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Rapid Point of Care Tests in Zimbabwe – Immunological and Sociological Perspectives



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1. Abbreviations List

3,3',5,5-tetramethylbenzidine.....	TMB
Antibody detection assays.....	ADA
Apostolic Women's Empowerment Trust.....	AWET
Arbitrary Units.....	AU
Bacille Calmette-Guerin.....	BCG
Basic Local Alignment Search Tool.....	BLAST®
Coronavirus Disease 2019.....	COVID-19
Dendritic Cells.....	DC
Enzyme-linked immunoabsorbent assays.....	ELISAs
Epidemic Preparedness Index.....	EPI
Horseradish peroxidase.....	HRP
Human Immunodeficiency Virus.....	HIV
Interferon Gamma.....	IFN-γ
Interferon-Gamma Release Assays.....	IGRAs
Immunoglobulin A.....	IgA
Immunoglobulin E.....	IgE
Immunoglobulin G.....	IgG
Immunoglobulin M.....	IgM
Interleukin 2.....	IL-2
Interleukin 4.....	IL-4
Interleukin 5.....	IL-5
Interleukin 9.....	IL-9
Interleukin 10.....	IL-10
Interleukin 12	IL-12
Interleukin 13.....	IL-13
Interleukin 18.....	IL-18
Interquartile range.....	IQR
Latent Tuberculosis Infection.....	LTBI
Lateral flow tests.....	LFTs
Lipoarabinomannan.....	LAM
Nasopharyngeal.....	NP

Natural killer cells.....	NK cells
Non-pharmaceutical interventions.....	NPI
Non-Tuberculosis Mycobacteria.....	NTM
Optical density at 450nm.....	OD ₄₅₀
Parasite Immunoepidemiology Group.....	P.I.G.
Pathogen-associated molecular patterns.....	PAMPs
Peripheral blood mononuclear cell.....	PBMC
Point-of-care.....	POC
Polymerase chain reaction.....	PCR
Preschool-aged children.....	PSAC
Purified Protein Derivative.....	PPD
Recombinant BCG.....	rBCG
Severe Acute Respiratory Syndrome Coronavirus-2.....	SARS-CoV-2
Sustainable Development Goals.....	SDG
T-helper type 1.....	Th1
T-helper type 2.....	Th2
Toll-like receptors.....	TLRs
Transforming growth factor β	TGF- β
Tuberculin skin test.....	TST
Tuberculosis.....	TB
Tumour Necrosis Factor α	TNF- α
Understanding Schistosomiasis among Children under Five.....	USaCuF
Village Health Workers.....	VHWs
Water, hygiene and sanitation.....	WASH
World Health Organisation.....	WHO

2. Abstract

Rapid point-of-care (POC) tests overcome many of the limitations imposed by traditional diagnostics, with respect to turnaround time, expenses and laboratory infrastructure. This is particularly beneficial in resource-limiting environments, such as rural Zimbabwe, where this study analysed the use of POC tests from immunological and sociological perspectives. Preliminary work was carried out to establish proof-of-concept for rapid TB diagnostics, in a *Schistosoma haematobium*-endemic region. This project aimed to quantify the levels of specific immunoglobulin M (IgM) and immunoglobulin G (IgG) against five unique *Mycobacterium tuberculosis* peptides, thereby understanding the patterns of humoral immunity between differing sex and age groups, and the effects of schistosome infection. The number of *S. haematobium* eggs were counted in stool and enzyme-linked immunoabsorbent assays (ELISAs) were performed on sera to quantify peptide-specific antibody levels. The results were then stratified by sex and schistosomiasis status of participants, prior to analysis of antibody titres with age. *M. tuberculosis* sensitisation was prevalent throughout the cohort and various sex-, age- and schistosomiasis-associated differences were identified. Peak female IgM titres were experienced higher and at a younger age with respect to males. As for IgG titres, females displayed a biphasic spectrum, seeing antibody levels peak twice in adulthood. By contrast, males experienced one peak IgG titre in middle age. Schistosomiasis disease status was associated with peak titre expression in younger age groups and elevated IgG levels; however, it exerted inconsistent effects on the magnitude of IgM responses. This demonstrated how serodiagnostics can be used to quantify bacterial exposure and characterise the immunomodulatory effects of parasite infection. Additionally, attitudes towards the use of POC tests were investigated. This aimed to establish whether participants were willing and able to self-diagnose COVID-19 using lateral flow tests (LFTs), as well as to ascertain whether they were cognizant of undertaking self-isolation when appropriate. Village health workers (VHWs) counselled participants on the correct usage of LFTs, which they then completed independently. A questionnaire was then conducted to capture information on the demographics of the cohort, COVID-19-associated behaviours and testing preferences. Descriptive statistics for the responses were reported and demographic factors associated with willingness to test identified. Most participants (90.8%) were willing to use LFTs and primarily reported preferences for self-testing as it was easier, pain-free, convenient and confidential. Farmers and followers of the Apostolic faith were found to be overrepresented among those unwilling to test, indicating that these individuals would be less likely to respond to LFT distribution. Participants displayed high levels of health literacy, demonstrated by questionnaire responses to COVID-19 transmission-mitigating and sanitary behaviours.

Taken together, immunological and sociological analysis inform context specific POC diagnostics, which is essential for implementation of novel health interventions.

3. Point of Care Tests: Immunological Perspectives

3.1. Literature Review

3.1.1. Worldwide Burden of TB

The infectious disease Tuberculosis (TB) is most commonly caused by *Mycobacterium tuberculosis* (*M. tb*), an aerobic, non-motile bacillus transmitted between individuals by means of airborne mucus droplets (World Health Organisation, 2021b). Following infection, most individuals will only develop latent TB (LTBI), an asymptomatic state that allows the persistence of *M. tb* in the body without further transmission. However, 10% of infections lead to active disease, characterised by symptoms of chronic cough, fever, weight loss and the presence of blood in expectorated mucus. In the majority of active cases, the disease exclusively affects the lungs (pulmonary TB), with a minority undergoing bacterial proliferation beyond the respiratory tract (extrapulmonary TB) (World Health Organisation, 2021b).

Its staggering effect on worldwide mortality is best exemplified by its position as the 2nd most deadly communicable disease, equivalent to 1.5 million people in 2020 (World Health Organisation, 2021b). Consequently, TB is a burden on health services and exerts financial pressure at national and international levels. It was declared a global emergency by the World Health Organisation (WHO) in 1993, since then driving the implementation of ambitious targets outlined in the End TB Strategy and Sustainable Development Goals (SDGs). The End TB Strategy was adopted by WHO member states in 2014-2015 and proposes to reduce the absolute number of TB deaths, incidence rate and households affected by TB-related costs. The WHO measures progress towards these goals in high TB burden and watch list countries using milestone targets. Wherein predefined markers of TB morbidity are quantified to ascertain whether the current interventions are sufficient or require additional resources. The first milestone targets took place in 2020, with a further assessment planned for 2025. By 2020, the End TB Strategy aimed to reduce TB deaths by 35% and TB incidence by 20%, concerning the 2015 baseline. This is reinforced by SDG Target 3.3, which aims to “end the global TB pandemic” and measures the success of this target by number of incidences per 100,000 people.

The targets implemented by the WHO focus specifically on rate of morbidity and mortality, making them effective at confronting active cases, although insufficient as indicators of TB burden and future disease. This is because the current metrics do not account for the global LTBI burden (23.0%), which is sufficient to prevent actualisation of targets if no further actions are taken (Houben and Dodd, 2016). Therefore, for the End TB Strategy to be successful at reducing the impacts of worldwide TB disease, there needs to be a transition towards a record

of TB infections for surveillance. More research is currently needed to obtain effective diagnostics to address this need across a variety of healthcare settings.

3.1.2. The Impact of the COVID-19 Pandemic on Global Targets

As a result of the Coronavirus Disease 2019 (COVID-19) pandemic, many of the 2020 milestones were not achieved, resulting in greater mortality and morbidity than predicted. The mounting number of COVID-19 cases causing concern for many countries took precedence, reflected in a reduction of global budget towards TB eradication from \$5.8 to \$5.3 billion US dollars (World Health Organisation, 2021b). This translated to a reduction in testing capacity, more limited access to treatment and other necessary TB services. The Global Tuberculosis Report (2021) showed that this reduction in testing capacity resulted in a decrease of novel diagnoses from 7.1 million in 2019 to 5.8 million in 2020. Treatment for multi-drug resistant TB fell by 15% and preventative solutions were reduced by 21% (World Health Organisation, 2021b). These factors lead to a higher number of TB-associated deaths in 2020 (1.3 million) compared to 2019 (1.2 million). This increase was also observed in deaths of Human Immunodeficiency Virus (HIV)-positive individuals, amounting to a further 214,000 in 2020 and 209,000 in 2019. Limited success was achieved in the 33 high TB burden and TB watchlist countries as measured by the 2020 milestones of the End TB Strategy. For example, only 9 of the 33 countries on the high TB burden and TB watch list achieved the targeted reduction in new TB cases and only 7 of these countries were able to reduce TB mortality (World Health Organisation, 2021b).

3.1.3. The BCG Vaccination

Since 1921, administration of the Bacille Calmette-Guerin (BCG) vaccination has been used as a preventative measure for TB. A hundred years later, it remains the only method of immunisation against the disease (Dye, 2013). It also has the highest coverage of any vaccine, administered to an estimated 85% of the global population (Dye, 2013; World Health Organisation, 2021a). Across World Bank Income Groups, a trend emerges wherein the average low-income country has the lowest BCG coverage (79%), with this value increasing until countries reach upper-middle income status (89%) (World Health Organisation, 2021a). This may highlight the disparity of resources, infrastructure or health care personnel between countries at different income levels. Public health legislation for the BCG vaccination varies across countries depending on population-wide TB incidence, guided by WHO recommendation. The WHO endorses a universal vaccination strategy at birth for countries with a high TB burden and a selective vaccination strategy for countries less affected by the disease. Revaccination is implemented in some countries, although is not recommended as there is no evidence that it improves upon existing immunity (Zwerling *et al.*, 2011).

3.1.4. BCG-Induced Immunity against TB

Following BCG vaccination, *Mycobacterium bovis* BCG is identified by the body through the interaction of pathogen-associated molecular patterns (PAMPs) with Toll-like receptors (TLRs) and accessory pathways initiated by complement and mannose receptors (Tsuji *et al.*, 2000; Moliva, Turner and Torrelles, 2017). Common PAMPs detected during this stage are fragments of the mycobacterial cell wall, including peptidoglycans, arabinogalactan and mycolic acids. This recognition triggers intracellular pathways, amplifying the original signal and ultimately resulting in stimulation of the T-helper type 1 (Th1) response. Dendritic cells (DC) expressing *M. bovis* BCG antigens migrate to nearby lymph nodes where they interact with naïve CD4⁺ T cells. In the presence of Interleukin 12 (IL-12) and interferon gamma IFN- γ , clonal expansion and differentiation take place, inducing T cell proliferation. These in turn synthesise tumour necrosis factor α (TNF- α), Interleukin 2 (IL-2), Interleukin 18 (IL-18) and increased circulating IFN- γ (Ravn *et al.*, 1997). The role of humoral immunity has been historically downplayed in the literature, as *M. tb* is an intracellular pathogen, assuming some protection from circulating antibodies (Orme, 1988; Kaufmann *et al.*, 2014; Jasenosky *et al.*, 2015). Nevertheless, some publications have rebuked this trend, arguing for the importance of *Mycobacterium*-specific antibodies (Chen *et al.*, 2016; Lu *et al.*, 2016; Zimmermann *et al.*, 2016; Li *et al.*, 2017). As with T cells, depleted B cell activity is correlated with a higher likelihood of contracting TB (Flynn *et al.*, 1993; Vordermeier *et al.*, 1996). Additionally, *M.tb*-specific antibodies have been identified in granulomas, implicating that some level of humoral response is important for immunity (Phuah *et al.*, 2012; Rahman *et al.*, 2015; Rao *et al.*, 2015). Methods of antibody-mediated protection involve interplay with various immune cells such as macrophages, immune complexes and T cells. At the time of writing, five distinct mechanisms of antibody action against *M. tb* have been identified. These were reviewed by Li and Javid (2018) and include neutralisation, stimulation of phagocytosis by macrophages, elevated phagolysosome formation, enhanced inflammasome activity and induction of natural killer (NK) cells.

3.1.5. Observed Trends in Heterogeneous Profiles of Immunity

Since the introduction of the vaccine in 1921, ongoing efficacy studies of the BCG vaccine have demonstrated heterogeneous effects on population immunity. The variability of outcomes was analysed by Mangtangi and colleagues (2014), who captured the reported efficacy range to be between 28% and 68%. BCG efficacy has been observed to correlate with age when vaccination took place, time since vaccination and region.

It is well established that neonatal vaccination imparts the highest levels of protective efficacy against TB (Rodrigues, Diwan and Wheeler, 1993; Colditz *et al.*, 1995; Roy *et al.*, 2014; Scriba

et al., 2016). This translates to a reduction of 69-92% in childhood miliary TB and contrasts with data compiled on individuals vaccinated as adults or adolescents, who display substantially more variable effects (Fine, 1995; Mangtani *et al.*, 2014). BCG-induced immunity wanes over time, however, studies disagree on the duration of protection (Sterne, Rodrigues and Guedes, 1998). The longest duration of BCG-induced immunity was recorded in a longitudinal study in a native American population between 1935-1998. At the beginning of the study (1935-1998) an average efficacy of 77% was calculated, which declined to 52% towards the end of the study period, signifying a high retention of immune memory (Aronson, 1948; Aronson *et al.*, 2004). Hart and Sutherland (1977) indicated a higher initial efficacy of 84%, although this decreased to 59% between 10-15 years following vaccination. Further studies conducted in the UK, Brazil and Norway also found BCG-induced protection lasting for shorter periods at 15, 15-20 and 30-40 years respectively (Sterne, Rodrigues and Guedes, 1998; Barreto *et al.*, 2005; Nguipdop-Djomo *et al.*, 2016). Interpretation of study conclusions can be complicated due to confounding with age and location where vaccination took place and should therefore be interpreted with caution. However, most studies implicate significant reduction in immune protection over time which is substantially shorter than the average lifespan.

Since BCG vaccine was licenced for use, many investigations have been conducted across populations from different regions highlighting significant variability. Zodepy and Shrikhande (2007) conducted a meta-analysis on BCG efficacy, examining 80 studies between 1942-2007 and found an efficacy range of 56% to 98%. They concluded that greater protective efficacy was associated with higher latitudes, calculating that this represents 15% of variance. Later studies have since clarified this conclusion, attributing the differences in efficacy between temperate, tropical and subtropical environments. These results are exemplified by the findings of two investigations, where the first demonstrated that Malawian individuals, living at a lower latitude, had reduced vaccine-induced responses in comparison with their British counterparts (Black *et al.*, 2002). Secondly the lowest value of BCG-induced efficacy was recorded in the tropical region of South India, where no significant differences in protective efficacy were observed between vaccinated and unvaccinated individuals (Colditz *et al.*, 1994).

3.1.6. Hypotheses for Observed Differences

The BCG vaccine has been shown to be extremely effective in animal models TB (Fine, 1989), however, ongoing efficacy trials in humans have indicated variable outcomes (Fine, 1995). Observations of heterogeneous immunity have been characterised across age and location in BCG-vaccinated populations and likely arise through the culmination of various factors. Different hypotheses have been proposed to understand these observations, such as genetic

variability of strains or manufacturing processes (Hart, 1967), genetic variability of populations influencing immune function (Fine, 1995), non-tuberculosis mycobacteria (NTM) exposure (Fine, 1995) and parasite burden. Evaluation of these hypotheses is vital to assess reasons for vaccine failure, to mitigate ramifications of elevated TB susceptibility and inform research for improved immunisation efforts.

3.1.7. The Genetic Variability of Strains and Vaccine Manufacturing Process

Different manufacturers use various preparation methods and strains of *M. bovis* BCG which may exhibit a range of immunogenic abilities, virulence and viability. The initial *M. bovis* BCG strain was maintained in culture over a significant period, prior to the implementation of conservation methods. As a result, genetic variation emerged following distribution to disparate locations, wherein isolates diverged further from the original strain, as indicated by deletions arising in genetic material (Abdallah *et al.*, 2015; Bottai and Brosch, 2016). Eventually, the integrity of the strains was somewhat protected by means of lyophilisation. Some commonly used strains include BCG Pasteur, Glaxo, Japanese, Russian and Copenhagen varieties (Ritz *et al.*, 2008; Kozak, 2011). Lagranderie *et al.* (1996) investigated protective antibody titres induced by different BCG vaccine strains in mice models and found that BCG Pasteur and Russian strains were correlated with the highest Immunoglobulin G (IgG) and Immunoglobulin A (IgA) purified protein derivative (PPD)-specific concentrations. In contrast, the Japanese strain resulted in the lowest titres of anti-PPD antibodies. To demonstrate their effects on adaptive immunity, beta-galactosidase assays were used to estimate removal of recombinant BCG (rBCG) particles from the cellular and intracellular environments. The results of this experiment showed that the strain-specific differences in antibody titre had no observable consequences on this response, highlighting the complex multi-factorial response towards *M. bovis* BCG (Lagranderie *et al.*, 1996). Other studies such as conducted by Li *et al.* (2016) supported that B cell epitopes had strain-specific differences, which poses questions about differential immunogenicity of antigens. Later studies suggested that different strains also impact T cell responses, as observed in *in vitro* peripheral blood mononuclear cell (PBMC) cultures (Andersen and Doherty, 2005; Ritz *et al.*, 2012). Comstock (1988) examined BCG efficacy in Indonesian and Colombian cohorts and observed that BCG efficacy declined sequentially as Japan, Glaxo, Paris and Danish bacillus strains were used. Subsequently, another study found that the Pasteur vaccine had a 40% more efficacy than the Glaxo strain when tested in 300,000 children over a six-year period (Hashimoto, 1997). This result was statistically significant; however, it contradicts the conclusions reached by the former study. Some caution regarding the interpretation of these results should be exercised, given the inconsistencies between experimental outcomes and differing efficacies which may arise *in vivo*. Population-wide studies would provide the most reliable results, however, this is

difficult to coordinate as countries often use a mixture of different strains, thus there is a paucity of research in this field (Ritz and Curtis, 2009). All considered, stratifying response by strain in international meta-investigation settings show that strains have consistent efficacy across different countries (Springett and Sutherland, 1994). However, when examining the effects of strain on efficacy between populations of different regions, the results are not consistent. Even as strain is kept constant, BCG efficacy is variable between populations, therefore, it is unable to fully account for observed differences. This was demonstrated by the fact that Glaxo was shown to elicit good protective immunity in the UK, but poor efficacy in Malawi despite use of the same strain (Sutherland and Springett, 1987; Pönnighaus *et al.*, 1992).

Different manufacturing procedures may also influence viability and growth rate. Difficulties arise trying to measure the effects of manufacturing and strain types on population-level immunity as public health systems use multiple batches simultaneously (Eisenhut *et al.*, 2009; Zwerling *et al.*, 2011). Despite this obstacle, various investigations have been conducted at a pre-clinical stage to understand any potential underlying biological factors. For instance, Petricevich *et al.* (2001) carried out a study to determine if the growth media of live-attenuated *M. bovis* BCG affected humoral immunity. It was found that Moreau strain bacteria cultured in Sauton-asparagine media gave rise to more colonies and elevated antibody production in mice, compared to Sauton-starch and bacto-peptone-enriched media (Petricevich *et al.*, 2001). In addition, Prados-Rosales *et al.* (2016) hypothesised that manufacturing and production differences can influence the encapsulated state of the bacillus, which affects the BCG-induced immune response. This is important due to plethora of immunogenic antigens associated with the capsule of the bacillus, which may affect the protective immune response (Tanner *et al.*, 2019). For instance, *M. bovis* BCG cultured in detergent degrades the capsule inducing a predominantly Immunoglobulin M (IgM)-mediated response. It was also found that the encapsulated bacillus induced IFN- γ and elevated T cell activity. Furthermore, more undetected differences may appear between batches because the vaccine requirements use a specified range of colony forming units, which does not account for varying proportions of dead bacteria in each vaccination (Prados-Rosales *et al.*, 2016). A study by Biering-Sørensen *et al.* (2015) showed how lagging growth in BCG batches was correlated with increased positive PPD test results and BCG scarring, indicating a greater inflammatory effect (Biering-Sørensen *et al.*, 2015).

3.1.8. Genetic Variation between Populations

Genetic differences within and between populations has been hypothesised to account for some variability in BCG-induced immunity. This was examined within South Indian and Cambodian populations, where it was found that select polymorphisms in the genes encoding

HLA-DR, HLA-DQ, vitamin D receptors and IFN- γ receptors make individuals more vulnerable to TB (Brahmajothi *et al.*, 1991; Goldfeld *et al.*, 1998). These elements partake in cell-mediated mechanisms of immunity, an important part of the body's defence against TB. As genetic differences influencing BCG efficacy has been shown to operate on an intra-population level, other studies have sought to understand the effects of genetic similarities between populations. This premise was investigated by quantifying BCG efficacy in British children with Indian ancestry and comparing this to the efficacy in Indian children. They found that BCG efficacy of the British children (64%) was consistent with the British average and therefore, does not support genetic variation as the main reason for population differences (Packer and Innes, 1988). Overall, there is an absence of clinical data to support widespread population-level effects and more research is needed to understand the prevalence of the identified polymorphisms enabling induction of immune responses to the BCG vaccine.

3.1.9. Prior Exposure to NTM

Differences in efficacy can be widely addressed by understanding the mechanisms by which NTM sensitise the immune response prior to BCG vaccination. NTM are a family of over 150 related species, under the genus *Mycobacteria*, which are unable to cause TB and leprosy. Data on the distribution of mycobacteria is not comprehensive, however, past trends suggest higher prevalence as latitude decreases (Edwards, Comstock and Palmer, 1968; Edwards *et al.*, 1969; 'Trial of BCG vaccines in south India for tuberculosis prevention: first report', 1979; Falkingham, Parker and Gruft, 1980). Mangtangi *et al.* (2014) conducted a systematic review of BCG-induced protection against TB in NTM-exposed populations. The conclusions of the study were as follows: cohorts that tested for previous NTM exposure using the Tuberculin test were the more protected than those who were not tested; there was a negative correlation between BCG administration in older age groups and efficacy; finally, there was increased BCG efficacy in higher latitude regions coinciding with reduced NTM exposure risk. The effects of NTM were shown to account for 40% of BCG-induced immune response variability (Colditz *et al.*, 1994).

There are two prevailing hypotheses regarding the mechanism of this phenomenon, namely masking and blocking. The masking hypothesis suggests that prior infection by mycobacteria induces an immune response and BCG vaccination does not improve upon existing immunity. This idea was introduced by Palmer & Long (1966) who conducted experiments on mycobacterial exposure in guinea pig models, finding that pre-sensitised guinea pigs had reduced BCG-induced protective immunity. This was supported by the results of a comparative efficacy study of British and Malawian adolescents undertaken by Black and colleagues (2002). Prior to vaccination, both cohorts had their IFN- γ responses to PPD

quantified to determine baseline levels of immunoreactivity to mycobacteria, wherein, Malawian individuals displayed elevated responses. After BCG administration, IFN- γ responses were measured a second time. Malawian participants did not display any significant changes from their baseline unlike the British cohort, who demonstrated an increase which greatly surpassed the Malawian average. This case study supports the blocking hypothesis in practice as it revealed that baseline immunity exists in pre-sensitised population, yet it is not improved with vaccination. Given that there is known cross-reactivity between antigens of *M. bovis*, *M. tb* and *M. leprae*, it is a reasonable assertion that other Mycobacteria may imbue some protection (Pönnighaus *et al.*, 1992; Brosch *et al.*, 2000; Brosch *et al.*, 2000). The masking hypothesis may also help to explain the discrepancy between high BCG-induced immunity in animal models and variability in responses of human populations. Animal models are raised in an NTM-absent environment, but exposure in humans is seen to vary globally (Andersen and Doherty, 2005). Under this hypothesis, select human populations without NTM exposure are therefore seen to mimic the environments of animal models thus exhibiting vaccine-induced immunity. The environments of pre-sensitised human populations differ substantially, changing factors of baseline immunology prior to vaccination resulting in suboptimal outcomes.

The blocking hypothesis posits that vaccination reactivates the NTM-induced immune response, preventing *M. bovis* dissemination and subsequent immunisation. When faced with virulent *M. tb*, the NTM-induced immune response is then not sufficient to suppress bacterial proliferation (Brandt *et al.*, 2002). This hypothesis was established in response to the results of a pre-sensitised mouse infection model of BCG vaccination (Brandt *et al.*, 2002). Prior exposure to *Mycobacterium avium* caused rapid identification of common antigens in *M. bovis* BCG and suppressed replication. Dissemination is an obligatory stage in the process of immune response induction by live attenuated vaccines, therefore, pre-sensitisation prevented the protective mechanism from taking pace (Siebenmann, 1951; Francis, 1956). The blocking hypothesis has formed the basis of studies aiming to improve BCG efficacy in animal models. Buddle *et al.* (2002) generated novel *M. bovis* strains displaying greater virulence than *M. bovis* BCG and administered both to NTM sensitised cows. High IFN- γ and IL-2 responses towards mutated *M. bovis* demonstrated consistent immunoreactivity. When exposed to an *M. tb* challenge, cows receiving the novel *M. bovis* strains mounted a more robust response than their BCG-vaccinated counterparts. Due to elevated virulence, the *M. bovis* mutant was able to evade the previously activated NTM-induced immune response in the cow models, triggering a more effective immune memory prior to *M. tb* infection (Buddle *et al.*, 2002). Similarly, Demangel and colleagues (2005) specifically introduced RD1 antigens to *M. bovis* BCG, causing the bacillus to become more virulent and offset any effects due to mycobacterial

pre-sensitisation. These experiments have been successful in improving vaccine-induced immunity in NTM-exposed animal models, demonstrating that the fundamental concepts of the blocking hypothesis are well-founded.

Mangtangi *et al.* (2014) conducted a systematic review of the literature about the effects of NTM pre-sensitisation on BCG and determined that the evidence was not sufficient to confirm masking or blocking mechanisms which limit vaccine-induced protective immunity. Future research should seek to establish a stronger immunological basis for the observed differences in efficacy, including investigation into NTM-specific antigens to fully understand peptide residues as targets of immune cells (Checkley *et al.*, 2011).

3.1.10. Parasitic Infection

In addition to NTM exposure, areas of low latitude indicative of poor BCG efficacy correlate with areas with high helminth burden (Hori, Watanabe and Sakurai, 1993; Chan, 1997; Elias *et al.*, 2001). Long-term helminth infections have been shown to have significant implications on host biology, namely by immunomodulation, a mechanism employed by parasites to reduce tissue damage, allowing persistence with the host organism (Maizels and Yazdanbakhsh, 2003). As a consequence of this process, the immune system reacts differently to the helminth in question and produces immunological side effects. Previous studies have informed that chronic helminth infection affects the ability of the immune system to be stimulated by unrelated antigens (Stewart *et al.*, 1999), resulting in altered responses to other pathogens (Curry *et al.*, 1995) and immunisation (Kilian and Nielsen, 1989a, 1989b; el Ghorab *et al.*, 1992; Sabin *et al.*, 1996; Malhotra *et al.*, 1999). Immunomodulation due to helminth exposure, particularly schistosomiasis, can occur indirectly during gestation or directly when established in the body (Elliott *et al.*, 2007).

Mice models consistently demonstrate the negative effects of schistosomiasis on BCG efficacy, however, clinical and population-wide studies still present a mixed picture, where some studies implicate significant immunomodulatory effects (Ghaffar *et al.*, 1990; Riner *et al.*, 2016; Nono *et al.*, 2018; Tweyongyere *et al.*, 2019) and others do not identify a relationship (Bassily *et al.*, 1992; Wajja *et al.*, 2017). Elias *et al.* (2005) investigated the effect of chronic infection with *Schistosoma mansoni* on BCG-induced protection in murine models. BCG efficacy was assessed by the capacity of *S. mansoni*-infected mice to protect against an *M. tb* challenge, measured by suppression of bacilli replication in lung and liver sections. *S. mansoni*-infected mice displayed a higher bacterial burden in these sections and a decrease in lung capacity. The spleen was also found to be affected, as lower concentrations of IFN- γ and nitric oxide were released following PPD exposure. Taken together, these responses demonstrate vaccine failure associated with parasitic infection. This effect has also been

observed at a population level, where a relationship between schistosomiasis disease and developing active TB was established (Brown *et al.*, 2006). Equally, other population studies dispute this. Chatterjee and colleagues (2014) reported no significant differences between patient outcomes of helminth co-infected and non-helminth-infected individuals over a 10-year period. However, these findings should be interpreted with caution as the authors failed to account for helminth re-infection (Babu and Nutman, 2016).

BCG is given neonatally in many high-TB burden countries; therefore, it is likely administered prior to direct helminth exposure. Hence, it is crucial to establish the effects of gestational exposure to schistosome antigens. In murine models, transgenerational exposure to schistosomes have a sustained effect on the immune system as a result of fetomaternal cross talk through placental blood (Lacorcía *et al.*, 2021). The impact on human foetuses is mediated through various mechanisms including immunological imprinting due to schistosome exposure of the pregnant person (Cortes-Selva *et al.*, 2021; Lacorcía *et al.*, 2021). In addition to this, accessory factors such as the parasite-altered microbiome of pregnant people may play a role, as it influences the child's microbiome, having consequences on future immune responses (Zaiss *et al.*, 2015; Lacorcía *et al.*, 2021).

The research presents a mixed picture, although some proportion indicates that BCG vaccine efficacy is reduced upon interaction with helminths directly or *in utero* (Ganji *et al.*, 2018). The mechanisms by which this takes place was reviewed by Nono *et al.* (2022). The process is initiated when immunogenic epitopes of the schistosome parasite are recognised by the host. Subsequently, activation of naïve T cells occurs in the secondary lymphoid organ, stimulating the Th1 or T-helper 2 (Th2) pathway depending on relative concentrations of surrounding interleukins. Helminths have been shown to polarise the host immune system to Th2 type responses, characterised by release of interleukins IL-4, IL-5, IL-9, IL-10 and IL-13 and synthesis of Immunoglobulin E (IgE) and IgG4 antibodies (Allen and Maizels, 2011; Grecis, 2015). After BCG administration, upregulated regulatory T cells (Tregs) inhibit BCG-induced Th1 and Th2 pathways through elevated secretion of IL-10 and Transforming growth factor beta (TGF- β) (van Riet, Hartgers and Yazdanbakhsh, 2007; Angeles, Mercado and Rivera, 2020). A study conducted by Toulza and colleagues (2016) supported this by demonstrating correlation of *S. mansoni* with reduced CD4⁺ IFN- γ secreting T cells and elevated CD4⁺ Tregs in people with Helminth-latent TB co-infections. A population-wide study in a Ugandan cohort disputes the implications of these findings, as transgenerational exposure to *Schistosoma* species was not observed to exert any effects on BCG efficacy (Elliott *et al.*, 2007), in spite of reducing Th1 activity (Williams *et al.*, 1994; Sabin *et al.*, 1996). In addition, the presence of schistosomes has been shown to inhibit host humoral immunity. Upregulation of regulatory B cells (Bregs) leads to elevated release of IL-10 that reduces B cell activation and subsequent

positive selection. As a result, there are fewer circulating memory B cells, which reduces the infected person's capacity to mount responses to *M. tb*.

The differences between study conclusions demonstrate the complexity of the schistosome-host interface and interaction with the immune system in the context of heterogeneous vaccine responses. Inconsistencies may also arise due to other factors such as the assessment method of vaccine efficacy, the accuracy or reliability of diagnostics, randomised versus non-randomised sampling methods and demographic differences of research cohorts. Future research should address these shortcomings by matching test and control groups for demographic factors and standardisation of measured variables to minimise these effects (Nono *et al.*, 2018).

3.1.11. Additional Hypotheses

UV light exposure has been suggested as another factor exerting an effect on BCG efficacy. This effect was observed in mice models, which displayed impaired immune responses to mycobacteria, likely due to the susceptibility of dermal Langerhans cells (actors in cell-mediated immunity) to UV-induced damage (Jeevan and Kripke, 1990). Although it is improbable that this is responsible for all the differences in BCG efficacy between populations of subtropical versus more temperate environments, exposure to UV light may be an accessory factor to vaccine-induced protection. However, other evidence disputes this effect as BCG-induced protection against mycobacteria-caused leprosy is not correlated with UV-dependent factors (Fine *et al.*, 1999). Furthermore, poverty and poor nutritional status are widely recognised determinants of BCG vaccine failure and subsequent TB progression. This is likely a result of reduced capacity to mount immune responses to the vaccine, causing impaired protection in the event of *M. tb* exposure.

3.1.12. Assessing BCG-Induced Protection

The BCG vaccine imparts variable protection against *M. tb* infection, hence the continuous disease burden and associated mortality. Better surveillance is required to monitor the presence of *M. tb* before symptoms of the disease appear, to prevent further bacterial transmission and reduce the global impact of TB. Currently, TB can be tested for using the Mantoux tuberculin skin test (TST) and Interferon-Gamma Release Assays (IGRAs). Both tests possess acceptable sensitivities for the detection of active TB, however, studies have identified deficiencies in predicting future disease incidence. This is exemplified by the fact that less than 5% of cases were successfully anticipated over a 2-year period (Diel *et al.*, 2011; Diel, Loddenkemper and Nienhaus, 2012). Additionally, the nature of these tests necessitates laboratory infrastructure and trained healthcare personnel, presenting a challenge in low-

resource environments. Moreover, some patients are discouraged from testing due to the requirement for a second appointment to receive results after incubation.

These practical challenges can be addressed through the introduction of point-of-care (POC) serodiagnostics, which ensure rapid turnaround time and do not require extensive equipment or training. This considered, no TB serodiagnostics are currently licenced for use as they do not meet appropriate sensitivity and specificity thresholds (Abebe *et al.*, 2007). Some progress has been made in the characterisation of immunogenic complexes which are associated with various stages of TB exposure and disease. These have been assessed from different sources including PPD (Barrera *et al.*, 1989), mycobacterial filtrates (Rosen, 1990; Laal *et al.*, 1997) and fractions of chromatography (Daniel *et al.*, 1986). To this end, several mycobacterial antigens present promising results including antigen 5, antigen 60 and Lipoarabinomannan (LAM) (Daniel and Debanne, 1987; Chan, Heifets and Iseman, 2000; Garg *et al.*, 2003). Although these complexes have been shown initiate measurable antibody responses in pre-clinical TB patients, there is a strong risk of confounding results with NTM or *M. bovis* BCG-sensitised patients. Therefore, future research should seek to validate unique *M. tb*-specific antibodies as candidates for sensitive, specific serodiagnostics.

3.1.13. Aims and Objectives

This investigation aims to characterise the heterogeneous profiles of humoral immunity to *M. tb* within a BCG-vaccinated population inhabiting a Schistosomiasis-endemic region in North-Eastern Zimbabwe. This was carried out by performing enzyme-linked immunoabsorbent assays (ELISAs) on sera to quantify antibody titres towards five novel peptides originating from *M. tb* proteins. The broader aims of this study were as follows: (i) to understand the relationship between age and antibody titre, thus defining how humoral immunity changes over time (ii) to define any sex-based differences in antibody responses and (iii) to elucidate the relationship between *M. tb* antibody titres and Schistosomiasis disease intensity.

3.2. Materials and Methods

3.2.1. Fieldwork Study Design and Ethical Approval

The samples used in this project originate from a 2019 study conducted by the Parasite Immunoepidemiology Group (P.I.G.) in collaboration with the University of Zimbabwe: 'Understanding Schistosomiasis among Children under Five (USaCuF)', which obtained blood, urine and stool from participants residing in the Murewha and Shamva Districts of North-Eastern Zimbabwe. The study was conducted across 20 operational health facilities, using Village Health Workers (VHWs) to survey the local population within a 10km radius of these centres. Each health facility took samples from 100 randomly selected preschool-aged children (PSAC) (children under five years of age). Participants of other ages were recruited

for the study separately. Additional samples were obtained from a 2016 study based in Chipinda, Murewha district, where participants were selected in a similar manner, although including further adult participants. Control samples were selected from another field study conducted in 2016, which investigated Schistosomiasis in Murewha and Shamva districts. However, unlike the USaCuF study, PSAC were surveyed exclusively. Sample collection and urinalysis for the presence of *S. haematobium* were carried out prior to the author's involvement in the research group. Ethical permission for the three studies was granted by the Medical Research Council of Zimbabwe before undertaking fieldwork.

The aims of the study were written in English and translated into the local language (Shona) to acquire informed consent from participants. Parental consent was given in cases where participants were under 18 years old. Recruitment to the study was on a voluntary basis and participants were permitted to withdraw at any stage of the process. Each participant was assigned a sample ID, assuring anonymity during analysis and demographic information was recorded (age, sex, etc.).

3.2.2. Sera and Urine Collection

During fieldwork, approximately 5ml of venous blood was taken from consenting participants. The samples were incubated at room temperature for 30 minutes, allowing clotting to occur. Following this stage, they were stored at 4°C up to 4 hours before serum acquisition. The samples were centrifuged at 3000 rpm for 10 minutes and the supernatant (sera) was transferred to cryotubes. Sera was temporarily stored in the field at -20°C before transit to final laboratory sites (The University of Zimbabwe and The University of Edinburgh) at -80°C for long-term depository.

Participants gave urine samples over three consecutive days. Urine samples were first tested for microhaematuria using urinalysis reagent strips. The urine was then mixed and passed through a filter. The filter was stained with iodine prior to examination under a light microscope and the number of *S. haematobium* eggs per 10 ml was recorded.

3.2.3. Inclusion Criteria and Study Design

For inclusion in this study, samples had to meet the following criteria: recorded demographic information (age and sex of participant), recorded *S. haematobium* egg count over a three-day period and a minimum volume of 300 µl viable serum. Where samples did not have associated age, school grade was accepted as a proxy.

3.2.4. Novel Peptide Microarray and Peptide Selection Criteria

This study aims to build on the preliminary research conducted by former P.I.G. members at The University of Edinburgh. An initial study using the PEPperCHIP® microarray screened seven serum samples from Zimbabwe for immunoreactive peptides, in which 5614 peptides between 5-18 amino acids were identified. A second study was carried out, in which 130 peptides were selected for further analysis using the PEPperPRINT® microarray in 26 serum samples of infants between the ages of 11-23 months. The pre-defined peptides originated from the twelve most common disease-causing pathogens targeted by the Zimbabwean immunisation programme, or the twelve most common disease-causing peptides affecting Zimbabwean children under 5. These peptides were ranked according to average immunoreactivity and analysed for potential cross-reactivity using the Basic Local Alignment Search Tool (BLAST®), where cross-reactivity was defined as the perfect alignment of at least five consecutive amino acids with another peptide (Pujol Hodge, unpublished). The five peptides exhibiting the greatest humoral response without cross-reactivity were selected for each vaccine schedule pathogen and high-burden disease-causing pathogen. After characterisation in the microarray (Intavis Peptide Services), selected peptides were lyophilised for storage purposes and refrigerated to extend their longevity.

Thus, the peptides examined in the following investigation (*Table 1*) were the most immunoreactive epitopes originating from *M. tb*, the aetiological agent of Tuberculosis, a high-burden disease and target of the preventative BCG vaccination.

Peptide	Protein of Origin	Amino Acid Sequence
MB1H37	Mce-Family Protein Mce1F	SEWRSPADTSPRPLP
MB2H37	Mce-Family Protein Mce1D	VTFHYQNKYKVPAN
MB3	Ppe Family Protein	VPPSWAAPSTRPVSA
MB4	Probable Molybdopterin-Guanine Dinucleotide Biosynthesis Protein A	VCAVDMPYLTVELIE
MB5	Secretion Protein Espe	IPIPGLPDIPGLPDF

Table 1: Characteristics of the unique *M. tuberculosis* peptides

3.2.5. Sources of Experimental Controls

PEPperPRINT® microarray data on samples from the Thrasher study (2016) informed the composition of controls used during the titration and ELISA experiments. The negative control consisted of 4 pooled samples, which were previously shown to be unreactive towards all selected peptides. Likewise, 4 samples exhibiting strong immunoreactivity towards multiple peptides were pooled to produce the positive control.

3.2.6. ELISA Titration Assays

Serum titration assays were carried out to define the optimal serum and peptide concentrations for IgG/IgM antibody responses to the each peptide. These were performed using sandwich enzyme-linked immunoabsorbant assays (ELISAs) on the positive and negative pools described above, in addition to a random selection of USaCuF samples, to capture the average immunoreactivity within this cohort.

Prior to titration assays, the lyophilised peptides were solubilised in a 6% (v/v) solution of dimethyl sulfoxide (Sigma Aldrich) in distilled water to achieve a final concentration of 1 mg/ml and stored in 20µl aliquots at -20°C. Optimisation of the assay conditions were carried out for IgG and IgM antibodies by titrating each peptide against the three aforementioned sample pools under differing sera concentrations. All peptide-antibody combinations of each sample pool were run in duplicate and the use of blank wells (consisting of identical assay reagents, excluding sera) were employed to measure background absorbance of the plate surface and reagents. For the purpose of this assay, the peptide solutions were made to a final concentration of 1 µg/ml in wash buffer (1% Bovine serum albumin factor V (Melford), 0.05% Tween (National Diagnostics) and 1% Tris-buffered saline solution). Each peptide was assigned a 96-well streptavidin-coated plate (Thermo Scientific), applied at a volume of 50 µl per well and incubated for 2 hours on a plate shaker. Following each incubation period, the plates were washed 3 times with wash buffer and blotted. The sample pools were then run vertically down the plates in a serial dilution, using concentrations of 1:10, 1:20, 1:50, 1:100, 1:200, 1:400 and 1:800 and incubated at approximately 4°C overnight.

Each plate measured the IgG and IgM response to a single peptide within the sample pools. Horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-human IgG (Dako, Denmark A/S) and HRP-conjugated goat polyclonal anti-human IgM (Bethyl Laboratories Inc.) were diluted in wash buffer to achieve a concentration of 1:1000. 50 µl of the anti-IgG and anti-IgM solutions were deposited in the appropriate wells and underwent an incubation period of 1 hour at room temperature. 100 µl of 3,3',5,5-tetramethylbenzidine (TMB) liquid HRP substrate solution (Rockland, Inc) per well was applied to the plates and incubated at room temperature for 15 minutes in the absence of light. The assay was completed with the addition of 100 µl of BioFX 450nm liquid stop solution for TMB substrates (Surmodics IVD, Inc.) per well. The optical density was measured at 450nm (OD₄₅₀) using the Emax plus microplate reader (Molecular Devices) and converted to a readable format using Softpro Max Version 7 software. To ensure reliability, similarity of sample duplicates on each plate were assessed. Curves for each peptide were plotted on line graphs using GraphPad Prism 8.2.0. The optimum dilution factors for sera were determined by locating the midpoint of the curve, equivalent to half-

maximal saturation, with adjustments to peptide and antibody dilutions where high saturation was observed. The optimum concentrations of peptides, sera and antibodies are reported in *table 2*.

Peptide	Optimum Conditions for IgG ELISAs			Optimum Conditions for IgM ELISAs		
	Peptide Concentration (µg/ml)	Sera Dilution	Antibody Dilution	Peptide Concentration (µg/ml)	Sera Dilution	Antibody Dilution
MB1H37	1.0	1:100	1:1000	0.5	1:400	1:1000
MB2H37	1.0	1:100	1:1000	0.1	1:800	1:2000
MB3	1.0	1:100	1:2000	0.5	1:400	1:2000
MB4	1.0	1:100	1:2000	0.5	1:400	1:2000
MB5	1.0	1:100	1:2000	0.5	1:400	1:2000

Table 2: The optimum conditions for each peptide according to titration assays

3.2.7. Peptide-Specific IgG and IgM Titres

Once the optimum conditions for each assay had been specified (*Table 2*), sandwich ELISAs were performed on serum samples to quantify the IgG and IgM levels of each peptide as a function of OD₄₅₀. These were carried out using the same steps as the titration assay previously described in this report. Each serum sample was run in duplicate and the two sets of controls were applied to each plate to validate consistency within each assay. As in titrations, blank well duplicates were used to determine background absorbance and were subtracted prior to analysis.

3.2.8. Data Analysis

Statistical analysis was carried out using GraphPad Prism 8.2.0 and Rstudio v4.2.1. The statistical analysis conducted in this study defined the significance level at $\alpha = 0.05$, wherein $p < 0.05$ were not considered sufficient evidence for the rejection of the null hypothesis. Thus, it is unlikely that the observed differences arose due to type I errors. Graphs were also produced using GraphPad Prism 8.2.0 and Rstudio v4.2.1. Where there are tests comparing multiple groups, post hoc Dunn tests were used to elucidate relationships between pairs of groups. Stated p -values are two-sided and the Bonferroni correction was used to avoid inflation of p -values. In many cases, normal distribution was not observed; therefore, non-parametric tests were employed and descriptive statistics are presented as medians with interquartile range (IQR). The data often violated the principle of homogeneity of variance.

Absorbance at 450 nm was used as a proxy value for *M. tb*-specific antibody titre during analysis as actual concentration could not be calculated without standard curves. Relationships between IgG and IgM antibodies for each peptide were established using Spearman's non-parametric test of correlation, calculating the correlation coefficient and p -value. The correlation between the peptide-specific antibody responses can be interpreted as

weak, moderate and strong depending on the value of Spearman's rank coefficient (ρ). For instance, the correlation can be said to be weak when $\rho = 0.25 < x < 0.5$, moderate when $\rho = 0.5 < x < 0.75$ and strong when $\rho > 0.75$. Correlation coefficients under 0.25 were not considered evidence for a relationship between the variables.

The study cohort was separated into males and females to account for any sex-based differences and then further stratified into participants with and without schistosomiasis to capture the effects of the parasitic infection in different age groups. OD₄₅₀ of IgM and IgG, as well as the IgM to IgG ratio, were plotted on the y-axis and age on the x-axis. Curves were fitted using local polynomial regression in the ggplot2 package in RStudio v.4.2.1. The details of this method are as follows: "for the fit at point x , the fit is made using points in a neighbourhood of x , weighted by their distance from x (with differences in 'parametric' variables being ignored when computing the distance). The size of the neighbourhood is controlled by α (set by functions 'span' or 'enp.target'). For $\alpha < 1$, the neighbourhood includes proportion α of the points, and these have tricubic weighting (proportional to $(1 - (dist / \max dist)^3)^3$). For $\alpha > 1$, all points are used, with the 'maximum distance' assumed to be $\alpha^{1/p}$ times the actual maximum distance for p explanatory variables" (Cleveland et al., 1992; DataCamp, n.d.). Shaded regions on the graph depict 95% confidence intervals (CIs), calculated as part of the 'geom_smooth' function in the ggplot2 package. Statistical significance between groups can be determined visually by the proximity of CIs, where the absence of overlapping intervals denotes significant differences ($p < 0.05$). Where pronounced distinction between study populations was observed, Mann-Whitney U tests were conducted to determine whether there was a significant difference overall. Spearman's correlation was then used to ascertain the potential association between age and antibody titre in the male and female cohorts of differing schistosomiasis disease statuses. Stated absorbances and ages are approximated from the curve and expressed in arbitrary units (AU) per 50 μ l of diluted sera used in the assay. Note that arbitrary units for each antibody (IgG or IgM) and peptide response were assay-specific, corresponding to differing conditions referred to in section 4.2.6. Therefore, arbitrary units referred to throughout cannot be used as a measure of antibody concentration when comparing assays of differing peptides or antibodies. To understand the relationship between schistosomiasis burden and antibody production, Spearman's non-parametric test of correlation was performed on mean *S. haematobium* egg count and OD₄₅₀ of peptide-specific IgM, IgG and IgM to IgG ratio.

3.3. Results

3.3.1. Descriptive Statistics and Participant Demographics

A total of 243 participants from 2 separate cohorts met the inclusion criteria and were thus involved in the study. This study cohort comprised 95 males (39.1%) and 148 females (60.9%),

with ages ranging from 2 to 76 years of age. More detailed demographic information is included in *tables 3 and 4*. As neonatal BCG vaccination is mandated by Zimbabwean law, all participants were of an age where they should have received it. Where information on age was unavailable, results were removed from age-dependent analysis.

Age Group	N	Percentage (%) $\pm$ 95% CI
Male	95	39.10 (33.00-45.20)
Female	148	60.90 (54.80-67.00)

Table 3: Sex of participants in the study population

Age Group	N	Percentage (%) $\pm$ 95% CI
2-5	18	7.41 (4.11-10.70)
6-10	56	23.00 (17.80-28.30)
11-15	68	28.00 (22.30-33.60)
16-20	19	7.82 (4.44-11.20)
21-25	5	2.06 (0.27-3.84)
26-30	12	4.94 (2.21-7.66)
31-35	10	4.12 (1.62-6.61)
36-40	4	1.65 (0.05-3.25)
41-45	7	2.88 (0.78-4.98)
46-50	5	2.06 (0.27-3.84)
51-55	4	1.65 (0.05-3.25)
56-60	6	2.47 (0.52-4.42)
61-65	1	0.41 (-0.39-1.22)
66-70	3	1.23 (-0.15-2.62)
71+	5	2.06 (0.27-3.84)
Unspecified	20	8.23 (4.77-11.70)

Table 4: Age of participants in the study population

3.3.2. Titration Assays

The purpose of the titration assays was two-fold: to identify the optimum conditions for the assay and to determine the validity of the sample pools for use as controls. The titrations found that the OD₄₅₀ of presumed-negative samples (see 4.2.5. Sources of Experimental Controls) was consistently higher than the presumed-positive and study population pools for all analysed peptides. Therefore, it could not be used as an effective cut-off point for the assay. For future analyses during ELISAs, the controls were used instead to confirm intra-assay consistency.

3.3.3. Excluded Samples

Mean absorbance of blank samples described in section 4.2.7, quantified the base absorbance attributed to the assay reagents in the absence of sera for each plate. Beyond

this threshold, absorbance increases due to the presence of peptide-specific antibody in sera. In some assays, a minority of samples did not exceed this threshold (*Table 5*). These samples were removed during analysis because absorbances did not account for assay reagents.

Peptide	Antibody	Sample ID	Age	Sex
MB2H37	IgG	1008	M	13
		1067	M	5
	IgM	M408	M	67
		M443	F	35
		M478	F	42
MB3	IgM	M408	M	67
MB5	IgG	971	M	6
		1008	M	13
		1067	M	5

Table 5: Excluded samples from analysis of respective assays. Samples were excluded from analysis where mean OD₄₅₀ did not exceed the plate-specific threshold of mean blank OD₄₅₀.

3.3.4. Relationships between *Mycobacterium tuberculosis* IgM and IgG responses

Peptide	Males	Females
MB1H37	0.140	0.177*
MB2H37	0.233*	0.102
MB3	0.002	-0.169*
MB4	0.577***	0.378***
MB5	0.265*	0.174*

Table 6: Spearman's non-parametric correlation between IgG and IgM titres for each *Mycobacterium tuberculosis* peptide. Spearman's rank correlation coefficient (ρ) is indicated and significance is denoted by asterisks: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

Spearman's non-parametric correlation was used to identify whether IgM and IgG responses for each peptide were correlated in males and females (*Table 6*). In both sexes, there was strong evidence to suggest positive correlations between MB4-specific IgG and IgM, although they differed in the strength of these correlations, with males and females displaying moderate ($\rho(20) = 0.577$, $p < 0.001$) and weak ($\rho(53) = 0.378$, $p < 0.001$) effects respectively. A weak relationship between MB5-specific antibodies was demonstrated exclusively by males ($\rho(20) = 0.265$, $p < 0.05$). Some peptide-specific antibody responses did not meet the threshold of $\rho \geq 0.25$ to establish a correlation. These included female responses to MB1H37 ($\rho(53) = 0.177$, $p < 0.05$), MB3 ($\rho(53) = -0.169$, $p < 0.05$) and MB5 peptides ($\rho(53) = 0.174$, $p < 0.05$) and the male response to the MB2H37 peptide ($\rho(19) = 0.233$, $p < 0.05$). The remaining antibody responses did not meet the criteria to establish correlation and were not statistically significant. No strong correlations between peptide-specific antibody responses were observed in this analysis.

3.3.5. The Spectrum of MB1H37-Specific Antibody Titres

Schistosomiasis-unexposed populations display some variability in MB1H37-specific IgM responses across age and sex demographics (*Figure 1*). Healthy males experience the lowest IgM titres at 3 years of age (1.25 AU), which increases rapidly over time until peaking at 24 (3.40 AU). Antibody titres were seen to decline at a similar rate until 45 years, after which it continues to decrease much slower. On the other hand, females do not experience a surge in antibody levels from a young age, starting at a much more moderate 2.25 AU, increasing until the age of 17, at which the trend line levels out at 2.75 AU. IgM titre began to fall slightly at the age of 60. With the exception of the period between 3 to 11 years old, healthy male IgM levels exceed those of healthy females. 95% CIs do not overlap between 3 and 6 years old, demonstrating that the males of this cohort had significantly lower IgM levels during these ages. A Mann-Whitney U test was performed and failed to identify significant differences between sex-associated IgM levels in healthy participants across all ages.

Further variability is observed in patterns of MB1H37-targeted IgG antibody expression between males and females (*Figure 1*). Overall, the spectrum of average IgG titres in healthy males is represented by a single peak and trough structure. Males display relatively low IgG levels in children under 5 (2.45 AU) which then increases with age, peaking at 22 years old (2.95 AU). They then experience a decrease in IgG titres until 60 years of age (2.40 AU), which is beneath the levels expressed by young children. Henceforth, this trend reverses direction, demonstrating a slight elevation in elderly males. Although the range of anti-MB1H37 IgG titres expressed by healthy females is similar to males, the pattern of titre with age differs substantially. Firstly, the peak antibody titre in males is not visible in the female profile, instead, the highest titres were observed in young children (2.80 AU). Secondly, females displayed a weak negative correlation between age and antibody production. Between 35 and 47 there is a small increase that interrupts this trend, yet IgG titre continues to decrease after 52 years of age.

The MB1H37-specific ratio between IgM and IgG is slightly variable between male and female participants of this cohort (*Figure 1*). Males displayed the lowest average antibody ratio in children under 5 (0.50) which rose until hitting its peak value at 27 years old (1.20). From this point, the ratio fell slowly with age. Females also exhibited the smallest ratio values in the youngest participants, although at 0.75, this was higher than in males of the same age. IgM/IgG ratio was observed to increase more slowly with age and reach a lower peak value (1.10) with respect to males. Following this, females also experienced a reduction in ratio with age.

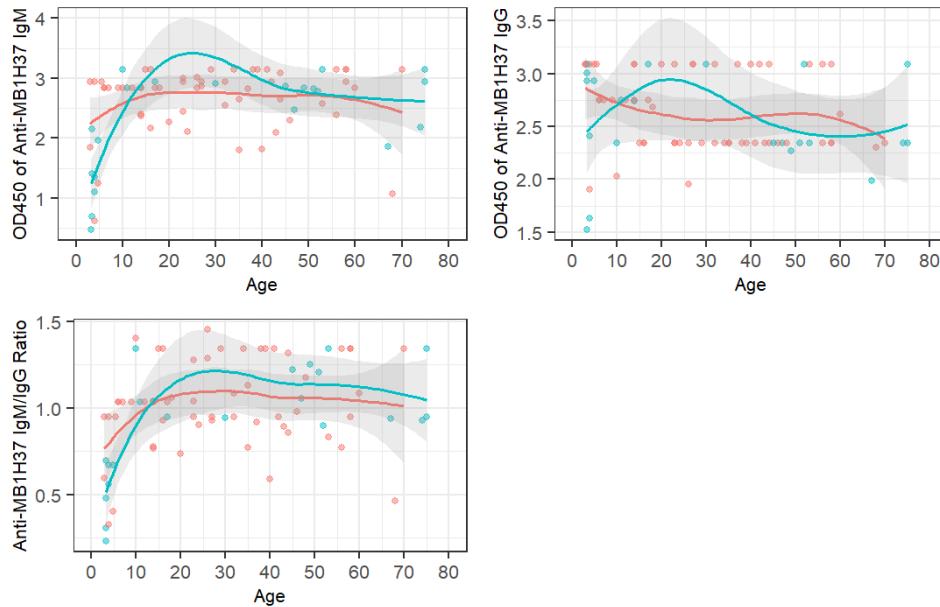


Figure 1: Antibody responses to Mycobacterium tuberculosis peptide MB1H37 in healthy participants. Fitted curves of absorbance at 450nm with age in individuals without schistosomiasis for MB1H37. Values and fitted curves of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB1H37 IgM in males (N = 22) and females (N = 55) with age. Top right: OD450 of anti-MB1H37 IgG in males (N = 22) and females (N = 55) with age. Bottom left: Anti-MB1H37 IgM/IgG ratio in males (N = 22) and females (N = 55).

Schistosomiasis-infected males and females experience immunological differences from their healthy equivalents and this is apparent in IgM responses targeted against the MB1H37 peptide (Figure 2). Average IgM titres begin at a low level (1.20 AU), as similarly expressed by uninfected males. However, infected males reach an earlier peak titre at 10 years old (2.40 AU), an absorbance which is much lower than the highest IgM titres observed in healthy males. There is a short period where antibody levels fall with time, although the trend begins to increase again from 15 years old, reaching similar IgM titres to the first peak at 20 years of age. Between 14 and 17 years of age, the spectra of IgM titres between infected and uninfected males diverged and 95% CIs were distinct. To determine whether schistosomiasis-infected individuals were significantly higher than healthy participants overall, a Mann-Whitney U test was performed, although was unable to recognise statistical differences between male participants of differing infection status. Females with schistosomiasis had higher IgM titres in participants under 5 (2.20 AU) than males with the infection. Despite this difference, a similar peak was reached at around 10 years of age (2.45 AU) and the anti-MB1H37 titre decreases until the age of 14. Notably, this peak was not present in healthy females. After the age of 14, IgM levels rose irregularly until reaching a second peak (2.50 AU) which corresponded to titres observed at the first peak. This was also seen in schistosomiasis-infected males, although the second peak occurred earlier in comparison with females. Finally, anti-MB1H37 IgM declined

slowly from 50 years old. Throughout the spectrum, uninfected females had higher antibody titres than those with the infection, particularly between the ages of 13 and 16 where CIs did not overlap. Significant differences between infected (median = 2.84, IQR = 2.41, 2.99) and uninfected females (median = 2.19, IQR = 1.99, 2.84) were identified across the entire cohort using a Mann-Whitney U test ($W = 1430$, $p < 0.0001$ two-tailed) (*Figure 3a*).

The trend of absorbance as a function of age in schistosomiasis-infected participants differed from the uninfected cohort (*Figure 2*). The youngest individuals have the lowest antibody titres (2.25 AU), which increase to form a peak before 10 years of age (2.70 AU). There was a similar rate of decline from the peak until approximately 15 years (2.45 AU), after which antibody titre increases at a slower rate. At 60 years old, antibody titre slowly declines. Key features of the graph that distinguish it from the uninfected participants are the peak observed in childhood and the lack of consistent negative association between absorbance and age. There is a deficiency of schistosomiasis-infected males in later years, which would be required to fully describe the effects of schistosomiasis on age-dependent antibody titres. Between 5 and 15 years of age, there are two small peaks at 2.6 AU and 2.70 AU, respectively. Females with schistosomiasis similarly begin with low levels of MB1H37-specific IgG, instead reaching a singular peak at around 2.70 AU before the age of 10. Between the ages of 12 and 18, females without schistosomiasis have significantly higher IgG titres than those without the infection. This was also found to be significant overall through the results of a Mann-Whitney U test ($W = 1981$, $p < 0.05$ two-tailed), where uninfected females (median = 2.62, IQR = 2.34, 3.09) expressed greater IgG levels than their infected counterparts (median = 0.72, IQR = 0.52, 1.09) (*Figure 3b*). At 18 years of age, there begins a gradual peak (the highest point at 2.75 AU), which begins to decline after 60. The antibody responses for children under 18 with schistosomiasis demonstrate several sex-based differences, primarily in the peak structures and a more prominent decrease following the point of highest antibody titres in females.

MB1H37-targeted IgM/IgG ratios demonstrate several key differences between schistosomiasis-infected and uninfected cohorts (*Figure 2*). Unlike healthy males, those with the infection displayed the highest antibody ratio in the youngest participants (1.60). It proceeded to decline with age until approximately 11 years of age (0.90), after which the ratio is seen to increase, closely following the trend line of uninfected males. Schistosomiasis-infected females display a distinct spectrum of ratios, particularly in the early ages of the cohort where high variability is observed. The high IgM/IgG ratios of infected females (1.30) are observed in females at 4 and 15 years old. The pattern of an initial peak ratio in young participants followed by a decline throughout childhood was shared between males and females with the disease. Prior to the age of 23, females with the parasitic infection displayed elevated ratio values compared to their healthy counterparts.

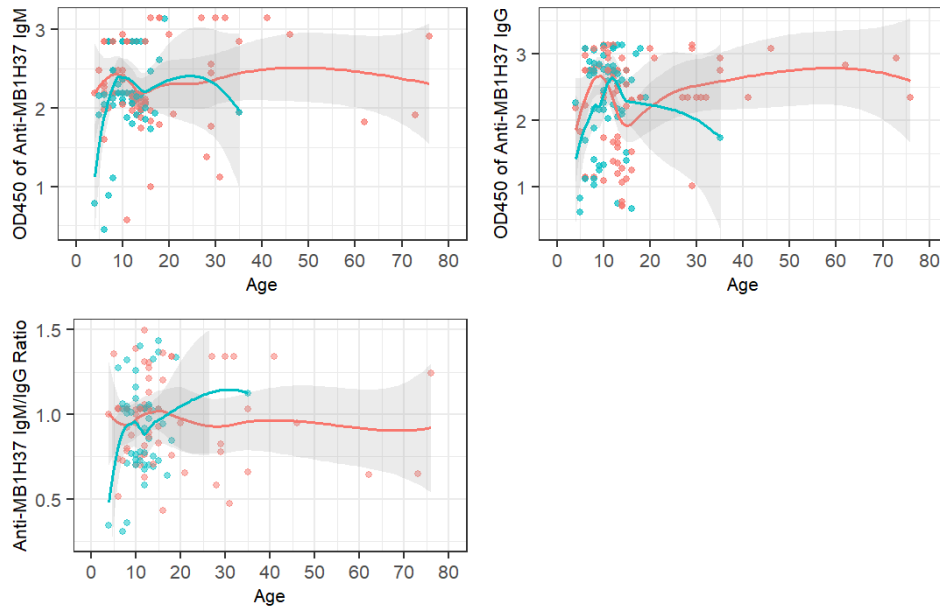


Figure 2: Antibody responses to *Mycobacterium tuberculosis* peptide MB1H37 in participants with schistosomiasis. Fitted curves of absorbance at 450nm with age in individuals with schistosomiasis for MB1H37. Values and fitted curves of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB1H37 IgM in males (N = 73) and females (N = 93) with age. Top right: OD450 of anti-MB1H37 IgG in males (N = 73) and females (N = 93) with age. Bottom left: Anti-MB1H37 IgM/IgG ratio in males (N = 73) and females (N = 93).

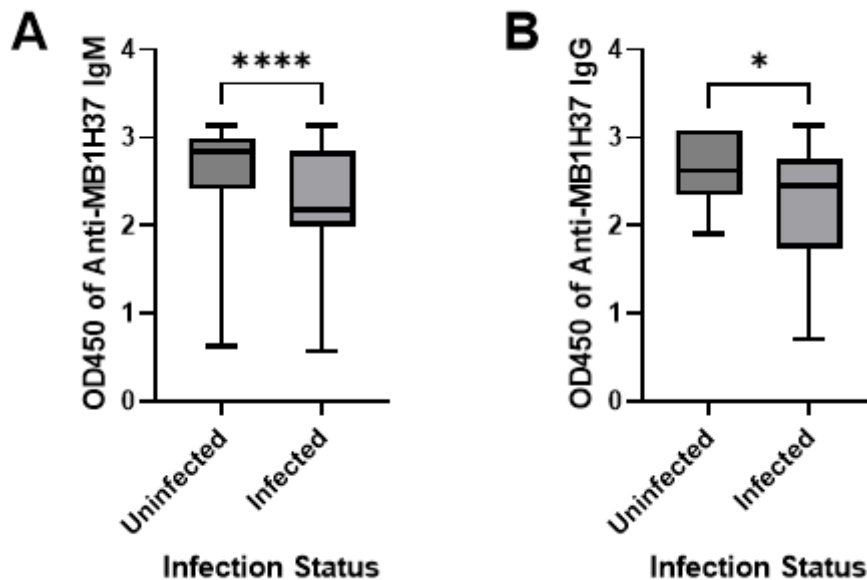


Figure 3 (a) Distribution of anti-MB1H37 IgM levels in females of differing schistosomiasis infection status. Uninfected females display significantly higher anti-MB1H37 IgM titres (N = 55, median = 2.84, IQR = 2.41, 2.99) than infected females (N = 93, median = 2.19, IQR = 1.99, 2.84) ($W = 1430$, $p < 0.0001$). The box represents the first third quartile and bars represent the range, excluding

outliers. The median is indicated by the interior line. Significance is denoted by asterisks: $p < 0.5 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

(b) Distribution of anti-MB1H37 IgG levels in females of differing schistosomiasis infection status. Uninfected females display significantly higher anti-MB1H37 IgG titres ($N = 55$, median = 2.62, IQR = 2.34, 3.09) than infected females ($N = 93$, median = 2.45, IQR = 1.74, 2.75) ($W = 1981$, $p = 0.0205$).

3.3.6. The Spectrum of MB2H37-Specific Antibody Titres

MB2H37-specific antibody responses differed in tandem with age, sex and schistosomiasis status (*Figure 4*). High IgM titres were observed in male children under 5 (0.85 AU), which declined over time until 20 years of age (0.70 AU), the lowest levels of the antibody observed in the cohort. The IgM titre begins to recover after 35, maintaining a steady increase until reaching levels previously observed in young children. A contrasting trend was discerned in healthy female IgM responses. Instead of the decreasing titre observed in males, females exhibit increasing titres between 5 (0.80 AU) and 25 (0.95 AU). Whilst males demonstrate a rise in IgM titres in adulthood, female IgM levels decrease from 25 and reach a short constant phase (0.70 AU) beginning at 47 for 10 years. However, between 60 and 70 years of age, the female IgM titre rises as in males, increasing to 0.80 AU, a level comparable to both female infants and males with an equivalent age.

Males without *S. haematobium* exposure experience the lowest IgG levels at around 5 years of age (0.75 AU), which increases at a somewhat regular rate until it peaks at approximately 2.20 AU (*Figure 4*). IgG titre was seen to decrease after 52 until the eldest ages which were analysed in the study. Sera originating from elderly members of the population exhibited an average IgG titre of 1.50 AU which was equivalent to that of 20-year-old participants. Females without schistosomiasis also experienced the smallest IgG titres in the youngest participants, wherein, the value of 1.10 AU at 5 was higher than that of the equivalent uninfected males. Females did not experience a similar IgG peak in middle-aged participants, as this occurs earlier in life at 20 years old (1.75 AU). The average IgG titre was seen to fluctuate about this point until the age of 55. As perceived in the male IgG profile, there is a reduction in IgG levels to a final recorded absorbance of 1.25 AU. This is smaller with respect to male IgG levels at this period and equates to titres present in uninfected females under 5.

The MB2H37-specific IgM/IgG ratio demonstrated variability with age in healthy participants (*Figure 4*). The highest ratio in males was observed in the youngest participants (1.50) before experiencing a dramatic decrease, reaching its lowest point at 30 years of age (0.25). The ratio recovers slowly with age reaching 0.60 in the eldest males. Similarly, females display the highest antibody ratio in participants under 5 (1.00) which decreases until 55 years old (0.40).

Although, there is a fluctuation resulting in a small peak at 25 years old, where the curve is seen to rise above the levels observed in males. The ratio between the antibodies begins to rise again later in life, displaying a value more similar to that previously seen in children (0.70).

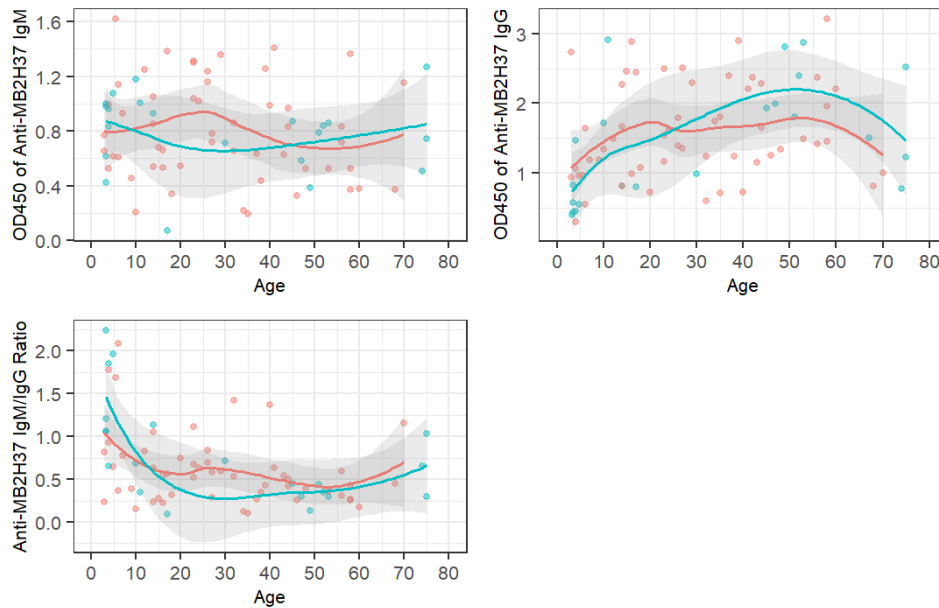


Figure 4: Antibody responses to Mycobacterium tuberculosis peptide MB2H37 in healthy participants. Values and fitted curves of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB1H37 IgM in males (N = 21) and females (N = 53) with age. Top right: OD450 of anti-MB1H37 IgG in males (N = 22) and females (N = 55) with age. Bottom left: Anti-MB1H37 IgM/IgG ratio in males (N = 21) and females (N = 53).

The spectrum of MB2H37-specific antibody titres with respect to age differs between *S. haematobium*-exposed and unexposed individuals (Figure 5). For instance, schistosomiasis-infected males experience a sharp increase in IgM titre with age between 5 (0.50 AU) and 9 (1.10 AU), where the curve oscillates near its highest point at 15 years old. In males without the infection, young children possessed higher titres than uninfected males of the same age. In addition, the gradient direction was reversed in uninfected males, presenting a decline instead of the IgM titre increase seen in schistosomiasis-positive males. As in infected males, females with parasite exposure saw an incline of IgM titre between 5 (1.00 AU) and 9 (1.25 AU). Infected females under 5 had substantially higher IgM; however, a smaller increase was observed in comparison with infected males, reducing the range between titres at the peak. The increase in uninfected females happened over a sustained period and possessed a lower titre throughout. This was shown to be statistically significant by the results of a Mann-Whitney U test (test (W = 1413, p < 0.0001 two-tailed), where infected females (median = 1.08, IQR = 0.86, 1.32) expressed greater IgG levels than their uninfected counterparts (median = 0.72, IQR = 0.52, 1.09) (Figure 6). After the peak, females experienced a reduction in average IgM

levels, wherein the titre at 16 years of age descended below the absorbances of children under 5. Subsequently, there were constant titres observed until approximately 25 years of age. The slow drop in MB2H37-targeted IgM from the ages of 25 to 50 imitated the response in uninfected females, concerning the age group and absolute antibody titres. Further similarities were observed in the seroreactivity of older adults as both schistosomiasis-positive and negative females showed evidence of elevated IgM after 57 years old, reaching comparable titres in elderly adults.

This study demonstrated that males with schistosomiasis have altered MB2H37-specific IgG seroreactivity compared to healthy individuals (*Figure 5*). The smallest average IgG titre is in children under 5 (0.40 AU), although, this increases rapidly and peaks at 12 years old (1.80 AU). These levels are substantially lower than healthy males at this age and IgG titre was shown to peak much later in life. Females with schistosomiasis initially possessed similar IgG titres at 5 years old (0.90 AU) to unexposed females (0.10 AU). In comparison with infected males, MB2H37-targeted IgG was more elevated in the youngest participants of the cohort and peaked earlier than males at 9 years old (1.75 AU). However, the peak antibody titre of females was marginally lower than males. A second peak at 27 was a prominent feature of this spectrum (1.75 AU), a similar titre present in sera of uninfected females. After the peak, IgG levels increased with age from 46 to 76. At 59 years old, the trend lines intersect, with infected females reaching elevated antibody titres in the elderly study population with respect to females without schistosomiasis.

Infected males had elevated MB2H37-targeted IgM/IgG with respect to their healthy counterparts (*Figure 5*). The youngest participants display the highest peptide-specific ratios at 3.00, although this rapidly declines, resulting in a reduced value of 1.00 at 9 years of age. MB2H37 antibody ratios in healthy males also experience this reduction from a peak value; however, this took effect over a longer period and was less pronounced than in those with schistosomiasis. Females with the parasite demonstrated more similar MB2H37 antibody ratios to unexposed females of the cohort, especially in children under 5 (1.00). This distinguished the curve from males of the same infection status, which began at a higher level. In contrast to healthy females, those with schistosomiasis experience the highest ratios (2.00) at the ages of 12 and 28, after which the value falls with age across the rest of the cohort. The average MB2H37 IgM/IgG ratio is predominantly higher in schistosomiasis-infected than uninfected females, in addition to males with the disease.

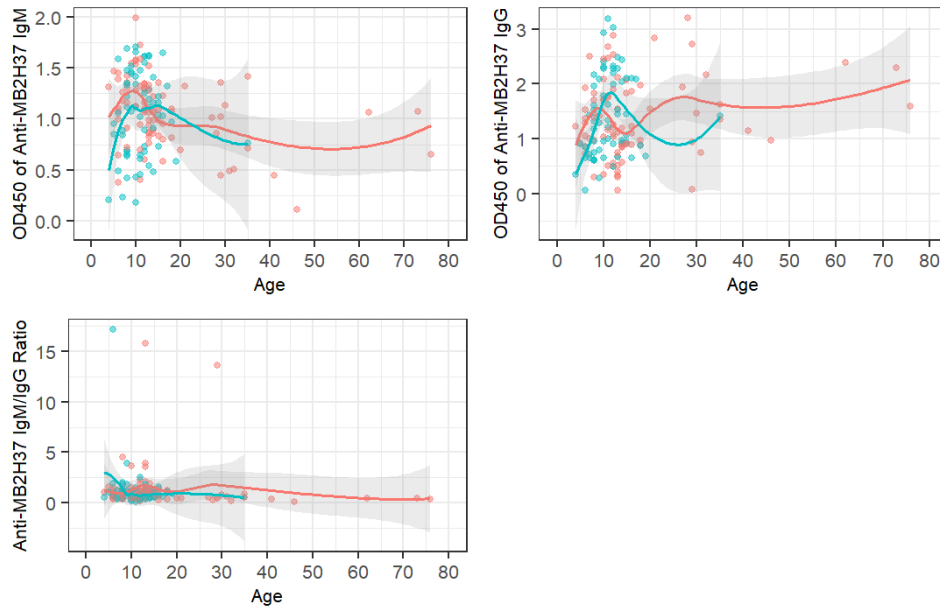


Figure 5: Antibody responses to *Mycobacterium tuberculosis* peptide MB2H37 in participants with schistosomiasis. Values and fitted curve of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB2H37 IgM in males ($N = 73$) and females ($N = 93$) with age. Top right: OD450 of anti-MB2H37 IgG in males ($N = 71$) and females ($N = 93$) with age. Bottom left: Anti-MB2H37 IgM/IgG ratio in males ($N = 71$) and females ($N = 93$).

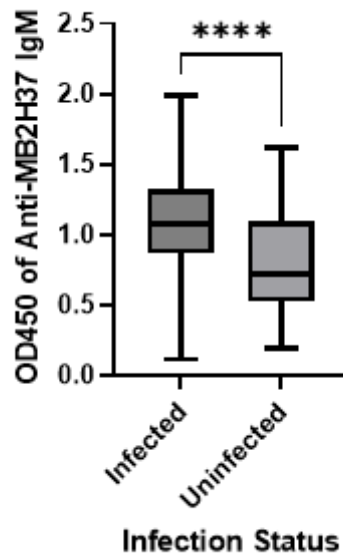


Figure 6: Distribution of anti-MB2H37 IgM levels in females of differing schistosomiasis infection status. Infected females display significantly higher anti-MB2H37 IgM titres ($N = 93$, median = 1.08, IQR = 0.86, 1.32) than uninfected females ($N = 53$, median = 0.72, IQR = 0.53, 1.09) ($W = 1413$, $p < 0.0001$).

3.3.7. The Spectrum of MB3-Specific Antibody Titres

Male participants who were not infected with *S. haematobium* had some MB3-specific IgM variability correlating with age (*Figure 7*). Young children had low levels of this antibody (1.50 AU) which increased with age until it reached a peak level at 20 years of age (3.00 AU). After this point, IgM titres trend downwards, falling at a fast rate between 30 and 45 years, at which it drops to childhood levels. IgM antibody levels against MB3 continue to slowly decrease, beginning to level off in elderly males at an absorbance of 1.40 AU. Healthy female participants also began with lower MB3-targeted IgM levels in children under 5 (2.00 AU). This rises to a high of 2.60 AU at 15 years old before decreasing and reaching a low of 2.00 AU at 37. This first peak structure was similar to healthy male equivalents; however, females had an overall smaller peak titre which occurred earlier in comparison. Following this, IgM titres increase slowly again with age until 60 years of age, when levels begin to flatten. Between the ages of 54 and 66, 95% CIs of average IgM absorbances did not overlap, with females expressing a higher IgM titre during this period. A Mann-Whitney U test was performed ($W = 386$, $p < 0.05$ two-tailed) and found significant differences between males (median = 1.85, IQR = 1.44, 2.43) and females (median = 2.23, IQR = 1.76, 2.97) in MB3-specific IgM (*Figure 8*).

The IgG response of the study cohort was also quantified (*Figure 7*). For uninfected males in the study, the profile of immunity varied with age. The youngest participants possessed the lowest levels of anti-MB3 IgG at 0.70 AU, which increased until peaking at 37 (1.75 AU). After the peak, IgG antibody levels gently decreased with age, culminating in an absorbance of 1.20 AU in elderly participants. Female children under 5 had an average titre of 1.20 AU which was higher than the average male. The overall trend differed from males without schistosomiasis as two peaks are observed in females where only one is visible in the male trend of antibody levels with age. The peaks are at the ages of 20 (1.60 AU) and 45 (2.00 AU) respectively, with a trough between these points at 27 years of age (1.40 AU). After this sequence, MB3-targeted IgG declined to 1.40 AU in individuals at the age of 70.

The graph in *Figure 7* depicts the highest ratio between IgM and IgG absorbances in male participants between the ages of 5 and 10 (2.70). This ratio decreases with age between 10 and 47, where it is valued at 1.00, before it increases from the age of 55 onwards. The curve showing the relationship between age and IgM/IgG ratio is smoother in males than in females, however, the overall trend is similar. Female children under 5 have the highest ratio (2.30) between the antibodies, largely decreasing until the age of 40 (1.25), with a slight fluctuation at 25 years old. This ratio begins to increase again at the age of 47, with elevated ratios in the elderly population as observed with males.

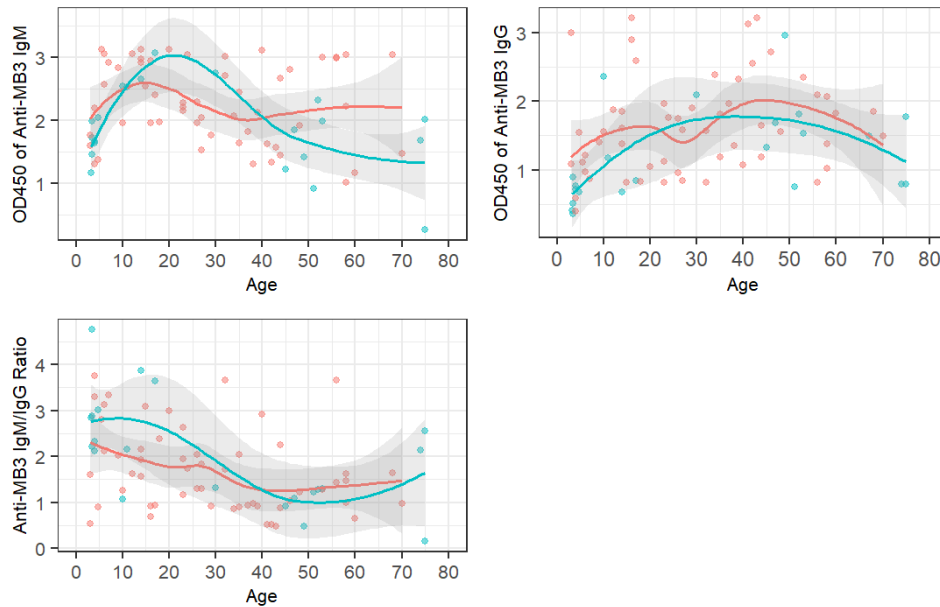


Figure 7: Antibody responses to *Mycobacterium tuberculosis* peptide MB3 in healthy participants. Values and fitted curves of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB3 IgM in males ($N = 21$) and females ($N = 55$) with age. Top right: OD450 of anti-MB3 IgG in males ($N = 22$) and females ($N = 55$) with age. Bottom left: Anti-MB3 IgM/IgG ratio in males ($N = 21$) and females ($N = 55$).

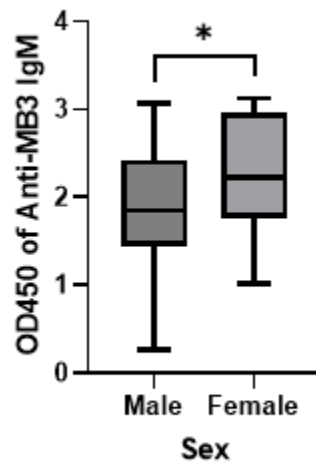


Figure 8: Distribution of anti-MB3 IgM levels in healthy individuals. Females display significantly higher anti-MB3 IgM titres ($N = 55$, median = 2.23, IQR = 1.76, 2.97) than males ($N = 21$, median = 1.85, IQR = 1.44, 2.43) ($W = 386$, $p = 0.0255$).

The trend of MB3-targeted IgM was distinct in schistosomiasis-infected males from their healthy counterparts in the study (Figure 9). Firstly, IgM levels were lower in males with this parasitic infection than those without. However, like healthy males, young children had low IgM titres (1.10 AU) with respect to older children. Antibody titre grows with age to form two

narrow peaks. As such, the highest antibody titre occurs at 12 years of age (2.25 AU). As with males with this parasitic infection, the lowest antibody levels are exhibited by females under 5 (1.30 AU), rising rapidly until 9 years of age (2.15 AU). At this early stage, similarities are apparent to the schistosomiasis-infected males as this initial increase in antibody levels was within a similar antibody range and age group. There is a brief period in early adulthood where IgM titres remain constant (1.50 AU) prior to a second peak forming at 2.20 AU. IgM antibody levels decline in elderly individuals close to those expressed by young children. Healthy females, in comparison, displayed an elevated, elongated peak with respect to infected females and demonstrated significantly higher IgM levels between 11-24 years old. The results of a Mann-Whitney U test ($W = 1567$, $p < 0.001$ two-tailed) determined that there were significant differences in anti-MB3 IgM titres between females with (median = 1.56, IQR = 1.20, 2.56) and without (median = 2.23, IQR = 1.76, 2.97) schistosomiasis overall (*Figure 11a*).

The MB3-specific IgG profile for males with schistosomiasis differed from the healthy males of the study cohort (*Figure 9*). The lowest IgG titres were expressed by children under 5 (0.90 AU), growing at a fast rate until the age of 10. Between 10 and 15, there was some oscillation resulting in a peak antibody titre of 2.50 AU. This contrasts with the healthy male response, which saw antibody titres rising more gently to form a smaller peak during middle age. As such, males with schistosomiasis (median = 2.38, IQR = 1.62, 2.87) were shown to express significantly higher IgG antibodies than their uninfected counterparts (median = 0.87, IQR = 0.71, 1.71) ($W = 281$, $p < 0.001$) (*Figure 10a*). Similarly, the youngest females of the cohort experience the smallest IgG titres (1.60 AU), increasing before 10 years old (2.20 AU), with peaks occurring in a similar region. Although, antibody levels started from a higher point than males and peaked lower. There was a second peak of high IgG levels at 22 years old (2.30 AU) after a low point recorded at the age of 15 (2.00 AU). Following the decline in anti-MB3 IgG titres, there is a short constant phase (1.70 AU) ending at 67, where antibodies begin slowly increasing again. Females of differing schistosomiasis statuses experience similar IgG titres from middle age onwards, with the highest distinction occurring during childhood and early adulthood. Overall, this results in a significant difference between the titre distributions of each state ($W = 1664$, $p < 0.001$), where infected females secrete increased levels of IgG antibodies (median = 2.05, IQR = 1.55, 2.63) against females without the infection (median = 1.56, IQR = 1.07, 1.96) (*Figure 11b*).

The MB3-targeted IgM/IgG ratio is much lower in schistosomiasis-positive individuals than in healthy individuals (*Figure 9*). The highest ratio between the antibodies is experienced at 5 years of age (1.80), after which it declines to 1.00. In uninfected individuals, males of this age had a constant ratio and the fall in antibody levels occurred later, over an extended period. Furthermore, there is a fluctuation between 10 and 15 years old, which is not visible in the

uninfected cohort. Between 8 and 18 years old, the ratio value was significantly lower in infected males. This difference was established on a cohort-wide level ($W = 334$, $p < 0.001$), demonstrating that healthy males displayed elevated antibody ratios (median = 2.14, IQR = 1.16, 2.86) with respect to schistosomiasis-infected individuals (median = 0.77, IQR = 0.54, 1.20) (Figure 10b). Females with schistosomiasis infection experience a peak ratio at the later age of 10 years old (1.20) in contrast with males. The subsequent fall in IgM/IgG ratio was a shared feature in both profiles, although this is delayed in schistosomiasis-infected females between 10 and 20 years old, resulting in the lowest point of 0.60. The subsequent increase then results in an elevated IgM/IgG ratio of 1.25. In comparison with equivalent males, infected females exhibited significantly lower values for antibody ratio in the age range of 5-27. This extended to both groups overall ($W = 1192$, $p < 0.001$), where females with the infection displayed chronically higher ratios (median = 2.05, IQR = 1.55, 2.63) compared to the uninfected baseline (median = 1.56, IQR = 1.07, 1.96) (Figure 11c).

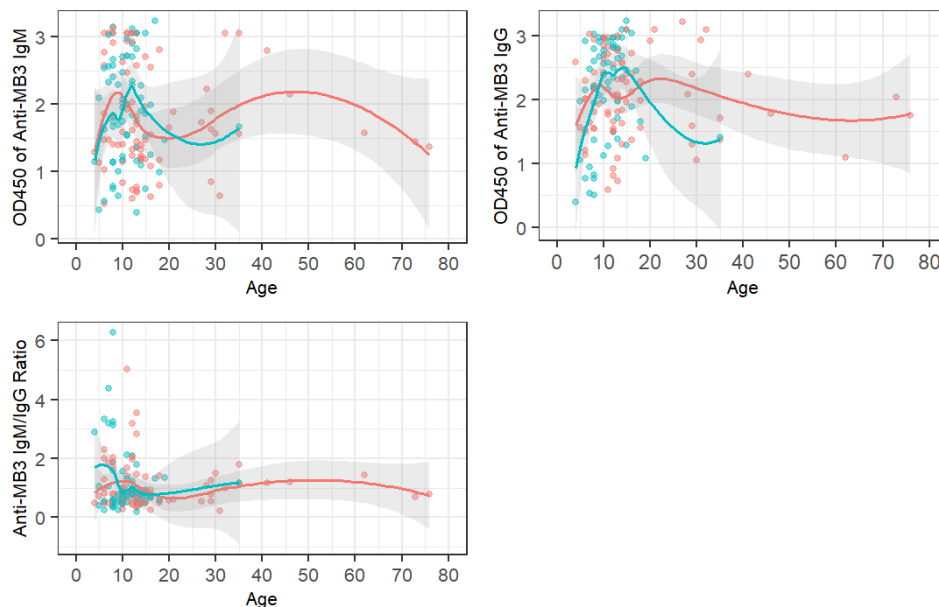


Figure 9: Antibody responses to *Mycobacterium tuberculosis* peptide MB3 in participants with schistosomiasis. Values and fitted curve of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB3 IgM in males ($N = 73$) and females ($N = 93$) with age. Top right: OD450 of anti-MB3 IgG in males ($N = 73$) and females ($N = 93$) with age. Bottom left: Anti-MB3 IgM/IgG ratio in males ($N = 73$) and females ($N = 93$).

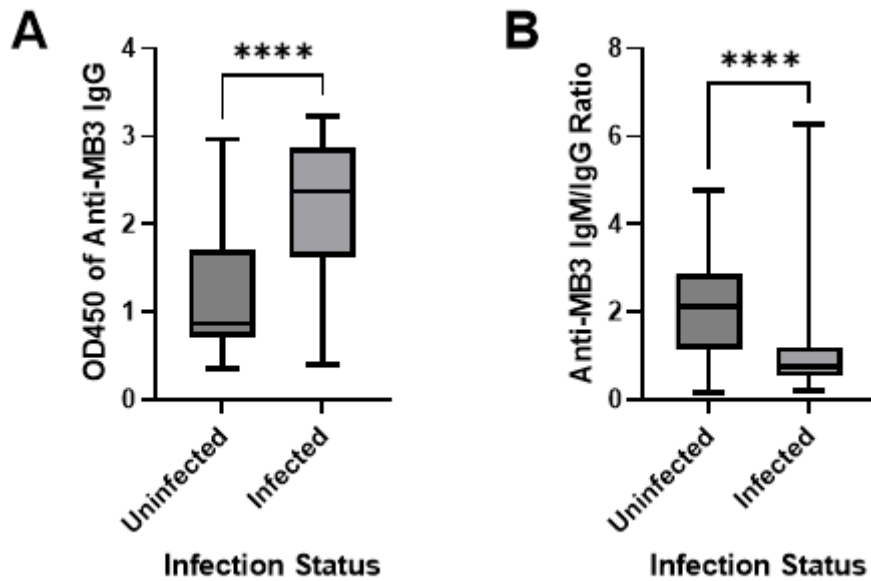


Figure 10 (a) Distribution of anti-MB3 IgG levels in males of differing schistosomiasis infection status. Infected males display significantly higher anti-MB3 IgM titres ($N = 73$, median = 2.38, IQR = 1.62, 2.87) than uninfected males ($N = 22$, median = 0.8720, IQR = 0.71, 1.71) ($W = 281$, $p < 0.0001$). **(b) Distribution of anti-MB3 IgM/IgG ratios in males of differing schistosomiasis infection status.** Uninfected males display significantly higher anti-MB3 IgG titres ($N = 21$, median = 2.14, IQR = 1.16, 2.86) than infected males ($N = 73$, median = 0.77, IQR = 0.54, 1.20) ($W = 334$, $p < 0.0001$).

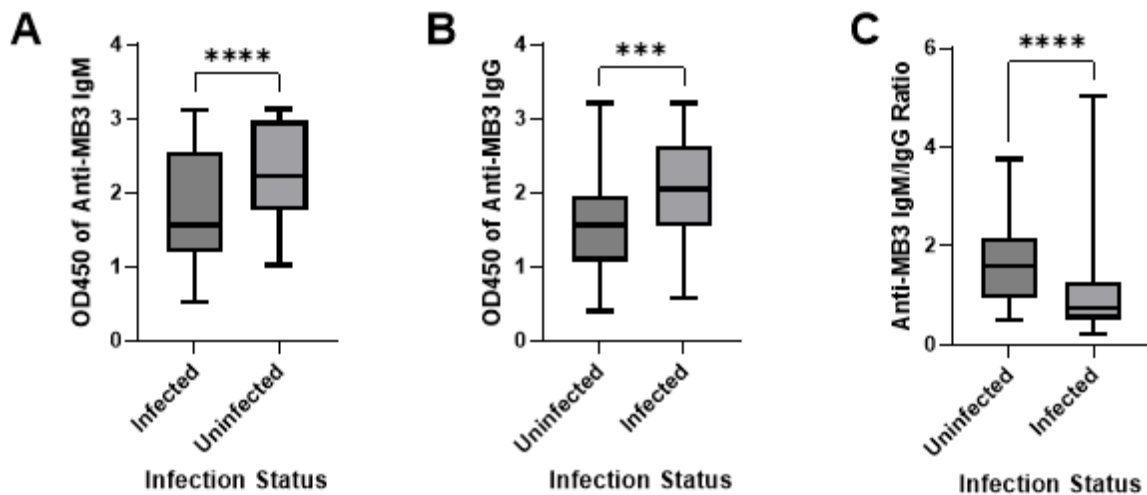


Figure 11 (a) Distribution of anti-MB3 IgM levels in females of differing schistosomiasis infection status. Uninfected females display significantly higher anti-MB3 IgM titres ($N = 55$, median = 2.23, IQR = 1.76, 2.97) than infected females ($N = 93$, median = 1.56, IQR = 1.20, 2.56) ($W = 1567$, $p < 0.0001$).

(b) Distribution of anti-MB3 IgG levels in females of differing schistosomiasis infection status.

Infected females display significantly higher anti-MB3 IgG titres ($N = 93$, median = 2.05, IQR = 1.55, 2.63) than uninfected females ($N = 55$, median = 1.56, IQR = 1.07, 1.96) ($W = 1664$, $p = 0.0003$).

(c) Distribution of anti-MB3 IgM/IgG ratios in females of differing schistosomiasis infection status.

Uninfected females display significantly higher anti-MB3 IgM/IgG ratios ($N = 55$, median = 1.57, IQR = 0.93, 2.15) than infected females ($N = 93$, median = 0.75, IQR = 0.51, 1.26) ($W = 1192$, $p < 0.0001$).

3.3.8. The Spectrum of MB4-Specific Antibody Titres

The spectrum of MB4-targeted IgM in uninfected males was distinguished by a rapid increase from 5 (1.10 AU) to 20 years old (1.95 AU) (*Figure 12*). Subsequently, anti-MB4 IgG decreased until the age of 45 (1.45 AU) and remained consistent for 10 years. Antibody titres were seen to gradually grow with age (1.55 AU). The graph depicting uninfected female IgM with age resembles the male equivalent in some respects. As with males unexposed to schistosomiasis, females expressed similarly low IgM levels in early childhood (1.25 AU), which increased until 18 years old (1.70 AU). After the peak, there was a slight reduction in IgM titres culminating in relatively static levels of approximately 1.50 AU between 30 and 50 years of age. Periods of constant IgM titres are observed across both sexes, however; this occurs sooner in females than in males. Females after 43 years old show a marked increase in antibody levels, which was greater than in healthy males, resulting in a higher final titre.

As seen with MB4-specific IgM, the smallest IgG titres in males were exhibited by young children (0.40 AU) (*Figure 12*). IgG titres proceeded to increase until reaching a peak at 25 years of age (1.60 AU). Between the ages of 40 and 52, there was a period of constant IgG levels amounting to 1.50 AU, before decreasing with age in the older participants of the study. Excluding children under 5 (0.60 AU), female IgG titres have lower IgG titres than males of equivalent ages. Female IgG levels peak earlier than males at 20 years old (1.20 AU) and there was a subsequent fall in antibody titres until 27 (1.15 AU), which was unique to the female profile. Female IgG titre increased again until 37 years old (1.35 AU), where it oscillated about this point until 52 years of age, imitating the constant period also observed in males. IgG titres in uninfected females decreased sharply after this stage, reaching an absorbance value of 1.80 AU at the age of 70. This feature was common to uninfected males, however, the titre reduction in females was greater.

Participants of this study exhibited variability in MB4-targeted antibodies which are reflected in the IgM/IgG ratio (*Figure 12*). A ratio of 3.20 is initially observed in male children under 5, which decreases until 30 years of age (1.00). From approximately 40 years old, IgM/IgG ratio increases over time, with the eldest members of the cohort reaching a ratio value of 2.10. The

male distribution of MB4-specific IgM/IgG ratio with age was similar to females, demonstrating the same fluctuations within the youngest and oldest members of the cohort in addition to the period of relative stability experienced during middle age. Although, females displayed a markedly increased ratio throughout all ages. The highest point was observed in the youngest participants (3.50) where it reduced until 37 years old (2.50). The IgM/IgG ratio experienced a constant before rising again after 50 years of age.

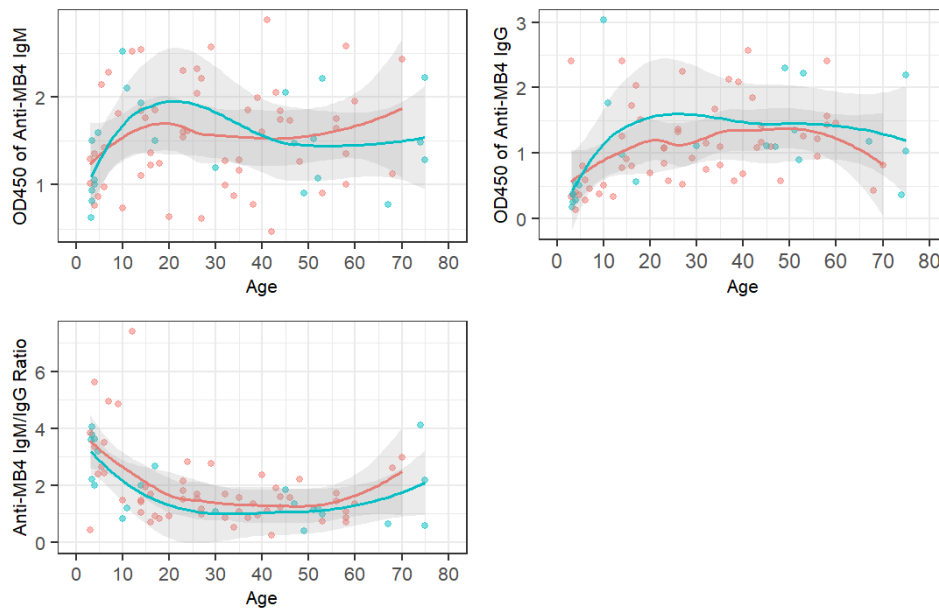


Figure 12: Antibody responses to *Mycobacterium tuberculosis* peptide MB1H37 in healthy participants. Values and fitted curve of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB4 IgM in males (N = 22) and females (N = 55) with age. Top right: OD450 of anti-MB4 IgG in males (N = 22) and females (N = 55) with age. Bottom left: Anti-MB4 IgM/IgG ratio in males (N = 22) and females (N = 55).

MB4-dependent IgM expression differs between healthy and schistosomiasis-afflicted males (Figure 13). Whilst there is a common increase in the early ages of the cohort, from the comparatively low titre of 0.80 AU at 5 years old, the peak structure differs. There are two narrow peaks forming between 9 and 15 years of age, with the highest point at 13 (1.95 AU). This differs from uninfected males, as infected males experience lower peak IgM levels at a younger age. As for females with schistosomiasis, distinction from uninfected females appears most prominent in the youngest and oldest participants of the study. Infected females showed an increase in IgM titres from 4 (1.75 AU) to 9 years old (2.00 AU). During this period, they displayed higher antibody titres than uninfected females, who expressed a comparatively delayed reduced peak titre. Conversely, the first peak IgG titre in males with schistosomiasis temporally aligned with infected females, although was associated with a lower absorbance. Nevertheless, the second peak titre observed in males reached comparable values with

infected females. Following the peak, infected female IgG levels undergo a rapid reduction until the age of 15. There is a short period where levels remain constant before the gradual decrease from 23 years old, causing the drop in IgG titres in relation to those without parasite infection. At the age of 62 (1.25 AU), this trend is slowly reversed, expressing a rise in antibody levels in later life.

Males with and without *S. haematobium* infection have overlapping trend lines between the ages of 3 (0.40 AU) and 7 (0.75 AU), expressing identical average IgG titres between the two cohorts (*Figure 13*). From the point of diversion, the titre begins to increase much more steeply prior to 10 years old (1.50 AU) where some fluctuation is experienced until 14. The IgG response of infected females is distinguished by two high titre peaks at 9 (1.40 AU) and 23 (1.45 AU). Between these peaks, there is a fall in IgG titres corresponding to 15 years of age (1.20 AU). This is where infected males undergo an increase in antibody levels, rising above levels expressed by females until adulthood where this is once more reversed. There is a subsequent decrease in the IgG titre of infected females until 42 years old resulting in lower antibody levels with respect to females without *S. haematobium* exposure. There is an elevation in antibody levels after 47 years old, which caused average titres of infected females to increase above uninfected females.

Infected males display distinct MB4-specific IgM/IgG ratios from healthy males of this cohort (*Figure 13*). Firstly, the moderate ratio of 1.50 in children under 5 is smaller than that identified in males without the disease. Instead, higher ratios are observed at two peaks appearing in quick succession at the ages of 8 (2.20) and 12 (2.10). However, these values are still lower with respect to uninfected individuals. Schistosomiasis-infected females also exhibited lower IgM/IgG with respect to their healthy counterparts. Although, like healthy females, the highest average ratio was observed in children under 5 (1.80). Similar IgM/IgG ratios were observed between the female cohorts of differing disease status until the age of 12 where infected females experience a drop in ratio value until early adulthood (1.30). Subsequently, there is a slow increase, seeing the ratio peak at the age of 40 and decline thereafter. Between the ages of 30 and 55, MB4-specific IgM/IgG ratio in females with and without the disease converge once more, demonstrating a resemblance between responses of healthy and infected individuals towards this peptide.

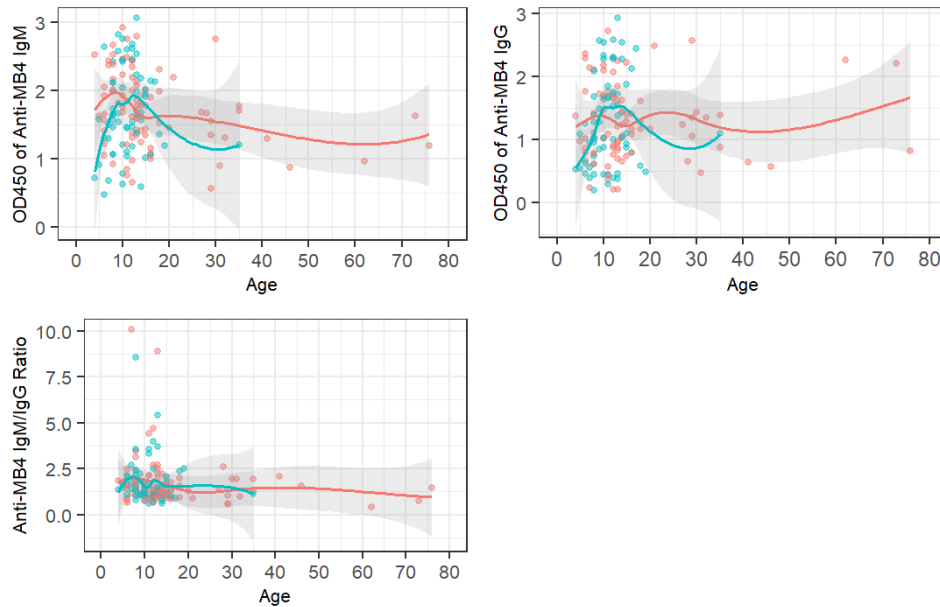


Figure 13: Antibody responses to *Mycobacterium tuberculosis* peptide MB4 in participants with schistosomiasis. Values and fitted curves of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB4 IgM in males ($N = 73$) and females ($N = 93$) with age. Top right: OD450 of anti-MB4 IgG in males ($N = 73$) and females ($N = 93$) with age. Bottom left: Anti-MB4 IgM/IgG ratio in males ($N = 73$) and females ($N = 93$).

3.3.9. The Spectrum of MB5-Specific Antibody Titres

The MB5 peptide elicits antibody responses that vary across age groups and infection statuses (Figure 14). For instance, males without schistosomiasis disease have moderate IgM titres of approximately 1.25 AU in children under 5, which slowly decreases to 1.15 AU at the age of 18. There is a rise in IgG levels with age until 44 years old, matching the titres present in children and subsequent decline when entering the elderly age group. Healthy female anti-MB5 IgM titres do not resemble the male curve. Firstly, it rises slowly between the ages of 5 (1.40 AU) and 25 (1.50 AU), where male equivalents had decreasing levels during this period. From 28 years old, females experience a fall in antibody levels until the age of 50 (1.20 AU), where again, the reverse is observed in males. From 50 years old, there is an exponential increase, achieving the highest IgM levels in elderly females (1.75 AU at 70 years old). While females generally display higher relative anti-MB5 IgM titres, the two sexes occupy a similar range captured within each other's 95% CIs.

As for MB5-directed IgG, uninfected males have low levels amounting to 0.60 AU in young children, constantly rising until the age of 40 (Figure 14). There is a peak at 1.75 AU at 47 years old, which then decreases with age. The oldest members of the cohort have exhibited a moderate titre of 1.25 AU. In the early ages of healthy females, increases in IgG levels resemble males. Females under 5 display similar titres of 0.65 AU, increasing until around 30

years old and levelling out to a near-constant titre in the range of 1.50-1.60 AU. Equivalent IgG titres are observed until profiles diverge at 30, where male titres continue to increase. That being considered, IgG levels between the sexes still operate within a similar region contained within CIs.

Males experience variable MB5-specific IgM/IgG ratios with age (*Figure 14*). This value is at a high point in the early years of this cohort (2.60) and sees a rapid decrease between 5 and 20 years old. The ratio continues to slowly decrease until the age of 45, culminating in the lowest ratio in relation to age (0.75). There is a marked increase in IgM/IgG ratio from 53 years old, however, this does not compare to that displayed by children under 5. As observed in uninfected males, the highest IgM/IgG ratio was found in children under 5 years of age (2.40) which fell quickly until 20. However, this decrease was not as consistent due to the fluctuation at 25 years old, distinguishing the female from the male response. Reduction of the ratio continued until 48, wherein the lowest ratio was observed at 0.80. After 56, healthy women displayed a rise in IgM/IgG ratio. Despite differences in IgM profile, the ratio between males and females is similar in shape and ratio values.

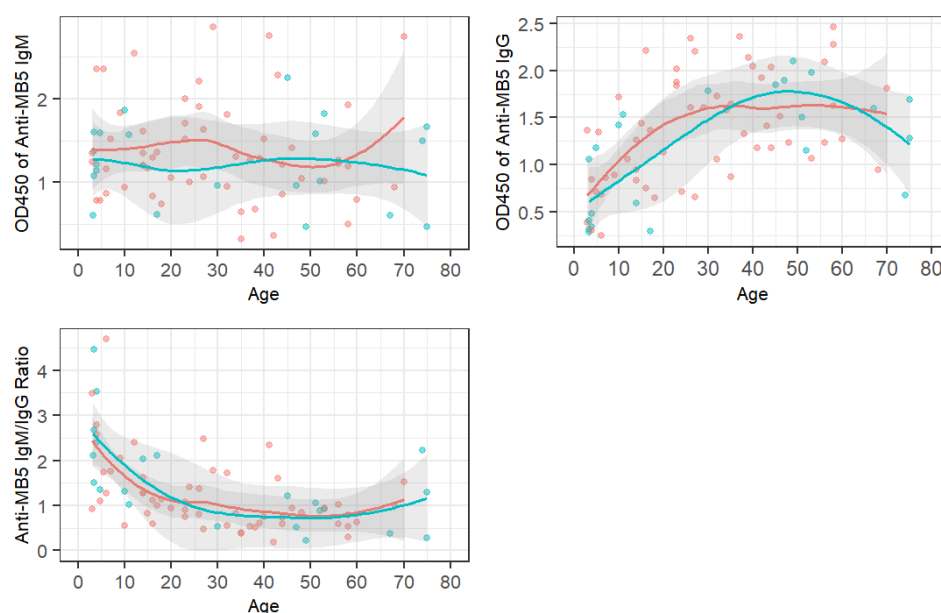


Figure 14: Antibody responses to Mycobacterium tuberculosis peptide MB5 in healthy participants. Values and fitted curve of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB5 IgM in males (N = 22) and females (N = 55) with age. Top right: OD450 of anti-MB5 IgG in males (N = 22) and females (N = 55) with age. Bottom left: Anti-MB5 IgM/IgG ratio in males (N = 22) and females (N = 55).

Males experiencing current Schistosome exposure had some differences from unexposed males in this study (*Figure 15*). The central feature of the IgM profile is the increase between the ages of 3 (1.00 AU) and 11 (1.75 AU). This is different from uninfected individuals as they

experience a small decrease in antibody levels instead. Initially, the exposed participants exhibited smaller antibody levels; however, these rose above the uninfected cohort. In females with the infection, there is an increase in antibody titres between 5 (1.50 AU) and 9 (1.80 AU). This profile is similar to males in relation to both time period and absolute antibody titre; however, this peak was not seen in uninfected females. There is a downward fluctuation at 15 years of age, experiencing similar IgM titres to those in young children, which was also not seen in the uninfected cohort. Following this, antibody levels increase slowly until the age of 32 (1.70 AU). Further variation occurred, seeing a decrease until 67 years old (1.25 AU) and IgG increase in elderly infected women. This variation took place in a similar age range to uninfected females, as well as the common increase in older participants of the study.

Males with schistosomiasis had a distinct MB5-targeted IgG profile with respect to age (*Figure 15*). The lowest levels of IgG are observed in the youngest participants (0.70 AU), resembling the levels found in uninfected individuals. However, the progress of MB5-specific IgG titres diverged soon after as schistosomiasis-positive males showed a steep elevation between the ages of 5 and 12 (1.50 AU), reaching peak levels much earlier than healthy male participants and demonstrating higher IgG activity in children. The female MB5-specific IgG response in schistosomiasis-positive individuals was characterised by two major peaks in antibody levels at the ages of 9 (1.25 AU) and 22 (1.60 AU). With respect to the male IgG response, the first peak occurred earlier and had a comparably low titre. In addition, uninfected females displayed a slower incline in IgG titres, resulting in a later peak than schistosomiasis-exposed females. Similarly, small titres of 0.90 AU were observed both in 5 and 13-year-olds, with the latter situated between the two peaks of high antibody levels. In comparison to females without the infection, schistosomiasis-positive females had higher IgG levels until the low average titre at 13. There is a gentle decline in IgG levels after the second peak which reverses direction at 49 years old, resulting in the high titres of 2.25 AU at 75 years old.

The MB5-determined IgM/IgG ratio profile of males with schistosomiasis contrasts with the ratio observed in uninfected males (*Figure 15*). Firstly, a ratio of 1.40 is observed in children under 5, which is lower than that observed in males without the parasitic infection. This value is elevated to its highest point at 7 years old (1.80), which is also smaller than the peak ratio in infected males. The ratio decreases quickly with age until the age of 10 as a result of the peaks and troughs of antibodies occurring at different points. Finally, there is an upwards fluctuation that was only present in the infected male cohort. In the early ages observed in the study, schistosomiasis-infected females exhibited similar IgM/IgG ratios to uninfected females though differing from their male counterparts. The largest ratio is in children under 5 (2.50) which begins to decrease with age from 7 years old. A low ratio of 1.25 was seen at 20, where it remains fairly constant until 48, contrasting slightly with uninfected females who experience

a ratio reduction in this time. After 48, the ratio decreases, which is dissimilar from the uninfected participants as the opposite is apparent.

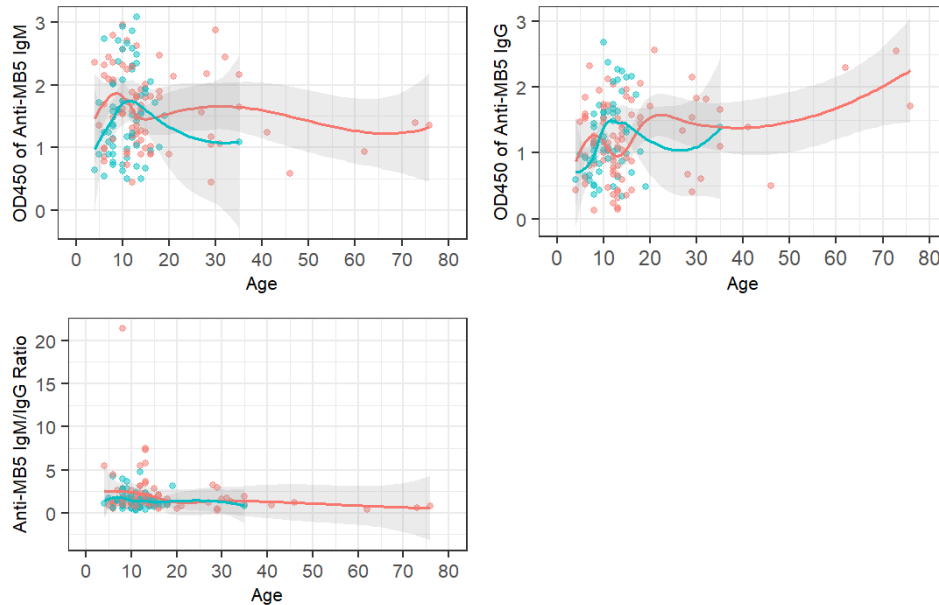


Figure 15: Antibody responses to *Mycobacterium tuberculosis* peptide MB5 in participants with schistosomiasis. Values and fitted curve of males and females are represented in blue and red respectively. Top left: OD450 of anti-MB5 IgM in males (N = 73) and females (N = 93) with age. Top right: OD450 of anti-MB5 IgG in males (N = 70) and females (N = 93) with age. Bottom left: Anti-MB5 IgM/IgG ratio in males (N = 70) and females (N = 93).

3.3.10. Correlation between Age and Peptide-Specific Antibody Responses

Peptide and Antibody	Uninfected		Infected	
	Males	Females	Males	Females
MB1H37 IgM	0.594*	0.176	-0.010	-0.071
MB1H37 IgG	-0.093	-0.225	0.104	-0.081
MB1H37 IgM/IgG	-0.621**	-0.191	-0.093	-0.001
MB2H37 IgM	-0.139	-0.124	0.123	-0.395***
MB2H37 IgG	0.587**	0.221	0.319*	0.051
MB2H37 IgM/IgG	-0.630**	-0.300*	-0.133	-0.273*
MB3 IgM	-0.014	-0.131	0.031	-0.106
MB3 IgG	0.544**	0.260	0.293*	0.021
MB3 IgM/IgG	-0.549**	-0.326*	-0.079	-0.036
MB4 IgM	0.269	0.121	0.127	-0.312**
MB4 IgG	0.564**	0.404**	0.262*	-0.027
MB4 IgM/IgG	-0.518*	-0.333*	-0.223	-0.147
MB5 IgM	-0.057	-0.101	0.139	-0.171
MB5 IgG	0.609**	0.471***	0.340**	0.159
MB5 IgM/IgG	-0.656***	-0.503***	-0.184	-0.175

Table 7: Spearman's non-parametric correlation between age and peptide-specific antibody titres or antibody ratio. Spearman's rank correlation coefficient (ρ) is indicated and significance is denoted by asterisks: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

To ascertain the variability of age with peptide-specific antibody titres, Spearman's correlation was conducted in males and females of varying infection statuses (Table 7). Regarding the cohort without parasitic infection, both sexes demonstrated statistically significant inverse correlations between age and IgM/IgG ratios in response to MB2H37, MB3, MB4 and MB5 peptides. The Spearman's rank coefficient indicated that the association between these variables was moderate in males. As for females, weak correlations were apparent in all responses excluding the MB5 peptide which was moderate. Females with schistosomiasis retained the inverse correlation between IgM/IgG ratio and age in response to the MB2H37 peptide, however, this was not observed in male equivalents. Further correlations were identified between age and IgG titres in healthy males towards peptides MB2H37, MB3, MB4 and MB5, again represented by moderate effect sizes, whereas in females, only MB4 and MB5 IgG titres were weakly associated with age. When infected by the parasite, association between age and male IgG responses were apparent for MB3, MB4 and MB5, although, females displayed no correlations between age and peptide-specific IgG. A minority of IgM responses were associated with age across all cohorts, where healthy males were only found to display moderate correlations to MB1H37-specific IgM. This was not replicated in uninfected female counterparts; however, weak correlations of contrasting directions were identified in responses to MB2H37 and MB4 peptides of schistosomiasis-positive participants.

3.3.11. Correlation between Schistosomiasis Burden and Peptide-Specific Antibody Responses

Peptide and Antibody	Males	Females
MB1H37 IgM	-0.145	-0.298***
MB1H37 IgG	-0.217*	-0.170*
MB1H37 IgM/IgG	0.170	0.048*
MB2H37 IgM	0.254*	0.302***
MB2H37 IgG	-0.114	-0.170*
MB2H37 IgM/IgG	0.217*	0.338***
MB3 IgM	-0.044	-0.179*
MB3 IgG	0.386***	0.275**
MB3 IgM/IgG	-0.350***	-0.330***
MB4 IgM	0.156	0.148
MB4 IgG	0.083	0.105
MB4 IgM/IgG	-0.070	-0.002
MB5 IgM	0.188	0.212**
MB5 IgG	-0.086	-0.200*
MB5 IgM/IgG	0.136	0.332***

Table 8: Spearman's non-parametric correlation between peptide-specific antibody titres or antibody ratio and *Schistosoma haematobium* egg count. Spearman's rank correlation coefficient (ρ) is indicated and significance is denoted by asterisks: $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$.

Spearman's non-parametric correlation was also used to determine association between the peptide-specific IgG and IgM responses and *S. haematobium* egg count, a proxy for Schistosomiasis disease burden, in males and females (Table 8). Similar correlations between MB2H37-specific IgM titres and egg count were observed in males ($\rho(19) = 0.254$, $p < 0.05$) and females ($\rho(51) = 0.302$, $p < 0.001$). Both sexes also experienced statistically significant correlations between schistosomiasis disease intensity and MB3 antibody responses. Males displayed weakly positive associations of egg count with MB3-specific IgG titre ($\rho(20) = 0.386$, $p < 0.001$) and the ratio of IgM/IgG titres against this peptide ($\rho(19) = -0.350$, $p < 0.001$). Analysis of female responses also showed evidence of a weak positive correlation between anti-MB3 IgG titre and parasite burden ($\rho(53) = 0.275$, $p < 0.01$). Contrasting with the male response, females displayed a weak negative correlation between IgM/IgG titre and *S. haematobium* egg count ($\rho(53) = -0.330$, $p < 0.001$). Further correlations between schistosomiasis intensity and peptide-specific antibody responses were unique to females, with MB1H37-targeted IgM antibody levels weakly correlating with schistosomiasis intensity ($\rho(53) = -0.298$, $p < 0.001$). IgM/IgG ratios of MB2H37 ($\rho(51) = 0.338$, $p < 0.001$) and MB5 peptides ($\rho(53) = 0.332$, $p < 0.001$) also demonstrated weak positive association disease burden. Although statistically significant, several antibody responses did not possess a sufficiently high correlation coefficient to establish an association. These included anti-MB1H37 IgG in both sexes, MB1H37 IgM/IgG, MB5 IgM and MB5 IgG in females and anti-MB2H37 IgM/IgG ratio in males. Other antibody responses showed p figures demonstrating a lack of correlation with schistosomiasis, these were also statistically insignificant. Only weak correlations were identified between antibody responses towards the five peptides and schistosomiasis disease intensity, no strong or moderate effects were shown.

3.4. Discussion

M. tb is the aetiological agent of TB, a disease which remains prevalent in Zimbabwe, justifying its place on the WHO-defined TB watch list in 2021. The BCG is the only licenced vaccine for immunisation against the disease and has been shown to have variable efficacy in different environments. In 1982, the Zimbabwean government codified access to the neonatal administration of the BCG vaccine in its Expanded Programme for Immunisation. This study aimed to characterise seroreactivity towards 5 unique peptides originating from *M. tb* in individuals from rural Zimbabwe of various ages, sexes and intensity of Schistosomiasis infections. It sought to understand this by quantifying IgG and IgM titres using ELISAs and analysing differences between these cohorts.

3.4.1. The Clinical Implications of *Mycobacterium tuberculosis*-Specific Titres

The BCG vaccine induces immune responses against *M. bovis* BCG, decreasing susceptibility to *M. tb* through immunological memory towards homologous antigens. Importantly, this means that antibody titres in this experiment did not arise as a result of BCG-induced immunity, instead signifying interaction with the pathogen itself. The sera used in these experiments originated from a Zimbabwean cohort and displayed varying levels of immunoreactivity towards the peptides, highlighting some evidence of host interaction with *M. tb*. Zimbabwe has a significant TB burden amounting to 193 infections per 100,000 people with an estimated 10-20% of the population afflicted with LTBI (Houben and Dodd, 2016), however, the prevalence of active TB and LTBI are not sufficient to explain the universality of humoral immunity markers towards *M. tb*-specific peptides.

Evidence of sensitisation by *M. tb* antigens does not uniquely implicate TB disease (Rickman et al., 2022). Instead, *M. tb*-specific IgM signifies any pathological state between current infection and disease. Similarly, *M. tb*-specific IgG can encompass an array of clinical manifestations, such as past infection and disease. For instance, immunoreactivity could arise following exposure to *M. tb* if bacilli are not rapidly cleared from the body by BCG-induced memory cells. This could explain the discrepancy between relatively low disease incidence in the context of population-wide TB sensitisation. Additionally, widespread expression of *M. tb*-specific antibodies could suggest a higher LTBI prevalence than previously estimated, herein reflecting a more accurate picture of infection in Zimbabwe. Alternatively, these communities may comprise LTBI hotspots, displaying elevated rates of infection with respect to overall disease levels countrywide. Some seroprevalence of *M. tb*-specific antibodies may be attributed to the presence of LTBI and active TB, although it is unlikely that this encompasses the majority of cases.

In summary, analysis of *M. tb*-specific titres does not discriminate between acquired *M. tb*-specific immunity, LTBI, subclinical TB and active TB. This spectrum of TB infection initiates some obstacles in the interpretation of immunological markers for TB diagnostics and BCG efficacy monitoring (Behr et al., 2019; Esmail et al., 2022, 2014). High *M. tb* antibody titre is not a determinant for vaccine failure, defined as the presence of a disease in spite of previous vaccination (Heininger et al., 2012). *M. tb*-antibody titre indicates a range of clinical presentations, where only current or past TB infection is evidence of BCG vaccine failure. Due to the range of clinical presentations represented by this metric, the study does not seek to make conclusions about *M. tb* antibodies as an indirect measure of BCG efficacy, only exposure.

3.4.2. Age- and Sex-Associated Differences of Peptide-Specific Antibody Titres in Healthy Participants

Among other isotypes, IgM antibodies are expressed earliest during primary exposure to an infectious agent. Upon secondary engagement with foreign antigens, IgM is re-expressed at lower concentrations. In this study, heterogeneity in IgM levels to *M. tb*-derived peptides was observed with age, signifying differences in recent exposure or infection to the bacteria within a BCG-vaccinated population. This variation in immunoreactivity was characterised in a cross-sectional approach towards males and females of varying ages, accounting for the effects of schistosomiasis. As such, a plurality of peptides (MB1H37, MB3 and MB4) evoked peak IgM titres in healthy males of approximately 20 years old, seeing pronounced elevation of antibody production from childhood. IgM responses were muted over time, either remaining constant after middle age (MB1H37 and MB4) or declining in the eldest members of the cohort (MB3). These patterns of immunoreactivity corresponded closely with those expressed by their female counterparts towards most epitopes (MB1H37, MB3, MB4 and MB5). In effect, IgM levels were expressed most highly in early adulthood across many of the investigated peptides, conveying recent *M. tb* exposure in these age groups. This is concordant with previous research suggesting elevated TB risk during adolescence and young adulthood which is seen to decline with age (Demkow et al., 2007). Equally, if the aforementioned hypothesis is correct, one may expect TB-protective IgM to decrease after this period of high exposure. This feature is not represented clearly by peptide-specific responses, aside from anti-MB5 IgM in males demonstrating a decisive decrease with age. Female IgM responses to most peptides (MB2H37, MB3, MB4 and MB5) oppose this conjecture through the expression of increased antibody titres with age. This may have arisen due to elevated disease susceptibility in elderly populations, increasing the likelihood of effective TB dissemination despite decreased exposure. Overall, no significant correlations were found between peptide-specific IgM and age. These results correspond with previous studies and demonstrate the high variability of IgM titres with age (Delacourt et al., 1993; Demkow et al., 2006). This heterogeneity may come about through interplay between different factors such as susceptibility, exposure and immune sensitivity within similar ages.

The youngest participants of the cohort displayed moderate IgM titres, with females exhibiting marginally higher levels of the antibody than males of the same age. This was typified by responses to the MB1H37 peptide, where females displayed significantly higher IgM titres between 3 and 6 years old. Until early adulthood, antibody levels increased in both sexes, however, this was more pronounced for males, resulting in higher peak titres. Following the expression of highest titres, male IgM levels reached similar or decreased levels in relation to female titres. Significant sex-associated differences manifested towards the MB3 peptide

between the ages of 54 and 66. When responses to this peptide were analysed across the entire cohort, females displayed significantly elevated IgM levels in comparison to males. A study carried out by Demkow and colleagues (2007) supports this observation, as *M. tb* antigen-specific IgM was found to be 1.4 times higher in females than in their male counterparts. Elevated *M. tb* immunoreactivity in females could signify sex-specific differences of the pathogen-immune system interface, prescribing changes in sensitivity. Alternatively, higher environmental exposure in females may arise from their social roles. Regardless, further research is required to ascertain the relative importance of these factors. Despite the results of previous studies, significant differences between sex-stratified IgM responses still represent the minority amongst investigated peptides. This may be due to other factors between study populations, such as differences in prior NTM exposure and TB infection status.

IgG antibodies are initially synthesised during late infection in response to a foreign antigen and are rapidly re-expressed during secondary exposure. In this regard, IgG antibodies comprise an important part of immunological memory, imbuing long-term immunity against infectious agents. Males displayed logarithmic growth of IgG titres towards numerous peptides (MB2H37, MB3 and MB5), which saw antibody levels peak during middle age and decrease somewhat in elderly participants. This demonstrates that participants are exposed to *M. tb* bacilli, inducing long-term humoral immunity, which is reactivated in response to secondary contact. The results therefore implicate that secondary exposure occurs during middle age. Subsequent falling titres may suggest reduced protective immunity in old age, due to thymic involution causing the decline in T-helper cell function and ensuing deterioration of IgG-producing B cells (Makinodan, 1980). One of the peptides (MB4) elicited an accelerated rise in male antibody levels resulting in an earlier peak IgG titre and saturation in older participants. This response displayed low variability in elder participants, with no clear reason for the discrepancy. Moreover, the trends of MB1H37-targeted IgG titres with age were distinct in both sexes, although this likely arose due to experimental errors as disproportionate antibody titres populated two OD₄₅₀ values, which is not replicated across other peptides. The female antibody spectra consisted of an IgG peak during middle age as previously observed in males, as well as an additional peak in early adulthood (MB2H37, MB3 and MB5). This biphasic spectrum suggests that females generally experience secondary exposure to *M. tb* antigens at two principal ages. As previously discussed, this may manifest due to a combination of biological or sociological factors. Despite varying patterns of antibody expression, there were no significant differences between average sex-stratified titres with age. Elevated IgG synthesis has not previously been shown to be associated with a particular sex, so this result is representative of the greater literature (Demkow et al., 2007). Overall, males displayed moderate positive correlations between age and IgG titre across all peptides, whereas females

displayed weak relationships with limited responses (MB4 and MB5). Increasing IgG with age reflects the building number of individuals who have experienced secondary exposure to *M. tb*. This result diverges from other studies, where *M. tb*-specific IgG has been inversely correlated with age (Demkow et al., 2007). It is possible that this difference has come about owing to the use of alternative mycobacterial antigens in previous investigations. Other studies often do not clarify whether the epitopes were species-specific, which introduces the risk of cross-reactivity with closely related bacteria. This is particularly important in TB diagnostics, as it reduces the capacity to distinguish between interactions with *M. bovis* BCG, NTM and *M. tb*. This study sought to address this by identifying unique *M. tb* responses, which has prevented confounding of results, however, in doing so, has reduced the ability to compare with other investigations.

3.4.3. The Effects of Schistosomiasis on Peptide-Specific Antibody Levels

The effects of schistosomiasis and other helminths on TB pathology are inconclusive with various studies implicating a beneficial (du Plessis et al., 2013), disadvantageous (Monin et al., 2015; Potian et al., 2011) or neutral effect (Hübner et al., 2012; Rafi et al., 2015). Due to the bias in TB literature towards cell-mediated immunity, the impacts of parasitic infection on humoral responses to the disease are rarely examined. This investigation addressed this research gap by examining the effects of schistosomiasis on *M. tb* immunoreactivity in a BCG-vaccinated population. Importantly, the results for schistosomiasis-positive males were affected by low sample size, considering that there is only 1 individual over the age of 20 years old. The values recorded for this individual affect the curve trajectory disproportionately; therefore, the results of this group should be interpreted with caution. As previously stated, high levels of peptide-specific IgM titres represent recent interaction of the immune system with TB-causing bacteria. All peptide-specific IgM responses were seen to peak in male children of approximately 10 years old. This is substantially earlier than observed in the uninfected cohort and implicates earlier exposure to the bacterium. Several possible interpretations can be taken from this, including schistosome-mediated reduction of vaccine efficacy or interference with *M. tb*-specific responses, promoting dissemination. Alternatively, participant behaviours such as WASH practices could influence schistosomiasis status and increase the likelihood of *M. tb* exposure. Therefore, it remains a possibility that these differences may not arise from parasite-induced immunomodulatory effects. As far as average IgM levels are concerned, no consistent effect associated with schistosomiasis infection was observed. For instance, equal numbers of peptide-specific IgM responses in schistosome-infected males displayed elevated (MB2H37 and MB5) and reduced (MB1H37 and MB3) peak titres with respect to healthy counterparts. However, these differences were only significant for limited responses (MB1H37 and MB3) during adolescence. This result differs from existing

literature on TB-helminth co-infection, which clearly depicts impaired B cell proliferation, reducing effective antibody titres (Anuradha et al., 2017).

The majority of peptides induce similar profiles of humoral immunity in females with schistosomiasis, whereby IgM levels peak in childhood, experiencing a decrease through middle age and subsequent rise in the elderly participants (MB2H37, MB4 and MB5). Differences between infected and uninfected cohorts manifested during childhood, with earlier peak IgM levels indicating that primary exposure to the bacterium is amplified in younger age groups with schistosomiasis. This difference was previously observed in males, with similar factors likely responsible, related either to schistosome-dependent immunomodulation or WASH practices affecting parasitic infection and *M. tb* exposure. Females displayed more spectra similarities during middle age, with some differences in peptide-specific IgM expression in elderly participants (MB3, MB4 and MB5). Interestingly, IgM responses of infected females were seen to increase earlier in childhood than males, although the peak IgM titres appeared to be similar between the sexes. Sex-associated differences in ages of elevated immunoreactivity may reflect distinct sources of exposure from social roles. Although, seeing that peak IgM occurs during puberty for both infected cohorts, there is a possibility that differential changes to participant endocrinology account for shifts in antibody synthesis within the context of environmental *M. tb*. As previously observed with males, there was some variability in the effects on peak IgM titre associated with schistosomiasis infection. The same peptides were seen to elicit smaller IgM levels in infected females (MB1H37 and MB3) and significant differences were found between responses from adolescence to early adulthood. Other peptides induced greater antibody expression in females with schistosomiasis (MB2H37 and MB4), which hindered straightforward analysis of parasite consequences on humoral immunity. A possible explanation for this is that relative peptide immunogenicity is different in the immunomodulated environment of schistosome-infected participants. Additionally, contrasting correlations were observed between a minority of peptide-specific IgM responses with age in schistosomiasis-positive females (MB2H37 and MB4). These were not reflected by healthy females or males with a similar infection status. The weak size, differing directions and absence of replicability of these correlations in other groups do not support a definitive effect of parasitic infection.

As previously stated, the presence of elevated IgG signifies previous or long-term exposure to *M. tb*. Males with schistosomiasis expressed the highest IgG titres to the majority of peptides at approximately 10 years old. As seen with IgM, IgG levels peaked sooner in schistosome-infected individuals, signifying earlier secondary exposure and interaction with *M. tb*. After these peak values, multiple peptide-specific IgG titres underwent an immediate decrease, suggesting that schistosomiasis affects the longevity of the immune response to the bacterium.

Most peptides induced higher antibody levels in infected males in comparison with the healthy baseline (MB2H37, MB3, MB4 and MB5), whilst the response to MB1H37 remains an outlier, where the uninfected cohort displayed higher anti-MB1H37 titres throughout. In particular, anti-MB3 IgG levels were demonstrated to be significantly higher in males with the disease between childhood and adolescence. Higher overall IgG titres may come about for a multitude of reasons pertaining to parasite infection. For instance, immunomodulation may cause higher sensitisation to other antigens, resulting in elevated IgG expression; however, this effect has not previously been reported by existing literature, making this explanation somewhat unlikely. An alternative, more probable justification for this result is higher incidences of chronic *M. tb* sensitisation or infection, of which IgG is indicative. This suggests that the altered immune system originating from schistosome infection could be associated with poorer TB outcomes, related either to reduction in BCG-induced efficacy or dampened responses that would otherwise clear *M. tb* from the body. As in the healthy cohort, age and most peptide-specific IgG titres (MB2H37, MB3, MB4 and MB5) in male participants with the parasitic infection were correlated. However, this relationship was weaker, prescribing greater antibody variability in responses of individuals with the parasitic infection. In this context, increased heterogeneity may have arisen as a result of biological differences between participants influencing the degree that immunomodulation affects humoral responses to foreign antigens.

The female IgG response was illustrated by two successive peak titres at 10 and 20 years old, trending upwards with age (MB2H37, MB4 and MB5). Females typically displayed earlier peak titres of similar magnitudes with respect to males, which is concordant with previous observations between IgG expression in healthy cohorts. This implies that schistosomiasis does not exert sex-specific immunomodulatory effects translating to the recognition of foreign antigens and subsequent antibody release. There were some distinctions between spectra of uninfected and infected female IgG levels with age, where the healthy cohort saw titres decrease in the elderly populations and experienced a comparatively delayed biphasic peak structure. It is probable that elderly participants afflicted with schistosomiasis display greater IgG levels as they experience increased susceptibility to secondary *M. tb* infection, exacerbating the effects of ageing immune systems. The responses of females with differing infection statuses display more similar titres, with substantial overlapping during childhood (MB2H37 and MB5). Some peptide-specific responses in females were seen to emulate the differences expressed by males, with infected participants exhibiting higher titres than those without the parasitic infection (MB3 and MB4). This was exemplified by the profile of anti-MB3 IgG, where schistosome-infected females displayed significantly greater average antibody levels during childhood and early adulthood. As previously described, this shift may be

associated with schistosome-affected features of the immune system, altering the capacity to mount an appropriate response.

The effect of schistosomiasis disease burden on humoral responses to *M. tb* was assessed by understanding correlations between peptide-specific antibody titre and mean schistosome egg count. All significant associations were weak and in some cases contradictory, indicating that the relationship between antibody responses and schistosomiasis is small. For instance, anti-MB3 IgG responses were correlated with schistosome burden in females, although the inverse was shown to be true in males. As for the IgM isotype, females displayed inverse correlations with the MB1H37 peptide, although both sexes were positively associated with the MB2H37 peptide. This absence of consistency between responses complicates interpretation of the effects of schistosomiasis burden on *M. tb* exposure, thus, no association between these variables can be defined.

3.4.4. Study Limitations

Over the course of this investigation, some limitations became apparent within the study design, methodologies and analysis. Considering these, some caution should be exercised in the analysis of results and subsequent conclusions. Firstly, there was an absence of appropriate positive and negative controls for ELISAs detecting peptide-specific antibody titres. As defined under section 4.2 of this report, the contents of pooled controls were determined by the results of a microarray screen, which quantified sample immunoreactivity to the peptides. The positive and negative controls were composed of these analysed samples with high and zero immunoreactivity respectively. However, during ELISA optimisation, the assumed negative control had consistently high absorbance, exceeding both the positive control and pooled study samples for all peptide-specific antibody responses. From the outset, this suggests that the control was not truly negative. This phenomenon has been observed by former P.I.G. members in the analysis of other peptides, therefore, it is appropriate to appraise the sensitivity of the microarray and subsequent validity of using control samples from this study (Hanton, unpublished; Minter, unpublished; Parkinson, unpublished). This discrepancy could be attributed to the differences in sera dilution used between the microarray (1:500) and ELISAs (1:100) for IgG quantification. The higher sera concentrations in ELISAs may have led to the underestimation of detected *M. tb*-antibody titres. However, evidence from this study disputes this as high dilution factors of 1:800 (MB2H37) and 1:500 was also used (MB1H37, MB3, MB4 and MB5), owing to high IgM titres observed during optimisation. This indicates that there is some degree of non-specific binding, causing assay wells to retain other serum components. In particular, uncharacterised antibodies may be present in high concentrations, forming low-affinity complexes with the antigen. Evidence from previous investigations

suggest that this possibility is especially pertinent for IgM responses, where the isotype's high avidity permits association with weakly cross-reactive antigens (Jayasekera, Moseman and Carroll, 2007). This effect may have been enhanced by additives in lyophilised peptides, introduced for preservation purposes, altering the anticipated chemical environment of the assay. This may have increased background noise in OD₄₅₀ readings, making it difficult to differentiate between true positive and negative samples.

This complication has had ramifications on the generation of cut-off points, as most standard methods require values from known negative controls (Lardeux et al., 2016), limiting the reliability of experimental interpretations as samples were not conclusively determined as *M. tb*-sensitised through comparison of antibody titres with an unexposed individual. Although controls were not used to define assay cut-off points, they were successfully employed as a validation measure for consistency between 96-well plates surveying matching peptide-specific antibody responses.

Secondly, this study did not identify absolute antibody titres which limited comparison across experimental assays and existing literature. Standard curves can be used to establish the relationship between absolute absorbance at 450 nm measured in the assays and antibody concentration, however, these were not accounted for in the study design. This method is not consistently utilised in clinical contexts, where measures of AU/ml are equally reported, although, this would not be appropriate for these experiments given the differences in reagent concentrations between specified peptide-antibody assays. Therefore, knowledge of assay titres in µg/ml would facilitate analysis by allowing comparisons between antibody responses for the *M. tb*-derived peptides, which was unviable with this experimental design. It additionally presents complications for comparisons with replication studies required for diagnostic validation.

Thirdly, relevant information on participants was excluded from the field records since sample collection took place prior to the creation of this project. Specifically, there was an absence of vaccination data and paucity of clinical observations detailing history of TB, NTM or schistosomiasis exposure and disease. Evidence of BCG administration would have also been appropriate, given that it exerts variable effects on *M. tb* immunoreactivity. It was assumed throughout analysis that all participants were BCG-vaccinated, however, this may not be accurate considering that Zimbabwean BCG coverage only extends to 88% of eligible persons (World Health Organisation, 2021). Whilst estimations of the unvaccinated population may be deflated due to underreporting, a proportion can be attributed to vaccine-hesitant groups. Notably, followers of the Apostolic Church of Zimbabwe are largely opposed to vaccination and thereby abstain from the practice. This denomination is influential in the region where

participants were recruited, therefore, it is likely that some will be unvaccinated. Mixed vaccination status will presumably contribute to greater heterogeneity in profiles of humoral immunity and undermines conclusions on vaccine efficacy.

Moreover, supplemental information on prior TB exposure or infection would have allowed assessment of associated immunoreactivity levels in comparison with presumed unexposed individuals. This would elucidate the relationship between immune responses to *M. tb* and disease incidence stemming from heterogeneity of vaccine-induced protection. Difficulties assessing prior NTM exposure are common to TB research due to the absence of specific diagnostics. Recent contributions identifying distinct NTM antigens demonstrate the capacity to distinguish this group from *M. tb* and *M. bovis* BCG-associated markers.

Furthermore, the conclusions of this study are limited, given the exclusive characterisation of current schistosome infection on BCG-induced humoral responses. Although beyond the scope of this study, schistosome exposure can take place *in utero*, during the development of BCG-induced immunity, prior or during *M. tb*-specific immune responses. Research suggests that gestational antigen exposure has lasting immunological effects on protective vaccine induced immunity. Patients may also contract schistosomiasis during immune activation by *M. tb* affecting mounting IgM responses or longevity of IgG protection. Moreover, the nature of exposure may also differ depending on whether chronic, acute or recurring infection occurs. Patients may exhibit different effects on the immune response depending on when infection took place. The results of this study therefore do not categorically define the range of TB-associated outcomes influenced by schistosomiasis. Given the time and resources, a longitudinal study should be carried out to understand the effects of exposure and cure on *M. tb* antibody titres.

3.5. Conclusions and Future Directions

This study has successfully analysed IgG and IgM seroreactivity towards *M. tb*-specific peptides in a Zimbabwean cohort, elucidating differences between males and females of various ages and Schistosomiasis status. The heterogeneous nature of population antibody titres has been classified across five immunogenic antigens of different origin proteins, displaying the diversity of humoral responses that take place against a single pathogen. This study identified sex-associated differences in *M. tb*-specific IgM expression with age, where average female IgM titres peaked marginally higher at a younger age than their male counterparts. Differences were also identified in IgG expression, demonstrated by a biphasic spectrum in females, seeing antibody levels peak twice during adulthood. By contrast, males experienced one peak titre in middle age. The presence of schistosome infection was correlated with some changes in antibody release, such as peak titre expression in younger

age groups and increase in IgG titres. However, it exerted inconsistent effects on magnitude of IgM responses. In contrast, schistosomiasis disease burden was not found to be strongly correlated with differences in IgG or IgM antibodies. This investigation contributes to the recent interest in antibody responses within the TB research community, opening new avenues of study for *M. tb* diagnostics. Importantly, this study has used samples from a previously unexamined population in TB research and therefore addresses the paucity of disease surveillance in a high TB-burden country.

This study understands the techniques used here in the context of its potential as a diagnostic marker and therefore requires additional research prior to considering implementation. Firstly, there is a need for validation of peptide-specific antibody responses for clinical relevance. This would involve longitudinal research into the *M. tb*-induced humoral responses of the study population, with associated details of BCG vaccination, previous or current TB infection, known exposure to confirmed TB patients and results of comparative diagnostics. For a greater understanding of TB infection during immune system development, it would be beneficial to include sera from neonates and children under 3 in future studies. Secondly, cross-reactivity should be assessed between analysed *M. tb* peptides and homologous Mycobacterial equivalents. This would identify whether other sources of infection such as NTM are interfering with the interpretation of *M. tb* exposure in the study population. Thirdly, additional antibody responses should be analysed to better capture the diverse range of immunogenic *M. tb* components.

4. Point of Care Tests: Sociological Perspectives

4.1. Introduction

4.1.1. The COVID-19 Epidemic in Zimbabwe

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is the causal agent of COVID-19 and has resulted in widespread mortality and morbidity worldwide (Kavenga et al., 2021; Mashe et al., 2021). COVID-19 emerged as a zoonotic disease in December 2019 in Wuhan, China and the outbreak was granted pandemic status on 11th March 2020. The WHO currently estimates the death toll at 6.58 million and the number of cases at 628 million, with Zimbabwe having declared a total of 5,606 deaths. When this value is expressed as a function of 100,000 people, Zimbabwe's COVID-19 morbidity is comparatively smaller than that of LMICs but higher than reported by the WHO African region (see *table 9*).

Region	Total Cases	Total Cases Per 100,000 Population	Total Deaths	Total Deaths Per 100,000 Population
Global	623,893,894	8,004.24	6,553,936	84.08
LMIC	81,687,011	30,023.37	1,029,957	294.45
Africa	9,345,860	833.01	174,637	15.57
Zimbabwe	257,893	1,735.14	5,606	37.72

Table 9: Recorded cases and deaths due to COVID-19 experienced worldwide, by World Bank classified LMICs, by the African WHO region and by Zimbabwe. (WHO, 2021)

The COVID-19 Epidemic in Zimbabwe

Zimbabwe's COVID-19 epidemic has been characterised by four distinct waves, associated with different SARS-CoV-2 variants. The index COVID-19 case in Zimbabwe was detected on the 20th March 2020, which preceded the first wave, culminating in over 8000 cases by October 2020 (Kavenga et al., 2021). The second wave took place in January 2021 and was driven by SARS-CoV-2 (Beta) variant B.1.351. Unlike the previous wave, the majority of these cases were a result of local transmission, instead of originating abroad and were responsible for approximately 10,000 cases at the peak (Mashe *et al.*, 2021; Murewanhema & Mutsigiri-Murewanhema, 2021; Murewanhema *et al.*, 2021). The third wave in Zimbabwe was associated with the importation of the B.1.617.2 (Delta) variant and peaked in July 2021 with 120,000 cumulative cases (Fryatt *et al.*, 2021; Murewanhema & Mutsigiri-Murewanhema, 2021). Finally, the fourth wave was initiated by B.1.1.529 (Omicron), which saw a dramatic increase in the rate of transmission. This was accountable for the largest number of cases, equating to 4031 daily cases at the peak in December 2021 (Sochas et al., 2017).

4.1.2. Control Measures in Zimbabwe

From the onset of the pandemic, there was a lack of information on this novel pathogen with an absence of appropriate vaccines and anti-viral drugs. Thus, precautionary measures were adopted to mitigate the negative impacts that high COVID-19 burden exerts on health systems and individuals. Zimbabwe followed the WHO's advice on pandemic control mechanisms, which were predicated on reducing SARS-CoV-2 transmission. This advice was seen to be particularly important in the context of Zimbabwe's health system which was previously deemed unprepared for a pandemic in a global context. This was highlighted by Oppenheim *et al.* (2019) who incorporated factors such as public health infrastructure, capacity and financial access to generate an Epidemic Preparedness Index (EPI). Zimbabwe's EPI was determined to be 4, where scores 1 and 5 are most and least prepared respectively (Oppenheim et al., 2019).

To tackle the pandemic threat in the context of a fragile health system, the Zimbabwean government established the COVID-19 National Preparedness and Response Plan in March 2020, imminently restricting non-essential movement (Price, 2020). Zimbabwe then announced a state of disaster on the 27th March 2020, followed shortly by a 21-day lockdown instigated on the 30th March to reduce the effects caused by high SARS-CoV-2 transmission ("BREAKING," 2020). During this period, Zimbabwean borders were shut to international travellers in order to limit the number of index cases coming from overseas. Zimbabwe then made use of a tiered approach to COVID-19, with levels 1 to 4 expressing different levels of severity and subsequent restrictions. Level 1 represented low alert and permitted the majority of normal activity provided that governmental advice was followed. Alert level was increased with tier 2 which implemented social distancing to reduce transmission, whilst allowing some work and socialising to take place. Level 3 was seen to introduce greater restrictions on most activities and level 4 communicated a severe public health threat due to high levels of transmission, informing strict containment measures (Chitungo et al., 2022). The first time these tiers were used was on the 17th of May 2020, where Level 2 interventions were implemented. On the 22nd July 2020, further measures were used to combat the first wave. These included the introduction of a curfew (from 6PM to 6AM), school closures and gatherings prohibited (Moyo, 2020). The second and third waves prompted the introduction of level 4 measures, however, the third wave permitted vaccinated individuals to return to work (Zinyuke, 2021). In the period between waves of infection, level 2 measures were maintained, with some targeted interventions in regions of high disease burden (Mutsaka, 2021; Xinhua, 2021a). The fourth wave enforced polymerase chain reaction (PCR) tests and quarantine for anyone entering the country and reintroduced the curfew. Vaccination was also required to access public venues such as restaurants and places of worship (Xinhua, 2021b).

4.1.3. COVID-19 Testing

Zimbabwe initially made use of pre-existing surveillance infrastructure in the public sector, created for TB and HIV testing. These were supplemented with private laboratories, using a mixture of PCR and lateral flow tests (LFTs) for disease confirmation (Chitungo et al., 2022). As of October 2022, 2,529,087 tests have been conducted in a population of 15.38 million people (Africa CDC, 2022). This means that only a small proportion of the population has previously been tested for COVID-19 which undermines the reliability of reported cases. Fryatt *et al.* (2021) investigated previous infection through detection of antibodies targeting the SARS-CoV-2 nucleocapsid. The number of individuals possessing antibodies indicative of past infection in the three districts of Harare was found to be greater than the total recorded infections within Harare (Fryatt et al., 2021).

4.1.4. COVID-19 Vaccinations

Vaccination against COVID-19 presents an important preventative intervention, which has been shown to reduce transmission, incidence of severe disease and death. The Zimbabwean vaccination programme began on the 18th February 2021, targeting healthcare personnel and elderly people (Banya, 2021; World Health Organisation, n.d.). The programme was extended sequentially to people with chronic conditions and key workers in essential professions (Murewanhema *et al.*, 2021). Remaining adults were then invited to be vaccinated after these prioritised groups (Murewanhema *et al.*, 2022). Vaccination rates were initially low due to safety and efficacy concerns in addition to poor government confidence (Mundagowa *et al.*, 2022), however, they have improved due to targeted community engagement. The Apostolic Women's Empowerment Trust (AWET) was particularly successful as it approached faith and community leaders to address vaccine hesitancy. This was important in creating COVID-19 awareness because adherents to the Apostolic Church of Zimbabwe have previously been opposed to vaccination and amount to a significant proportion of the population (Machekanyanga *et al.*, 2017). 31.3% of Zimbabwean adults are fully vaccinated due to these efforts, with 40.3% of the population having received one dose. This comprises a total of 12,222,754 administered vaccines as of October 2021 (Mathieu *et al.*, 2020). The vaccine rollout continued to experience difficulties because of an unexpected surge in uptake during the third wave, leading to shortages (Chitungo *et al.*, 2022). After this wave was surpassed, vaccine complacency took hold, resulting in reduced demand (Bulawayo 24 News, 2021).

4.1.5. Harms Resulting From COVID-19 Measures

The effect of successive lockdowns on Zimbabwean livelihoods has been significant and wide-ranging, including de-prioritisation of non-COVID-19-related healthcare, disrupted education and reduction in household income. The COVID-19 pandemic impacted delivery of other important healthcare interventions, increasing morbidity and mortality in these diseases (Chitungo *et al.*, 2022; Murewanhema and Makurumidze, 2020). The streamlined focus on the COVID-19 response resulted in the interruption of non-emergency medical care. For example, one study showed that 19% of HIV-positive patients were unable to receive refills of their medication in April 2020 (Jewell *et al.*, 2020). In addition to treatment, essential screening services were de-prioritised (Gavi *et al.*, 2021) as a result of pivoting TB and HIV testing equipment towards SARS-CoV-2 diagnostics (Mukwenha *et al.*, 2020) and there was a reduction in delivery of maternal and neonatal care (Bikwa *et al.*, 2021).

Education of Zimbabwean children was severely impacted by the COVID-19 pandemic due to extended periods of school closure. This policy took force in March 2020, remaining in place until September 2020. Schools opened at a reduced capacity during October 2020 ahead of

a full return in November and December 2020 (Rwezuva et al., 2021). From the outset, there were concerns in how restrictions on primary school education threatened the progress of literacy levels in the country (Moyo, 2021). To tackle this disruption, the Ministry of Primary and Secondary Education launched an online curriculum, however, a substantial proportion of the population did not have sufficient access to appropriate equipment or internet access, exacerbating differences in attainment between different socioeconomic groups (Chitungo et al., 2022; Hove and Dube, 2021). This was demonstrated by the percentage coverage of the programme, reaching 40% and 9% of children in urban and rural areas respectively (The World Bank, 2021). In addition, elite private schools had the capabilities to deliver education online, whereas government schools did not possess the resources to do so (Hove and Dube, 2021).

Finally, the population of Zimbabwe experienced a decline in disposable income over the course of the pandemic (Madhi et al., 2020). Household earnings were affected particularly badly since a high proportion of Zimbabwe's workforce is employed in the informal sector (Small, 2020). As such, many people rely on daily payment and are not in possession of savings (Chitungo et al., 2022). Although limited fiscal support was available for the worst affected, uptake was low as the programmes did not provide sufficient aid, culminating in reduced living standards for Zimbabweans (Chitungo et al., 2022).

4.1.6. Ability to Comply with Restrictions

Lockdown and social distancing guidance interfered with the ability of communities from low socioeconomic backgrounds to meet their basic needs. Firstly, some individuals do not have piped water and rely on community boreholes for access to clean water, necessitating daily movement from the household (Mackworth-Young et al., 2021). As it is a shared facility, exposure to others when accessing the borehole is likely, causing an antagonism between necessary daily practices and public health advice. Secondly, many do not possess savings, meaning that individuals may have to buy food on a daily basis, increasing contact with others from the community (Mackworth-Young et al., 2021). Moreover, financial insecurity incentivised people to forgo public health advice in favour of employment-assured stability. Due to economic factors, many people were unable to fully comply with the guidelines, which undermined the effects of implemented interventions, resulting in elevated transmission.

4.1.7. The Importance of COVID-19 Surveillance, Disadvantages of Centralised Testing and Benefits of Rapid POC Tests

Improved surveillance can be used to detect clusters of disease when they occur, allowing effective contact tracing to take place. This can inform targeted responses to reduce transmission, removing the necessity for harsh public health interventions. The knowledge of

COVID-19 infection status would enable individuals to isolate when appropriate, whilst allowing the rest of the population to carry out normal socioeconomic activity necessary to sustain their livelihoods and future prospects. Evidently this would require routine testing, which would have been unsustainable with the Zimbabwean testing capacity at the onset of the pandemic where PCR was exclusively used. PCR tests were regarded as the diagnostic gold standard for the detection of SARS-CoV-2 infection as they possess the highest degree of specificity and sensitivity among other methods. However, diagnostic PCR requires laboratory infrastructure and trained personnel, both of which were only accessible in secondary healthcare settings and above. Zimbabwe initially used a centralised approach, carrying out all COVID-19 at the National Microbiology Reference Laboratory (NMRL). This facility had limited personnel, which resulted in staff burnout and excessive turnaround times between 48 hours to over a month (Sochas et al., 2017). Testing was particularly inaccessible to the majority of Zimbabweans (68%) inhabiting rural areas, reinforcing the existing socioeconomic divide (Piot et al., 2019). These factors meant that the Zimbabwean testing capacity was not fit-for-purpose and did not address the requirements of community public health.

As of September 2020, the WHO supported the use of rapid POC tests to address many of the associated problems with PCR diagnostics (Mackworth-Young et al., 2021; Viceisza et al., 2020). These tests were cost effective and required less specialist equipment, allowing decentralisation of COVID-19 testing (Mackworth-Young et al., 2021). This has placed greater responsibility for testing on health workers in primary healthcare settings, democratising access and improving equitability in rural areas. In addition, LFTs reported results 30 minutes after testing, decreasing turnaround times to support improved surveillance and overall increased capacity (Mackworth-Young et al., 2021). Widespread access to LFTs in primary healthcare settings for COVID-19 has reduced the burden on national testing facilities and reinstituted capacity at the NMRL for HIV and TB diagnostics. That being considered, COVID-19 LFTs are administered exclusively by healthcare professionals and are not distributed for personal use within the community. In the context of further COVID-19 waves and future pandemics, it therefore pertinent to understand whether testing uptake can be improved with greater personal access to LFTs.

4.1.8. Study Aims and Objectives

Increased SARS-CoV-2 testing is important to reduce the necessity for future lockdowns and their subsequent impact on the Zimbabwean economy. LFTs are currently unavailable for personal use in the country, which could be limiting uptake. To understand whether implementation of self-testing would improve surveillance, the following study addressed

community attitudes and abilities to conduct self-testing for COVID-19 in a rural low-resource setting which previously comprised a research gap. This study was conducted at the Mupfure clinic, located in the Shamva district, Mashonaland Central Province, Zimbabwe. This clinic is responsible for providing primary healthcare to the population living in the catchment area encompassing a 7 km radius. The aims of the study were as follows:

- (i) to determine the prevalence of COVID-19 in the community served by the Mupfure clinic using lateral flow tests;
- (ii) to assess the community's enthusiasm to adopt routine self-testing for COVID-19;
- (iii) to assess the ability of the community to carry out LFTs for COVID-19 self-diagnosis;
- (iv) to determine whether participants were able to complete the appropriate steps following positive test results;
- (v) to understand the relationship of broader social behaviours to COVID-19 and demographic information.

4.2. Materials and Methods

4.2.1. Participant Recruitment and Community Self-Testing

Nine Village Health Workers (VHWs) were trained by the nurse in charge to test themselves for COVID-19 using the UNscience SARS-CoV-2 Antigen Rapid Test Kit (Wuhan UNscience Biotechnology Co., Ltd). Instructions for the test were conveyed in the local language, Shona, in accordance with the method described in the tests attached leaflet. The importance of good health and safety practice was emphasised, such as the safe disposal of the nasopharyngeal swab. The test was observed after developing for 10-15 minutes and the significance of the control and test lines were explained. The VHWs then carried out personal LFTs and the responsible nurse observed that these were being carried out correctly.

The VHWs recruited members of the community served by the Mupfure clinic. The community members were informed of the study aims in Shona and gave informed consent to be involved in the investigation. They were permitted to withdraw their consent at any stage of the process. Participants were assigned a unique study identification number to retain anonymity during analysis.

The study population attended the Mupfure clinic and they were taught how to use LFTs. They received individual instructions from the VHWs, allowing them to ask any questions they may have about LFTs and confirming that the test was being performed correctly. As described above, health and safety were emphasised and the test results were recorded after a brief incubation period. The test result was verified by the visibility of the control line.

4.2.2. Questionnaire on Social Behaviours Pertaining to COVID-19

After conducting personal LFTs, the participants then took part in a survey. The questionnaire was composed in English and translated into Shona; the participants were given the choice of their preferred language. The survey recorded the following demographic indicators: sex, marital status, occupation, religion, household size, source of drinking water, medical conditions, height and weight. COVID-19-associated symptoms experienced on the day of the survey and the previous two months were included. In addition, insights into COVID-19-associated behaviours were captured in the survey questions, such as self-isolation and mask-wearing. Quantitative and qualitative information was obtained using fixed choice and open-ended responses respectively. The questionnaire was conducted by four individuals, two of whom were native Shona speakers. The non-Shona speakers were aided by translators, both of whom were VHWs fluent in the language. Details of the full questionnaire are included in the appendix.

4.2.3. Data Analysis

Data was entered in Microsoft Excel and analysed using R v.4.2.1, with the Tidyverse packages for data analysis and visualisation. Figures were produced using GraphPad Prism v. 8.2.0. Descriptive analyses were carried out on data generated by the survey and presented as frequencies and percentages of responses. The Fisher's Exact Test was used to assess the independence of categorical variables, in order to identify factors affecting willingness to self-test.

4.3. Results

4.3.1. Participant Demographics

A total of 120 participants were recruited to this study by VHWs, from surrounding villages served by the Mupfure clinic. Of these individuals, 99 (82.5%) were female and 21 (17.5%) were male. A large proportion of participants were farmers (68.3%), with the remainder working as homemakers (7.5%), paid employees (6.7%), piece workers (4.2%), students (4.2%), vendors (5.0%) or VHWs (3.3%). Followers of the Apostolic faith (76.7%) comprised the majority of respondents, although followers of other religious denominations such as Pentecostal (19.2%) or Mainline Church (2.5%) were also present in the community. A minority (1.7%) of the community were not religious. Participants received clean water from various sources such as community borehole (66.7%), piped household water (24.2%), well (5.8%) and household borehole (3.3%).

4.3.2. COVID-19 Symptoms and Responses

Over half (59.2%) of survey respondents had experienced at least one COVID-19 related symptoms such as fever, dry cough, fatigue, aches and pains, nasal congestion, running nose, sore throat, diarrhoea and loss of smell or taste. Full details of the symptoms are outlined in the Appendix. Those with symptoms were asked an open-ended question on their response to these symptoms. A high proportion (55.1%) recalled that they attended a clinic or hospital for medication to address their symptoms. Some reported using the home remedy of “steaming” involving the steam inhalation of the *Lippia javanica* (zumbani) leaves, which is thought to possess anti-inflammatory properties (Mfengu et al., 2021). Sometimes “steaming” was used in conjunction with visits to the clinic (2.9%), however, more people performed this exclusively (15.9%). Some individuals accessed medicine such as painkillers at home (8.7%), with a minority (8.7%) not taking any steps to improve their symptoms.

On the days that the survey was performed, most individuals (57.5%) did not suffer from any symptoms. As with those who had previous symptoms within the past 2 months, there was some diversity in response to this question. The majority (66.7%) were coping with their symptoms without further intervention, although a substantial minority stated that they were seeking medical care at the clinic (19.6%). 2 individuals (3.9%) each relayed that they were self-isolating or using home remedies. 1 person (2.0%) informed that they had been steaming and had come to seek help from the clinic. A small number of respondents reported that they had only sought religious assistance (2.0%), with others using this in combination with hospital assistance (2.0%).

4.3.3. COVID-19 Related Behaviours

Behaviours which were relevant to SARS-CoV-2 transmission were assessed in the questionnaire. These behaviours included presence at a crowded place, frequency of mask wearing in public, frequency of covering the mouth whilst coughing, whether they took part in COVID-19 testing and the number of vaccines they had received against the virus. The results of these questions are recorded in table 10.

The participants who had chosen to abstain from vaccination were prompted to explain their reasons behind their decision. 1 individual believed that they were not at risk of catching the virus because they were always at home, having limited contact with others for transmission to take place. Another was pregnant at the time of vaccination and believed that the COVID-19 vaccine was not appropriate for them.

Behaviour	Frequency	Percentage (%) ($\pm 95\%$ CI)
Presence At a Crowded Place		
Yes	74	61.7 (52.4-70.4)
No	46	38.3 (29.6-47.7)
Frequency of Handwashing (Times Per Day)		
Never	1	0.8 (0.0-4.6)
1 Time Per Day	8	6.7 (2.9-12.7)
2-3 Times Per Day	58	48.3 (39.1-57.6)
>5 Times Per Day	34	28.3 (20.5-37.3)
After Contact	19	15.8 (9.8-23.6)
Frequency of Mask Wearing in Public		
Never	26	21.7 (14.7-30.1)
Sometimes	28	23.3 (16.1-31.9)
Always	65	54.2 (44.8-63.3)
Declined To Respond	1	0.8 (0.0-4.6)
Frequency of Covering Mouth When Coughing		
Never	2	1.7 (0.2-5.9)
Sometimes	16	13.3 (7.8-20.8)
Always	101	84.2 (76.4-90.2)
Declined To Respond	1	0.8 (0.0-4.6)
Number of Vaccines Received For COVID-19		
None	13	10.8 (5.9-17.8)
One	4	3.3 (0.9-8.3)
Two	52	43.3 (34.3-52.7)
Three	51	42.5 (33.5-51.9)

Table 10: Behaviours relevant to SARS-CoV-2 Transmission

4.3.4. Previous Use of Rapid Tests for the Diagnosis of Infectious Diseases

The community was assessed for the prior use of rapid tests for other diseases. Of this cohort, 4 individuals had previously conducted rapid tests for multiple conditions, with 1 having taken all tests and 2 each having undergone testing for malaria and HIV or hypertension. A proportion of 75.0% had a preference for the malaria test with the remainder favouring the HIV diagnostic.

Condition	Previously Tested		Not Previously Tested	
	Frequency	Percentage (%) ($\pm 95\%$ CI)	Frequency	Percentage (%) ($\pm 95\%$ CI)
Malaria	13	10.8 (5.9-17.8)	107	89.2 (82.2-94.1)
HIV/AIDS	14	11.7 (6.5-18.8)	106	88.3 (81.2-93.5)
Hypertension	11	9.2 (4.7-15.8)	109	90.8 (84.2-95.3)
Diabetes	1	0.8 (0.0-4.6)	119	99.2 (95.4-100.0)
Any Condition	33	27.5 (19.8-36.4)	87	72.5 (63.3-80.3)

Table 11: Rapid point of care testing for infectious disease that has taken place in the study population prior to this survey.

4.3.5. Previous SARS-CoV-2 Testing

Further questions were asked of individuals to ascertain the level of SARS-CoV-2 testing in the community. As shown in *table 12*, most individuals had been tested previously. These individuals were asked to recall the type of testing they had undergone to receive their diagnosis. Participants relayed that they had tested for SARS-CoV-2 using antibody detection assays (ADA), LFTs and PCR tests. In most cases, individuals were not aware of the specific test they had received, only that the test had used a nasopharyngeal swab.

Prior SARS-CoV-2 Testing	Frequency	Percentage (%) ($\pm 95\%$ CI)
Previously Tested For SARS-CoV-2		
Yes	95	79.2 (70.8-86.0)
No	25	20.8 (14.0-29.2)
Type of COVID-19 Test		
ADA	3	3.2 (0.6-9.0)
LFT	2	2.1 (0.3-7.4)
NP	68	71.6 (61.4-80.4)
NP and ADA	21	22.1 (14.2-31.8)
PCR	1	1.1 (0.0-5.7)

Table 12: SARS-CoV-2 testing that has taken place in the study population prior to this survey. Abbreviations of COVID-19 test types are as follows: ADA = antigen detection assay, LFT = lateral flow test, NP = unspecified test using a nasopharyngeal swab, PCR = polymerase chain reaction.

4.3.6. Community Prevalence of COVID-19

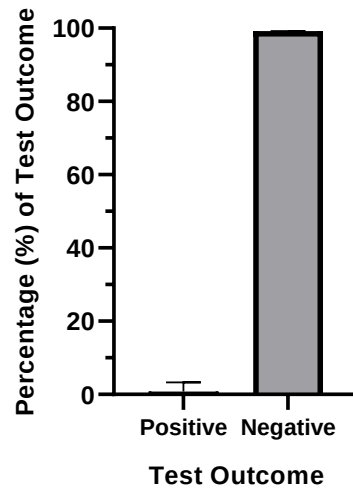


Figure 16: Percentage of SARS-CoV-2 LFT outcomes. Error bars represent 95% CIs.

The prevalence of COVID-19 was found to be very low in this community, as only 1 in a total of 120 surveyed (0.83%) tested positive (*Figure 16*). This positive result was confirmed by carrying out an additional LFT which was consistent with the original test.

4.3.7. Attitudes towards the Use of Rapid SARS-CoV-2 Tests

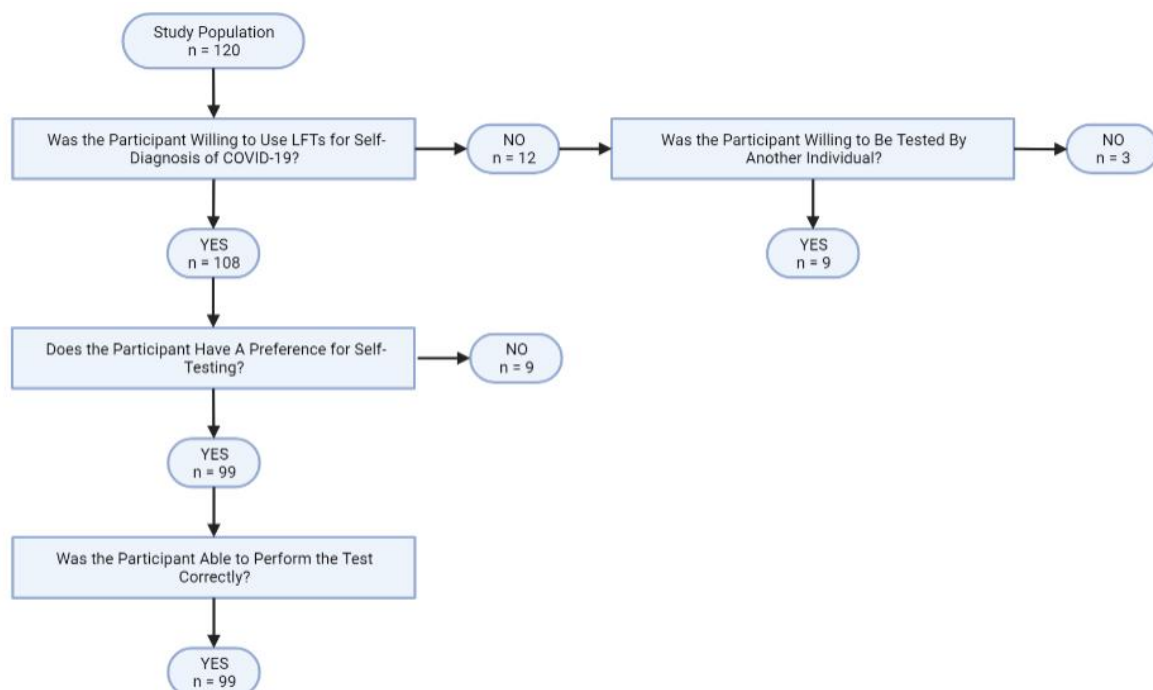


Figure 17: Flowchart depicting LFT testing preferences

The flowchart in *figure 17* displays how survey questions on the attitudes to LFTs were arranged during analysis to identify groups of individuals with differing testing preferences, in order to understand correlated demographic factors and identify the groups that would be suited to the introduction of differing targeted interventions: (i) participants who were willing and able with a preference of self-testing, (ii) participants who were willing to use LFTs, with a preference for testing by another individual, (iii) participants who were unwilling to carry out self-testing though willing to undergo testing by another individual and (iv) participants who were unwilling to be tested altogether.

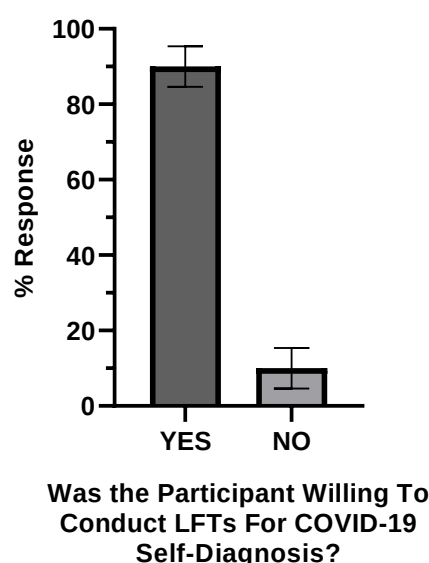


Figure 18: Percentage response to the survey question: was the participant willing to conduct LFTs for COVID-19 self-diagnosis? Error bars represent 95% CIs.

There was significant enthusiasm for the uptake of LFTs for the diagnosis of COVID-19, with 90% of participants stating that they would be willing to use them for self-testing (*Figure 18*). The groups of individuals answering “Yes” and “No” to this question concerning adoption of LFTs were separated to ascertain associated demographics, with the relevant factors of these groups (gender, occupation and religious affiliation) detailed in the table below (*table 13*). Males, farmers and followers of the apostolic faith are overrepresented in the group of individuals unwilling to carry out LFTs. However, fisher exact tests found no significant relationship between these demographic factors and willingness to use rapid SARS-CoV-2 tests.

Category	Total Participants	The Willingness of Participants to Self-Test For COVID-19		p-value
		Willing	Unwilling	
Overall	120	108	12	
Gender				0.2202
Male	21	17 (15.7%)	4 (33.3%)	
Female	99	91 (84.3%)	8 (66.7%)	
Occupation				0.3411
Farmer	82	72 (66.7%)	10 (83.3%)	
Non-Farmer	38	36 (33.3%)	2 (16.7%)	
Religious Affiliation				0.0663
Apostolic	92	80 (74.1%)	12 (100.0%)	
Other Affiliations	28	28 (25.9%)	0 (0.0%)	

Table 13: Relationships between Demographic Factors and Willingness to Use LFTs for Self-Diagnosis of COVID-19. Fisher Exact Tests were used to determine the independence of categorical variables, percentages are expressed as the number of willing or unwilling as a part of the population selection.

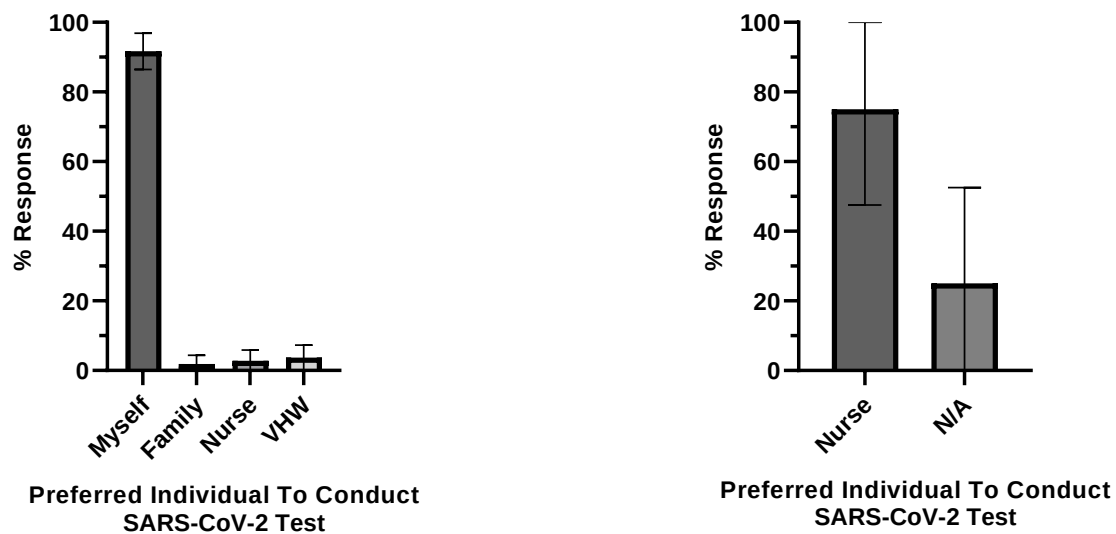


Figure 19 (a) Percentage response to the survey question: who was the participant's preferred individual to conduct the LFT? Attitudes towards others performing the test of those willing self-test for COVID-19. This was addressed to those responding "YES" to the question: was the participant willing to conduct LFTs for COVID-19 self-diagnosis? Bars represent 95% CIs.

Figure 19 (b) Percentage response to the survey question: was the participant willing to be tested by another individual? Attitudes towards others performing the test of those unwilling self-test for COVID-19. This was addressed to those responding "NO" to the question: was the participant willing to conduct LFTs for COVID-19 self-diagnosis? Bars represent 95% CIs.

Of those unwilling to self-test, 75.0% were satisfied to be tested by another individual, namely nurses (Figure 19a). When pressed for explanations of their preference, 66.7% stated reasons relating to the nurse's education and experience, assuring test reliability and 33.3% expressed their trust in nurses. The remaining 3 individuals preferred to self-test when given other options, saying that it was easier, quicker and prevented the procedure from being painful. Given their aversion to self-testing as answered previously, this group is likely to have arisen as an artefact of the questionnaire, which did not include an option to say that they didn't want to be tested at all. Hence, the graph in figure 19b and further analysis, characterises this group as N/A.

To gain greater insight into the group willing to self-test, they were also asked for their preferred method of testing. Participants were overwhelmingly in favour of self-testing, stating diverse reasons for their preference. The most common response commented on the ease or simplicity of the test, with 45.1% of individuals confirming this. Another 21.6% of respondents specified that conducting the test themselves was painless, in contrast to tests performed by others. Some 11.8% highlighted preferences for the use of personal LFTs for confidentiality

reasons and another 7.8% emphasised the convenience of being able to test more regularly without the need to return to clinic. In addition, 4.9% advocated for self-testing on the basis that it would create greater awareness of testing in the community. A minority of individuals expressed their preference for testing by family members, nurses and VHWs. As with those who would not be satisfied with self-testing, the preference for nurses was related to experience in a trusted health professional. Additional reasons included perceived greater ease if performed by another individual and the opportunity for advice or counselling when interacting with healthcare personnel. VHWs were uniquely favourable to this group, again related to experience, with high emphasis by participants on the convenience of having these individuals close to their community. The individuals that preferred testing to be carried out by family members did so for reasons of confidentiality and familiarity.

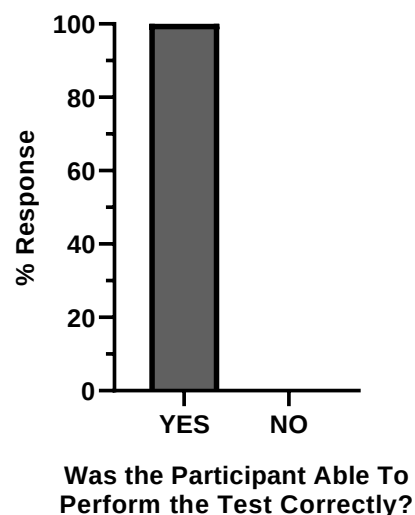


Figure 20: Percentage response to the survey question: was the participant able to perform the test correctly? Bars represent 95% CIs.

Every participant in this study was seen to perform the LFT correctly by VHVs and each test was shown to possess a control band, verifying the result (*Figure 20*)

4.3.8. Compliance of Self-Isolation

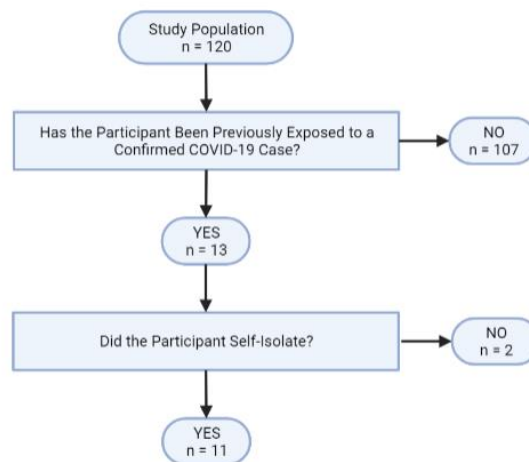


Figure 21: Flowchart depicting LFT testing preferences

The flowchart in *figure 21* displays the number of individuals in the study population who had previously self-isolated following contact with a confirmed COVID-19 case. A small proportion of participants (10.8%) were aware that they had been in exposed to SARS-CoV-2. Of these individuals, 84.6% observed self-isolation (*Figure 22*).

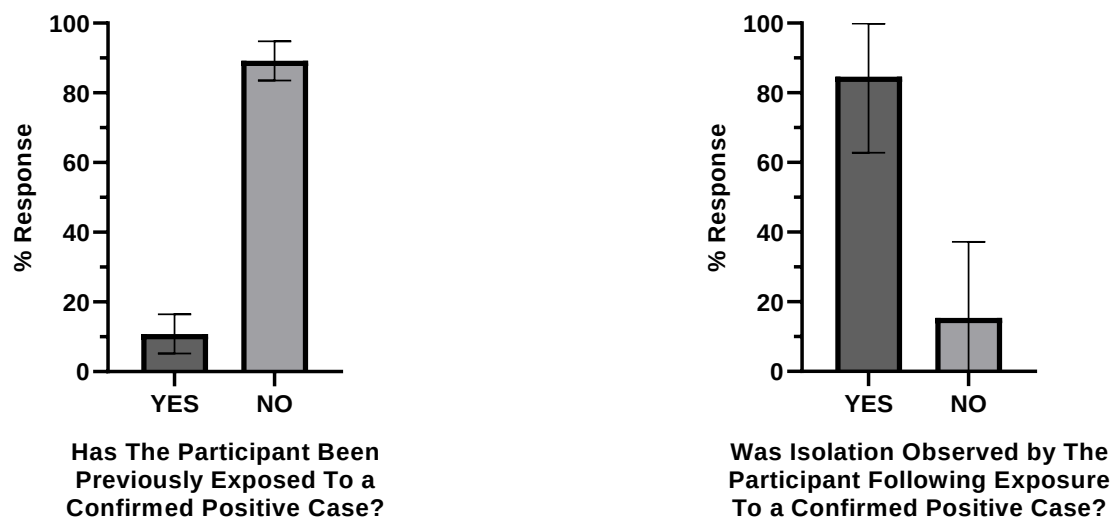


Figure 22 (a) Percentage response to the survey question: has the participant been previously exposed to a confirmed COVID-19 case? Error bars represent 95% CIs. (b) Percentage response to the survey question: was isolation observed following exposure to a confirmed positive case? Error bars represent 95% CIs.

4.4. Discussion

Other studies have shown the impact of implementing POC tests in primary healthcare settings on creating opportunities for diagnosis in low-resource settings (Ncube & Chataway, 2019). However, caution should be exercised when implementing these interventions, as positive clinical outcomes are not always translated between different settings. Therefore, the evaluation of interventions should be context-specific (Beisel *et al.*, 2016). This study sought to understand the attitudes to the use of LFTs for self-diagnosis of COVID-19 and assess other related behaviours in a low-resource setting in rural Zimbabwe.

4.4.1. COVID-19 Symptoms

A large proportion of individuals reported that they had experienced several COVID-19-associated symptoms in the last 2 months and on the day of the survey. Although many participants had carried out measures to mitigate the effects of their symptoms in healthcare settings and at home, no one disclosed attempts to procure COVID-19 tests. This could be due to multiple reasons, including a lack of testing availability, length of turnaround time, stigma on receiving a positive result, avoidance of mandated self-isolation and apathy towards disease harms. At the onset of the pandemic, rural communities were particularly affected by testing access and delays, making it a possibility that these factors may have indelibly reduced demand for testing in this region despite current availability. Another barrier to testing that may arise in this setting is the presumption that COVID-19 symptoms are caused by other factors, influencing a patient's likelihood to seek testing. This has been previously observed in other settings, where members of the public were only inclined to request testing for more distinguishable COVID-19 symptoms, such as loss of taste and smell (Fabella, 2020; Khalifa *et al.*, 2021). Further reasons for the absence of testing demand may be due to deficient knowledge of COVID-19 symptoms. This was demonstrated by a study carried out in Manicaland, Zimbabwe, where some participants were unaware of symptoms indicative of disease as only 46.5% identified shortness of breath as a signifier of infection (McAbee *et al.*, 2021). The demand for testing has also been shown to be variable, where symptoms were not severe or did not persist (Khalifa *et al.*, 2021). Understanding how symptoms of COVID-19 manifest and knowledge of the appropriate time to seek testing constitutes a major burden on individuals without formal health training. It may therefore be beneficial to introduce routine testing to this population to reduce complexity for patients and lower the subjective threshold of symptoms deemed appropriate to request testing.

4.4.2. COVID-19 Related Behaviours

Non-pharmaceutical interventions (NPI) have been shown to be effective in controlling the spread of SARS-CoV-2. For this reason, behaviours informed by NPI were analysed for

presence or frequency. This survey analysed several practices such as attendance in a crowded place, mask-wearing, hand hygiene and covering coughs with the inner elbow or cloth. Firstly, recent appearance at large gatherings were common within this study cohort, likely required to access shops, water and sell goods. In this context, widespread community exposure is unavoidable, therefore mitigation by other means is required in the event of high viral transmission.

Most participants adhered to basic hygiene practices such as handwashing and covering mouth whilst coughing, an important factor in reducing COVID-19 transmission. This is consistent with previous research on the WASH practices of Zimbabweans during the height of the pandemic (McAbee et al., 2021). McAbee et al. (2021) reported that 86.8% of participants washed their hands regularly to reduce the risk of succumbing to viral infection. This suggests that COVID-19 information campaigns were successful and continued to be influential even when transmission is low.

Mask-wearing is not the most impactful intervention in the context of other NPI, although it has been repeatedly shown to reduce transmission of SARS-CoV-2 across many different settings, particularly indoors (Mendez-Brito et al., 2021). Moreover, it is significantly less disruptive to normal socioeconomic activities than other NPI, which is of particular benefit to LMICs like Zimbabwe. Participants reported frequent usage of masks in public spaces, with over half declaring that they “always” took part in this behaviour. Similar proportions of participants relayed that they “never” or “sometimes” wore masks in public places. This high frequency of reported mask-wearing reiterates the findings of other studies in Zimbabwe and reflects population-wide awareness of reduced infection risk associated with the behaviour (McAbee et al., 2021). Nevertheless, it is likely that the proportion adhering to this behaviour is smaller than described because mask-wearing was not seen to be practiced in the wider community of the surrounding area. Additionally, several participants indicated that they “always” wore face masks in public places, despite not wearing one at the healthcare facility. Therefore, this may be a result of social desirability bias, which urges individuals to report inflated levels of positive behaviour, whilst minimising negative actions (Zerbe and Paulhus, 1987). The widespread awareness of benefits afforded by mask-wearing may therefore cause individuals to overreport the frequency of this behaviour.

A very high number of the community had received vaccines for COVID-19 amounting to a higher proportion than that of the general population. 42.5% of surveyed participants were fully vaccinated with 89.2% having received at least one vaccine, compared to 31.3% and 40.3% in Zimbabwean adults overall (Mathieu et al., 2020). Therefore, national schemes to target vaccine hesitancy were successful in this community.

4.4.3. Previous SARS-CoV-2 Testing In the Population

Prior to the introduction of further COVID-19 diagnostics, it is important to understand the role of existing tests in community surveillance. Where respondents were unable to identify which COVID-19 diagnostic they had undertaken, tests were classified as non-specific nasopharyngeal test, a category which described use of a swab to capture SARS-CoV-2 viral particles for detection using genetic material (RT-PCR) or proteins (LFT). Most people in this community were said to have been tested for COVID-19, with the majority of these people reporting an unspecified nasopharyngeal test, some of whom had both unspecified nasopharyngeal and antibody detection assay. Only a few participants were able to specify whether they had a confirmed LFT or PCR. Some had only received the antibody detection assay for the surveillance of past infections prior to the vaccination rollout. Analysis of testing methods in this community is difficult given the absence of discernibility between LFTs and PCR tests. The delivery of diagnostics could be better assessed if more knowledge was available so as understand the current role of health workers or facilities and to anticipate future needs.

4.4.4. Community Prevalence of COVID-19

This study identified low COVID-19 prevalence in the community amounting to 0.83%, which represented a single case in a cohort of 120 participants. Over the period the survey took place, Zimbabwean authorities recorded daily cases between 20.57 and 29.43 cases translating to 1.14-1.80 cases per 100,000 population (Mathieu et al., 2020). When adjusted for the size of the study population, official prevalence was lower than that recorded by this population. This difference may have come about because actual COVID-19 prevalence is likely higher than reported due to the low levels of testing in the population. Additionally, the official figures for case numbers are relatively low, therefore, a much higher sample size would be required to identify the reported value.

4.4.5. Attitudes towards the Use of SARS-CoV-2 LFTs

Most participants declared that they were willing to self-test and expressed enthusiasm about the introduction of LFTs for personal use for a variety of reasons, including that they were straightforward, pain-free, confidential and convenient. They also reported that the teaching of the testing process and knowing one's status promoted awareness of COVID-19. Evidently, the simplicity of SARS-CoV-2 rapid tests has positive implications on community usage as it increases patient confidence to perform the procedure independently of health workers. Thus, it can empower individuals to determine whether they have the disease and take responsibility to prevent further transmission. Many survey respondents had a preference for self-testing because it was relatively painless compared to previously taken COVID-19 tests. This irritation

caused by the nasopharyngeal swab has been well-documented in previous investigations and was also recounted in this study as the reason for favouring other rapid tests (HIV or malaria) over the LFT used here. However, this discomfort can be reduced when people complete the tests themselves, driving many individuals to favour this option. (Denford et al., 2021; Hofschulte-Beck et al., 2021; Kawamura et al., 2021; Kernberg et al., 2020; Zimba et al., 2020). Convenience was an important factor in the willingness to accept self-testing, with some participants citing the time-saving aspect as a major influence for personal uptake. This addresses a current limitation where test-seeking members of the community are compelled to spend extended periods of time travelling to a clinic, testing and in some cases returning later for results. This factor has a causal relationship to affordability where patients may have to forgo employment to access these services when available. This has been previously demonstrated with HIV testing in Zimbabwe, where real-time income loss and inconvenient location were prohibitive to testing uptake (Morin et al., 2006). The importance of confidentiality was apparent in individuals who favoured self-testing. Former studies have suggested that confidentiality is required to circumnavigate the negative impacts of disease-associated stigma and is crucial in settings where there is a trust deficit in healthcare personnel or the government (Blair et al., 2017; Ferree et al., 2021; Vinck et al., 2019; Wong et al., 2022). Studies on attitudes to HIV testing have reflected that individuals attending testing units or medical centres feared stigmatisation as they are seen as at risk of the disease (Morin et al., 2006). As self-testing can be conducted in the privacy of one's home, patients avoid the necessity of interfacing with these facilities, eliminating the effect of stigma in seeking diagnosis. Furthermore, personal POC diagnostics prevent the access to test results by authorities in communities where this relationship with the administration is unknown. Finally, the importance of knowing one's status has been a significant driver of enthusiasm for LFTs in this study, as well as uptake of COVID-19 testing in other countries (Clipman et al., 2020; Denford et al., 2021). Whilst this factor is not unique to self-testing, POC tests for COVID-19 can be carried out more regularly, providing accurate knowledge of current disease status given the speed of transmission.

A minority of participants did not favour self-testing with rapid COVID-19 diagnostics. However, this cohort was still shown to be largely receptive to testing in general, with a significant number expressing satisfaction for testing carried out by nurses. Pre-established trust of healthcare personnel was revealed to be important as some individuals were still concerned with result accuracy when conducting self-testing. These members of the community were reassured when offered the choice to have a nurse perform the test, thereby increasing the overall number of participants willing to either carry out their personal LFTs or be subjected to the test by a professional. It is reassuring that high trust in nurses was discernible as primary

healthcare settings such as this rural health centre comprise the majority of facilities and are usually managed by nurses, with doctors practicing at secondary healthcare and above (Björkman et al., 1998).

As detailed above, health-related stigma can increase the importance of confidentiality in disease-afflicted communities, causing individuals to favour self-testing. However, this stigma may also prevent individuals from testing altogether. For instance, 1 individual stated their preference for rapid malaria tests due to the reduced social consequences associated with the disease compared to COVID-19. This finding is clearly represented in a Malawian context, where 81% of respondents reported that they would experience COVID-19-associated stigma within their community if they tested positive for the disease (Ferree et al., 2021). The reduced stigma of malaria is testament to the long-established community awareness of the disease combined with knowledge that interpersonal contact will not result in transmission. Unfortunately, the spread of SARS-CoV-2 occurs via direct transmission and self-isolation from the community is an important mechanism by which to reduce the totality of infections. Despite the clear disadvantage upon hypothetically testing positive, the participant was still in favour of self-testing, suggesting that members of the community are still willing to sacrifice short-term isolation given the benefit to public health. All considered, efforts must be made to reduce the impact of COVID-19-related stigma, as these disadvantages may be enough to discourage other members of the community from taking up POC tests for COVID-19.

Various groups were identified for targeting with different interventions to increase COVID-19 community testing. Those who are willing and able to use personal LFTs for SARS-CoV-2 should be provided with these to carry them out at their own discretion. Those who are willing to test but have preferences to be tested by another individual and those who will only be tested together can be grouped together and be tested upon request by primary healthcare personnel given that there are sufficient resources to do so. If resources are not sufficient, then those who are unwilling to test should be prioritised. Those unwilling to test would not be receptive to increased COVID-19 surveillance and indicate that further education is required in these communities to encourage greater diagnostic uptake. These individuals were disproportionately likely to be males, apostolic and farmers, therefore, communities with high numbers of these demographics should be targeted for greater engagement with the health services.

4.4.6. Compliance of Self-Isolation

As previously discussed, ability to follow COVID-19 safety measures in Zimbabwe was specific to socioeconomic context and varied substantially (Mackworth-Young et al., 2021). The study population are largely subsistence farmers, sourcing clean water from community boreholes

and participating in the informal sector. Moreover, the financial instability driven by low income in the community may necessitate the daily purchase of essentials. Although there is no previous region-specific research on the subject, the aforementioned factors suggest that compliance with self-isolation may be difficult to achieve. This hypothesis was supported by 1 individual, relaying that the nature of their employment may prevent them from withstanding extended periods at home. Notwithstanding the evident difficulty of quarantining in this context, questionnaire responses suggested that self-isolation had been carried out by most individuals exposed to a known COVID-19 case. This diverged from the prediction hitherto considered, suggesting that there is more complexity to COVID-19-associated responses in rural Zimbabwe. Other undefined factors such as temporal context may provide additional insight into self-isolation behaviours, especially in the shifting landscape of societal norms over the course of the pandemic. Information on when self-isolation took place was not captured in this survey, although, it is unlikely to have occurred before the decentralisation of testing where rural communities had very limited access to diagnostics. All considered, isolation may have taken place at any given time period with social attitudes pertaining to the range associated with pandemic phase, from the fear-defined stages following introduction of the virus towards adoption of a “new normal”. More research is required to understand the discrepancy between self-isolation behaviours observed in this study with those reported elsewhere, with an emphasis on the means taken by individuals to ensure socioeconomic security.

4.4.7. Study Limitations

Various limitations arose over the course of the study, relating to delivery of the questionnaire, reliability and interpretation of long-form survey responses. Firstly, the average period spent on questions was limited by the length of the survey and number of participants at peak times. Consequently, there was a scarcity of time to clarify questions for each individual which may have contributed to misunderstandings. Some efforts were exercised to prevent this from occurring such as the absence of jargon-heavy questions and the opportunity to answer in the vernacular language (Shona) which reduced, although did not negate the impact of this limitation. In addition, time constraints reduced the capacity to gather further details from participants where vague answers were provided. This was apparent in the difference in information quantity extracted from questionnaires conducted at peak versus quiet periods. The first questionnaires were conducted on VHWs and coincided with a quiet period, allowing more developed answers from this group. This may have distorted responses to open-ended questions which were reflective of a demographic presumably more health-conscious than the general population.

Secondly, the survey included many fixed-answer multiple choice questions, which reduced the amount of qualitative data obtained from the participants. This approach was followed in order to distil information and facilitate data analysis, which successfully fulfilled the objectives of the study, however, further interviews would be required to elucidate the reasons behind social behaviours. Where open-ended questions were used, interpretation may have been affected by translation from Shona. Although, it remains difficult to appreciate the scale of this effect, given the absence of recorded information on the proportion of entries compiled in the vernacular language. Both question formats therefore introduced different limitations to data interpretation during analysis.

The reliability of these results are likely affected by social desirability bias, which is the inclination of survey participants to respond with answers that would be observed positively by others (Zerbe and Paulhus, 1987). This may have affected questions about COVID-19-associated behaviours, where respondents may answer questions in accordance with the stated health advice and previously enforced laws. This likely impacted many of the questions addressed by this survey, inflating incidences of mask-wearing, mouth-covering whilst coughing and hand washing practices. In addition, it may have influenced the reported responses to COVID-19 symptoms, driving individuals to relay increased frequency interfacing with the health system. Reliability was also subject to selection bias in the survey as participants were more likely to have attended the clinic and taken part if they have pre-established interests in testing or disease prevalence. This was introduced by the absence of random selection in the study design and subsequent recruitment to the survey, culminating in results which may not be representative of the community.

4.5. Conclusions and Future Directions

The implementation of lockdowns to reduce COVID-19 transmission and associated morbidity has had unintended consequences on the livelihoods of Zimbabweans, particularly in vulnerable communities of low socioeconomic status. It should therefore be a priority to promote targeted interventions to limit the spread of disease whilst minimising the impact on wider economic activity which sustains low-income households. Increased COVID-19 testing can be used to identify individuals with the disease, removing them from a context where they are able to transmit the infection, preventing disruption to other members of the community. This study demonstrated that most participants were willing and able to perform rapid tests for COVID-19 independent of healthcare personnel, correctly interpret results and undergo self-isolation when necessary. Further benefits of LFTs over other forms of testing were highlighted, such as ease of protocol, reduced irritation by the nasopharyngeal swab, convenience and the guarantee of confidentiality. This study has significant implications for the future of the

COVID-19 pandemic in a low-income environment, presenting how POC tests could be used to prevent widespread transmission given the potential emergence of novel strains with elevated transmissibility and pathogenicity. If LFTs for COVID-19 self-diagnosis were implemented in this region, further research would be required to understand uptake in practice and how this would affect COVID-19-associated behaviours. In addition to community attitudes, further consultation with healthcare professionals should be sought to understand the most effective distribution methods for LFTs and the greater impact that this intervention from a health service perspective.

5. Conclusions

The WHO Maputo declaration recognised the effects of poor laboratory infrastructure in many African countries as an obstruction to high quality infectious disease surveillance (WHO, 2008). To this day, upscaling remains difficult, as it requires significant, sustained investment and trained personnel, which is difficult to achieve in low-resource environments. This has a direct impact on community health of the region, as it hinders testing accessibility and subsequently delays treatment, contributing to preventable mortality and morbidity. These obstacles are pertinent in rural Zimbabwe, the region which constitutes the focus of these studies. Rapid POC tests confront many of the impediments associated with traditional diagnostic approaches. These include reduced costs and equipment with decreased burden on health professionals.

The studies that comprise this thesis have sought to address POC testing from immunological and sociological perspectives. Wherein, the analysis of *M. tb*-specific peptide immunoreactivity is proof-of-concept for rapid TB diagnostic prototypes, developed from a previously uncharacterised Zimbabwean population. This investigation also demonstrated how serodiagnostics can be used to understand the immunological side effects of parasitic infection, an important consideration when examining medical care in endemic regions. Furthermore, sociological analysis contextualised this research within the target population, through comprehending factors that promote testing uptake and reduce hesitancy, in addition to understanding demographics which pre-dispose these behaviours. Combined knowledge of the circumstances under which these health systems operate, alongside context-tailored diagnostics are crucial for successful implementation of novel interventions.

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