify the cytopathic effects and the quantal criterion used resulted in a failure to detect neutralisation. However, the EHV1 antiserum was known to have a 50 per cent neutralisation titre ($SN_{50}$) of $10^{2.4}$ $SN_{50}$ per ml to the RAC-H strain of EHV1 by conventional test and the absence of any evidence of neutralisation in this study indicated that the isolate was antigenically distinct from EHV1. The EHV2 antiserum was known to be of low titre (neutralising 2 logs in the neutralisation index test). Erasmus (1970) stated that difficulties are inherent in the conventional neutralisation test for EHV2 because complete neutralisation does not occur even at low dilutions of serum. An additional adverse factor may have been the lack of complement in the system; this absence would increase apparent antigenic differences (Plummer et al., 1973). The plaque number reduction test is alleged to reduce this type of problem (Plummer et al., 1969) but time did not permit its application in this study.

The equine origin of the isolates was supported by a lack of spontaneous degeneration in rabbit kidney cell cultures during prolonged observation and subpassage, the absence of inclusion bodies or virus particles in uninfected
cell cultures, complement fixation of infected cell culture antigen with horse antibody and failure of complement fixation with horse antibody and uninfected cell culture antigen.

My isolates, therefore, fulfilled the criteria laid down for herpesviruses but they also had some characteristics in common with the salivary gland viruses of other species, the so-called cytomegaloviruses. However, the term cytomegalovirus is currently controversial because, to quote Plummer (1973), 'there appears to be a gradation of properties between individual herpesviruses and information available at the moment does not allow an unequivocal taxonomic delineation between herpesviruses and cytomegaloviruses'.
CONCLUSIONS

Studies of prevalence, growth characteristics, virion morphology and antigenic relationships confirmed previous suggestions that EHV2 infections are endemic in normal horse populations. The prevalence of 89 per cent in adult horses in northern Britain, as judged by leucocyte sampling, agreed closely with the findings of an American survey.

Antigenic investigation, by the complement fixation reaction, demonstrated that the viruses obtained were related to the LK isolate of EHV2 and thus to many other isolates from different sources.
ACKNOWLEDGEMENTS

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(a) I Isolation and properties

(b) II Immunological studies of CEO virus.


