University of Venda

EFFECTS OF HOST GENETIC POLYMORPHISMS ON THE OCCURRENCE OF SCHISTOSOMIASIS AND CHLAMYDIA IN LIMPOPO PROVINCE

BY

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Declaration

I Tshepo Malesela Mafokwane, declare that this dissertation is my original work and has not been submitted for any degree at this or any other university or institution. The dissertation does not contain other person's writing unless specifically acknowledged in the form of reference list.

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Signed (Co-supervisor): …Ramalivhana…………………Date: …28/04/2016……………..

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Dedication

This dissertation is humbly dedicated to God almighty, who has been my eternal rock and source of refuge and for the love and favor that kept me all throughout the journey of completing this work. I also dedicate this work to my loving parents: Kwena David Mafokwane and Ramasela Rebecca Mafokwane, all my aunts and my siblings for they have always been my pillar of support.

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List of Abbreviations

CD14	: Cluster of differentiation 14
CD284	: Cluster of differentiation 284
CD4+	: Cluster of differentiation 4
CD8	: Cluster of differentiation 8
CDC	: Centers for Diseases Control and Prevention
CPAF	: Chlamydial Protease-Like Activity Factor
CSF	: Cerebrospinal fluid
DNA	: Deoxy ribonucleic acid
dNTP	: Deoxynucleotide
ELISA	: Enzyme linked immunosorbent assay
HCV	: Hepatitis C virus
HIV	: Human immunodeficiency virus
AIDS	: Acquired immune deficiency Syndrome
HSP60	: Heat shock protein 60
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IL13	: Interleukin 13
IL4	: Interleukin 4
IL5	: Interleukin 5
LNFPIII	: Lacto-N-fucopentaose II
MHC	: Major histocompatibility complex
NATSTAL	: National Survey of Sexual Attitudes and Lifestyles

NC	: Negative Control
NF-κB	: Nuclear factor kappa beta
NK	: Natural killer cells
NTDs	: Neglected Tropical Diseases
OD value	: Optical depth
PAMPS	: Pathogen associated molecular patterns
PBS	: Phosphate Buffer Saline
PCR	: Polymerase chain reaction
PID	: Pelvic inflammatory diseases
PC	: Positive control
PRR	: Pattern recognition receptors
RE	: Restriction enzymes
SNP's	: Single nucleotide polymorphisms
SPSS	: Statistical Package for social science
STAT 6	: Signal transducer and activator of transcription 6
STIs	: Sexually transmitted infections
TCR	: T cell receptor
TH1	: T helper 1 cells
TH2	: T helper 2 cells
TLR	: Toll like receptors
WHO	: World Health Organization

ABSTRACT

BACKGROUND

Genetic polymorphisms are responsible for inter-individual variation and diversity and therefore they have been recently considered as the main genetic elements involved in the development and progression of common and complex diseases. Chlamydia and schistosomiasis are among the most common and complex diseases throughout the world and in South Africa in particular. However, there are not enough studies on the role of host genetics on their occurrence. Studies have revealed associations between polymorphisms in Toll like receptor genes and human diseases as well as the role of Il-4 in disease progression and severity. Therefore, the present study aims at investigating the potential impacts of host genetic polymorphisms at TLR2 (-196 to -174 deletion) on the sero-prevalence of schistosomiasis and chlamydia and to investigate the role of IL-4 serum levels on the occurrence of the infections.

METHODOLOGY

Patients were recruited from different hospitals and clinics from three major Districts in Limpopo Province. About 650 blood samples were collected from HIV positive individuals and screened using ELISA for antibodies against *schistosoma* and *chlamydia*. DNA was extracted from buffy coat using the Gen-Elute TM blood genomic kit from Sigma Aldrich. Genotyping of TLR2 SNP'S was carried out using allele-specific PCR. PCR results were confirmed by sequencing and phylogenetic analysis. IL-4 serum levels were measured using commercially available ELISA for human Il-4 from MABTECH.

RESULTS

Out of the 650 samples tested, 69.5% were females. The age varied from 1 to 77 years with a mean of 35.4±12.8. The CD4 count from the studied participants varied from 5 to 4881 with a mean of 465.2 ± 415.1. The overall prevalence of *Chlamydia* IgG and IgM were 59.2% and 29.9% respectively, while that of *Schistosoma* IgG was 60.7%. The age group within which the highest prevalence for chlamydia IgG was found was between 26-45 years. Chlamydia IgM infection was significantly associated with female gender (p=0.002). For schistosomiasis IgG, the highest prevalence was found among participants who are 60 years and above. Chlamydia and schistosomiasis infections were not associated with any of the socio-demographic characteristics. A strong association was observed between chlamydia IgG and schistosomiasis IgG (p=0.002). The overall genotypic distribution of TLR 2 gene was 6.5% for del/del, 44.2% for ins/del and 49.4% for ins/ins. The genotypes obtained did not show any association with the demographic characteristics within the studied population as well as the reported infections. Higher IL-4 levels was associated with female gender (p=0.033). Higher IL-4 levels was also associated with higher Chlamydia both IgG and IgM although the difference was not statistically significant.

CONCLUSIONS

The present study demonstrated a high prevalence of both chlamydia and schistosomiasis in the Limpopo Province. Chlamydia infection was significantly much common among females compared to males. However, schistosomiasis is more common in males than in females. The Ins/Ins genotype was associated with weak chlamydia IgG infection. There is an urgent need to sensitise the community in order to encourage young people to get tested for chlamydia and get treatment on time considering the role of chlamydia on ectopic pregnancy. IL-4 cytokine could be protective for chlamydia but not schistosomiasis.

Keywords: Chlamydia, schistosomiasis, IgG, IgM, TLR2 polymorphism, IL-4 serum levels, sexually transmitted infections

CHAPTER ONE:

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Genetic association studies across the world have identified numerous polymorphisms which alter the susceptibility and outcome of infections (Hart and Tapping 2012). Many of the polymorphisms are within the genes that encode components of the innate and adaptive immune system. Thus the genetic variation in innate immune receptor gene contributes to individual differences in disease manifestation and the degree of complications upon infection (Van Well *et al*., 2012). Host defence against invading microbial pathogens is drawn forth by the immune system which consists of the innate and acquired immunity (Xue *et al.,* 2008).

Innate immune response is activated through recognition of pathogen associated molecular patterns (PAMPS) by specific germ-line encoded receptors on the host cells called Toll like receptors. Activation of these pattern recognition receptors leads to an immediate response to infection which can profoundly influence the development of an adaptive immune response (Van de kleij *et a*l., 2004). Toll-like receptors are a class of receptors that play an important role in the recognition of PAMPS. The first TLR ligands identified were all of the bacterial origin but it is becoming clearer that the recognition spectrum of TLRs is broad, because it includes also the viruses, yeasts and helminths (Xue *et al.,* 2008). There are at least 12 TLRs in which TLR1–10 have been found in human and the rest in mice (Mai *et al*., 2013). TLR2 is primarily involved in the recognition of peptidoglycans and lipoteichoic acid of gram positive bacteria and mycobacteria while TLR4 recognizes lipopolysaccharides (Lee *et al*., 2008). TLR2 and TLR4 are the most peculiar class of PRR because they are said to signal through a wide range of microbial peptides (Lester and Li, 2014).

Schistosomiasis and chlamydia are two of the most common diseases throughout the world, with schistosomiasis being the second most important parasitic infection after malaria and the first among the neglected tropical diseases (Van-Lume *et al*., 2013). Infections with schistosomiasis may result in urinary tract complications which includes fibrosis calcification, bladder cancer intestinal cancer and structuring (Bacelar *et al.,* 2007). Eggs may be deposited in the uterus, cervix and lower genital tract to cause female genital schistosomiasis which is linked to acquisition of sexually transmitted infections and HIV/AIDS (Mbabazi *et al.,* 2011). Globally, sexually transmitted infections (STIs) continue to pose a significant public health concern (Yirenya-Tawiah *et al*., 2011). However, surveillance remains a challenge especially in resource limited countries (Mohammed *et al.,* 2016). Schistosomiasis and chlamydia are inflammatory diseases in which cytokine levels correlates with disease severity (Loomis and Starnbach, 2002). They have a th2 biased immune response where IL-4, IL-5 and IL-13 are believed to be critical or defense against extracellular pathogens. IL-4 is a pivotal cytokine in cellular recruitment and subject to endogenous regulation. Therefore, the prediction would be that IL-4 is critical in the athogenesis of Th2-mediated diseases (Ruiter and Shreffler, 2012).

LR2 is an important sensor of parasites components and have been linked with Schistosoma fections (Thomas *et al*., 2003). There is strong evidence that TLR2 is critical for chlamydia diated host cell activation and pathology (Fraser *et al*., 2012). Numerous Single-nucleotide ymorphisms (SNPs) have been found in TLR2 gene. These include TLR2 R753Q and TLR2 - to -174delete polymorphisms. This polymorphism lead to variation in immune response, and are the most peculiar class of PRR because they are said to signal through a wide range of microbial peptides (Lester and Li, 2014).

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TLR2 is an important sensor of parasites components and have been linked with Schistosoma infections (Thomas *et al*., 2003). There is strong evidence that TLR2 is critical for chlamydia mediated host cell activation and pathology (Fraser *et al*., 2012). Numerous Single-nucleotide polymorphisms (SNPs) have been found in TLR2 gene. These include TLR2 R753Q and TLR2 -196 to -174delete polymorphisms. This polymorphism lead to variation in immune response, and alter susceptibility to various infectious diseases (Janardhanan *et al.*, 2013). TLR2 plays important role in recognizing specific pathogen-associated molecular patterns such as envelope proteins in viruses, lipoarabinomannan in Mycobacterium, LPS, peptidoglycan in bacteria, glycophosphatidylinositol in parasites as well as zymosan fungi, therefore it is of considerable interest to investigate the potential association between the functional impairments within these genes with infectious diseases (Zhang *et al.*, 2013). In this regard, the present study aims at investigating the impacts of host genetic polymorphisms at TLR2 (-196 to 174 Del) on the occurrence of schistosomiasis and chlamydia in Limpopo Province.

1.2. Rationale of the study

The recognition that host genetic variation alters host susceptibility to infections has led to multiple studies evaluating TLR gene polymorphisms in susceptibility to infections. With growing evidence, modern science has embraced Louis Pasteur and Robert Koch's "germ theory of disease," suggesting microbe–host interactions in the pathogenesis of several diseases, but their relationships have not been successfully disentangled (Shrestha, 2011).

Identification of the gene variants that affect susceptibility to diseases is an initial step towards defining the genes and the interactions among their products that determine susceptibility to diseases, this could be potentially translated into more efficient therapeutic strategies (VillaseÇor-Cardoso and Ortega, 2011). For a variety of infectious diseases for example malaria, hepatitis, meningococcal infections and cancer, it has been shown that host genetics play a crucial role in susceptibility to and severity of disease, but little attention has been paid to diseases such as chlamydia and schistosomiasis.

Chlamydia is the most common sexually transmitted bacterial pathogen and up to 4 million new cases are reported annually (Rizzo et al., 2015). Given that there is no effective vaccine available for Chlamydia till date, a better understanding of the immunological and molecular mechanisms mediated by TLRs will greatly aid in possibly exploiting these molecules as immunotherapeutic targets (Joyee and Yang, 2008). According to Wira *et al*., (2011), the innate and adaptive immunity in the female genital tract has been extensively reviewed but the interplay between hosts genetics with outcomes of infections remains to be elucidated, therefore the study of bacteria and host genetics in chlamydia infection is valuable.

Human schistosomiasis presents a classic, complex disease manifestation, with marked disparity in the intensity of infection, the immune response to infection as well as the development of schistosome-related pathology (Bethony and Quinnell, 2008). Schistosomiasis is one of the most neglected tropical diseases of high importance, and still remains a major problem in public health in Endemic countries (Hotez *et al*., 2008). There is no vaccine available yet for the prevention of schistosomiasis and according Bergquist and Colley, (1998) there is evidence in human populations for natural resistance and acquired immunity against *schistosoma* infections suggesting that the development of a vaccine is authentic to be envisaged. Therefore there is an urgent need to identify and develop candidate molecules for use in future vaccines for protection against schistosomiasis (Siddiqui *et al*., 2011).

Several association studies of schistosomiasis have focused on the Th2 gene cluster, especially IL4, IL5, and IL13 gene polymorphisms. However, these studies did not look at the association between the serum cytokine level and disease severity. Furthermore, few studies have been published regarding the association of toll like receptor polymorphisms with schistosomiasis and chlamydia in human (Ouf *et al*., 2012). Therefore, further investigations are needed to clarify whether the polymorphisms in TLR2 have an impact on the outcomes of infection with chlamydia and schistosomiasis and also the association between regulatory cytokine levels especially IL-4 with the disease severity.

Considering the importance of TLRs in the human immune system, it is interestingly important to observe the genetic distribution in the local population in order to improve our understanding on the occurrence of infectious diseases. In addition, studies around Limpopo province have only looked at the prevalence of schistosomiasis (Samie *et al*., 2010) and chlamydia (Dubbink *et al.*, 2012, Mafokwane and Samie 2016). Therefore there are no documented studies around the Limpopo province especially in Capricorn, Sekhukhune and Waterberg Districts which looked at the impacts of host genetic polymorphisms on the occurrence of chlamydia and schistosomiasis.

1.3 Study hypothesis

In this study, we hypothesize that host genetic polymorphisms at TLR2 (-196 to -174 deletion) is associated with susceptibility to chlamydia or schistosomiasis.

1.4 General aim of the study

To determine the potential impact of the host genetic polymorphisms at the Toll like Receptor 2 (-196 to -174 deletion) gene on the sero-prevalence of chlamydia and schistosomiasis.

1.5 Objectives of the study

- To determine the sero-prevalence of chlamydia and schistosomiasis in the Capricorn, Waterberg and Sekhukhune Districts.
- To determine the distribution of the TLR 2 (-196 to 174 Del) polymorphism in the study population and their potential association with schistosomiasis and chlamydia.

- To evaluate the relationships between interleukin 4 serum levels with schistosomiasis and chlamydia diseases.

CHAPTER TWO:

LITERATURE RIVIEW

2.1 Toll like Receptor 2 structure and function

Toll-like receptor 2 is an extracellular receptor that recognizes lipoteichoic acid (Yoshimura *et al.,* 1999) and meningococcal porin (Massari *et al.,* 2006). It is encoded by a DNA sequence and is composed of 2352 bases that specify 784 amino acids (Rock *et al.,* 1998). It is located at the long arm of chromosome 4 between q31.3q32.1 as indicated on figure 2.1 and has two 5' non-coding exons followed by a third exon (Mandal *et al.,* 2012). TLRs 1, 2, 6 and 10 are co-receptors that form a functional unit called the TLR2 subfamily encoded by genes in the chromosome 4, and play an important role in recognition of bacterial structures.

Human TLR2 plays a key role in response to peptidoglycan, lipoteichoic acid and a variety of macromolecules particularly gram positive bacteria. Thus TLR2 is considered as the most important receptor and is associated with the detection of PAMPS associated with fungi, protozoa, viruses and helminthes (Compton *et al.,* 2003). TLR2 gene is expressed most abundantly in peripheral blood leukocytes, and mediates host response to Gram-positive bacteria and yeast through the stimulation of NF-κB (Jensen and Thomsen, 2012).

TLR2 has been shown to form heterodimers with TLR1 and TLR6. Using TLR1, TLR2, and TLR6 knockout mice enabled scientists to determine that the different heterodimers recognize unique peptides. For example TLR2/6 recognizes diacylated forms of mycoplasmal lipoproteins and TLR2/1 recognizes triacylated bacterial lipopeptides (Farhat *et al*., 2008). These heterodimers are advantageous as they expand the range of ligands that may be recognized through TLR2 (Mogensen, 2009).

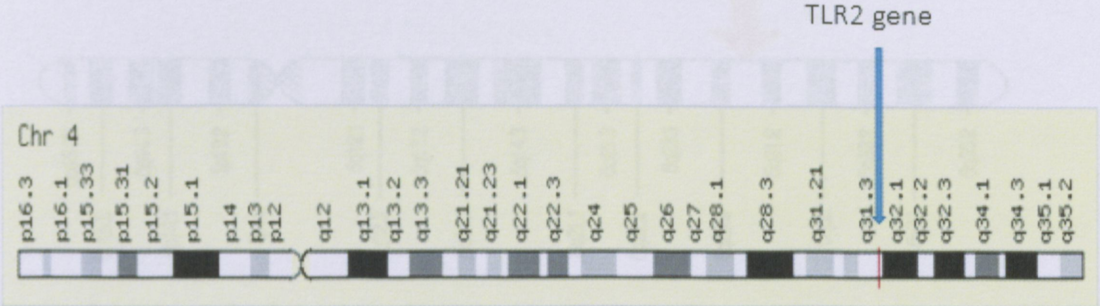


Figure 2.1 Chromosome 4 indicating TLR2 location (gene cards, 2016)

<u>2.2 Interleukin 4 gene structure and function</u>

Interleukin 4 is a protein coding gene that induces the differentiation of naïve T cells (THO cells) to TH2 cells. It is a ligand of the interleukin 4 receptor (Chang *et al.,* 2015). The interleukin 4 receptor binds to IL-13 making a contribution to many overlapping functions of the cytokine and IL-13. A signal transducer and activator of transcription which has been shown to play a central role in mediating the immune regulatory signal of this cytokine is called STAT-6. IL-4 gene together with IL-3, IL-5 and CSF2 forms a cytokine gene cluster located on chromosome 5q (between 5q33.1 and 5q32) as indicated on figure 2.2.

Some of the diseases that are associated with IL-4 include vernal conjunctivitis and transient acantholytic dermatosis. IL-4 is known to participate in several B cell activation processes as well as other cell types. This cytokine has a detrimental role in controlling *Heligmosomoides polygyrus* and *Trichuris muris* and can contribute to the protection against *Nippostrongylus brasiliensis* and *Trichinella spiralis* infections in mice independent of IgE (Finkelman *et al.,* 1997)

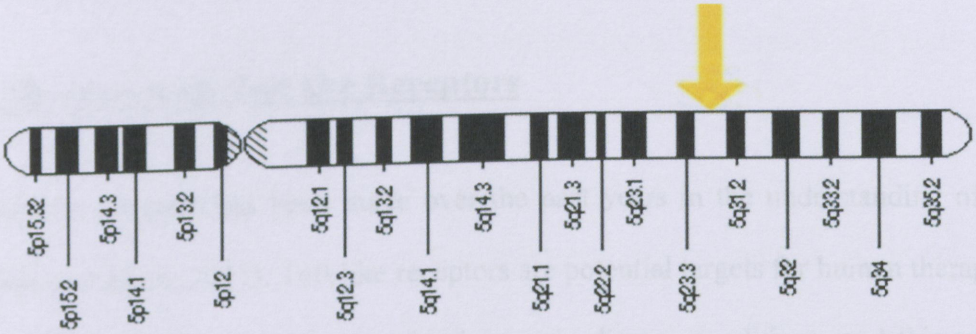


Figure 2.2 Molecular Location of interleukin (IL-4) on chromosome 5 (gene cards, 2016)

2.3 Effects of Toll like receptors genetic polymorphism on infectious diseases

The genetics of TLRs provide important insights in gene-environment interactions in health and disease, and it may enable scientists to assess patients' susceptibility to diseases or predict their response to treatments (Garantziotis *et al.*, 2008). Although TLR-mediated signalling is paramount in eradicating microbial infections and promoting tissue repair, the regulation must be tight. Toll-like receptors enable innate immune recognition of endogenous and exogenous ligands (Belkaid and Hand, 2014). TLRs together with their co-receptors and their downstream signalling patterns plays a crucial role in pathogen recognition and subsequent activation of the host immune response and therefore any polymorphisms that alters the protein structure and in so doing affect the ability of toll like receptors to bind to their respective ligands will most likely affect the host susceptibility towards infections (Mehta and Jeffrey, 2015). Polymorphisms or mutations to the downstream members confers resistance or increased susceptibility depending on their nature (Mogensen, 2009). Studies in humans have revealed associations between TLR polymorphisms and human disease (Cook *et al.,* 2004). For example the R753Q polymorphism in TLR2 is associated with decrease response to bacterial peptides and therefore predisposing individuals to life threatening bacterial infections (Lorenz *et al.,* 2000). Another polymorphism the TLR2 -196 to -174 del has been reported to increase the risk of gastric cancer (de Oliveira and Silva, 2012).

2.4 Therapy with Toll like Receptors

Significant progress has been made over the past years in the understanding of TLR function (Kawai and Akira, 2011). Toll like receptors are potential targets for human therapeutics (Horton *et al.,* 2010). Their roles are emerging in certain disease conditions, and this suggests that the selective targeting of TLRs might be useful therapeutically and also in development of vaccines (O'Neill, 2003). Extensive analysis of TLRs has revealed specificity in terms of ligand recognition, expression in different cell types and tissues, and importantly, a role for TLRs in the pathogenesis of multiple diseases involving both the innate and adaptive immune systems (O'Neill *et al.,* 2009). TLRs are implicated in a number of inflammatory and immune disorders and play a role in cancer (Rakoff-Nahoum and Medzhitov, 2009). TLR are the most promising targets for therapeutics, by virtue of their cell surface location, quick induction and the ability to mount a wide variety of inflammatory responses (Hennessy *et al.,* 2010).

2.5 General Introduction to schistoshomiasis

Schistosomiasis also known as bilharziasis or snail fever is a tropical disease caused by helminthes of the genus *Schistosoma* under the family schistosomatidae, order Digenea, class Trematoda, phylum Platyhelmints and kingdom Animalia (Webster, 2006, Beaumier *et al.,* 2013). It is a disease that is most common in areas of extreme poverty and poor sanitation in Africa, South America, Middle East and Asia where people come into contact with urine or feces contaminated water as part of their daily lives (Davis *et al.,* 2003). The three *Schistosoma* species namely, *S. mansoni*, *S. haematobium* and *S. japonicum* are of medical importance to humans due to their high prevalence rates, pathogenicity as well as an extensive distribution (Gryseels, 2013 and WHO, 2011). *Schistosoma mansoni* which affects the hepatic and gastrointestinal tracts while *S. haematobium* affects the urinary and reproductive system in its hosts. These two are the most common species of schistosoma in Africa. *Schistosoma haematobium* most commonly cause the disease known as urogenital schistosomiasis. In some areas of Sub-Saharan Africa were *S. haematobium* is endemic, there is a co-existence between schistosomiasis and sexually transmitted infections (Kjetland, 2008). The mortality rate is estimated to be more than 250,000 people worldwide, this makes the infection to be considered as one of the most neglected tropical diseases which poses a significant public health concern (Hotez *et al.,* 2008).

Helminthes infections are chronic in nature and provide a suitable model for studying the effect of repeated challenge of the innate immune system (Beaumier *et al*., 2013). Approximately 200 million people are infected worldwide (Macwilliam *et al*., 2012). Out of the 200 million people infected worldwide 80% live in Sub-Saharan Africa (King 2010). The most prevalent species in sub-Saharan Africa are *S. haematobium* which accounts for urogenital schistosomiasis and severe pathological conditions such as hematuria, hydronephrosis, and bladder cancer. *Schistosoma Mansoni* causes intestinal schistosomiasis (Ouf *et al*., 2011). Regarding the involvement of TLRs, controversial data have been reported. Lacto-N-fucopentaose III (LNFPIII), a Lewisx motif-containing carbohydrate present on schistosome egg glycoproteins, was reported to promote Th2 polarization in mice in vitro and in vivo in a TLR4-dependent manner (Caldasa *et al*., 2010). The heterogeneity in the clinical presentation of the disease depends on the factors related to the parasite and the hosts (Verma *et al*., 2010).

2.5.1 History and discovery of schistosomiasis

Schistosomiasis has been affecting human health for at least 4000 years (Adamson, 1976). It was first described in humans in Cairo in the year 1851. Schistosomiasis was initially called Bilharziasis, named after the German physician/pathologist Theodor Maximilian Bilharz (El-Saghier Mowafy and Abdel-Hafeez, 2015). The first species to be described was *Schistosoma haematobium,* it was described on the basis of the eggs with their peculiar pointed terminal projection (Coon, 2005). In 1904 another species *Schistosoma japonicum* was described after being discovered back in 1847 by Yoshinao Fujii in water (Tanaka and Tsuji, 1997). *Schistosoma japonicum* was described by Fujiro Katsurada, who was also a pathologist at Okayama Medical College in Japan. In 1907, another species was described by Luingi Westenra Sambon of the London school of tropical medicine, he named the species *Schistosoma mansoni* in honor of Patrick Manson who was known as the father of tropical medicine. In South Africa the first record of schistosomiasis was 12 years after the discovery made by Bilharz in 1863 among boys in Uitanage, Eastern Cape (Appleton, 2012).

2.5.2 Geographical distribution and genetic epidemiology of schistosomiasis

Schistosomiasis has a worldwide occurrence as shown on figure 2.3. It is a widespread helminthic infection that is endemic in 76 countries of America, Africa, and Asia. According to the WHO (2006), this disease affects at least 200 to 300 million people, in which 650 million are at risk of infection (Oliveira *et al.,* 2004). Globally, schistosomiasis is a major source of morbidity and mortality (Adenowoa *et al.,* 2015). Its unique life cycle limits endemic areas to tropical and subtropical zones, the disease may even increase with some agricultural practices, therefore the geographic spread continues because of the water resource engineering issues (Utzinger *et al.,* 2003). It has been said that 85% of the people who are at risk of the infection and those with severe disease are concentrated in Africa (Kjetland *et al.,* 2006 and Davis, 2000).

Schistosomiasis has been successfully eliminated in countries like Japan and Tunisia, however, Morocco and some Caribbean Islands countries have made significant progress in controlling the disease while Brazil, China, and Egypt are taking steps towards elimination of the disease (Utzinger *et al.,* 2009). From these Carlton *et al.,* (2015) reported a prevalence of 8.4% in rural villages of China. In DRC the average prevalence of *schistosomiasis* was found to be as high as 82.7% (Linsuke, 2014). A cross-sectional study in North West of Tanzanzia on school children between ages of 8–17 years in Sengerema District, revealed an alarming prevalence of 64.3%*S. mansoni* infection (Mazigo *et al.,* 2010). A nationwide survey by Augusto et al., (2009) on the prevalence of schistostomal infections and soil helminthes in school children from Mozambique reported a prevalence of 47% for *S. haematobium* and 1% for *S. mansoni* infections. Midzi *et al.,* 2014 reported a 22.7% prevalence of schistosomiasis in Zimbabwe while a pilot study in the Eastern Cape Province, South Africa among school aged students revealed a 73.3% prevalence (Mentees and Boyles, 2010).

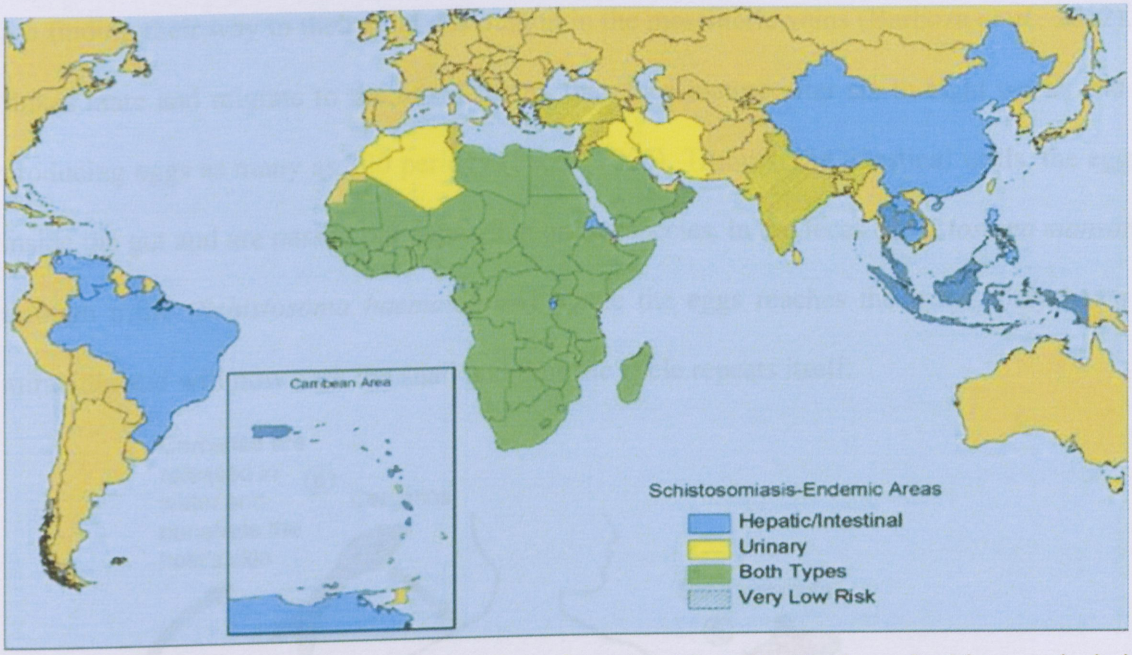


Figure 2.3: Diagrammatic representation of geographical distribution of schistosomiasis in the world (http://www.nathnac.org)

2.5.3 Life cycle of schistosomiasis

Schistosomiasis has a complex life cycle, mainly because it takes place in the human definitive host as well as in the fresh water snail as the intermediate host (Swartz *et al.,* 2015). Illustration of the life cycle is shown on figure 2.4. After the eggs of the human-dwelling parasite have been emitted from the feces into the water, the ripe miracadium hatches out of the egg. The miracadium therefore searches for a suitable snail as an intermediate host in the water and eventually penetrates it (Cromley, 2012). The miracadium develops through mother-sporocyst and daughter-sporocyst generation to become the cercaria (Barboza *et al.,* 2012). The cercariae is the infective stage of schistosomiasis and therefore upon an encounter with the human skin they penetrate it within a short space of time. The cecariae shed their forked tail and transforms into an endoparasitic larva called the schistostomulae (Nayak and Kishore, 2013). The schistosomulae migrate through several tissues and form male-female pairs in the liver as they are finding their way to their final destination in the mesenteric veins (Barboza *et al.,* 2012). The flukes mate and migrate to the bloodstream, they then mature after six to eight weeks and start producing eggs as many as 300 per day (Secor, 2005). Through the intestinal walls, the eggs get inside the gut and are passed out depending on the species, in the feces (*Schistosoma mansoni)* or through urine (*Schistosoma haematobium).* Once the eggs reaches the water, they hatch into miracidia and will now seek the snail and thus the cycle repeats itself.

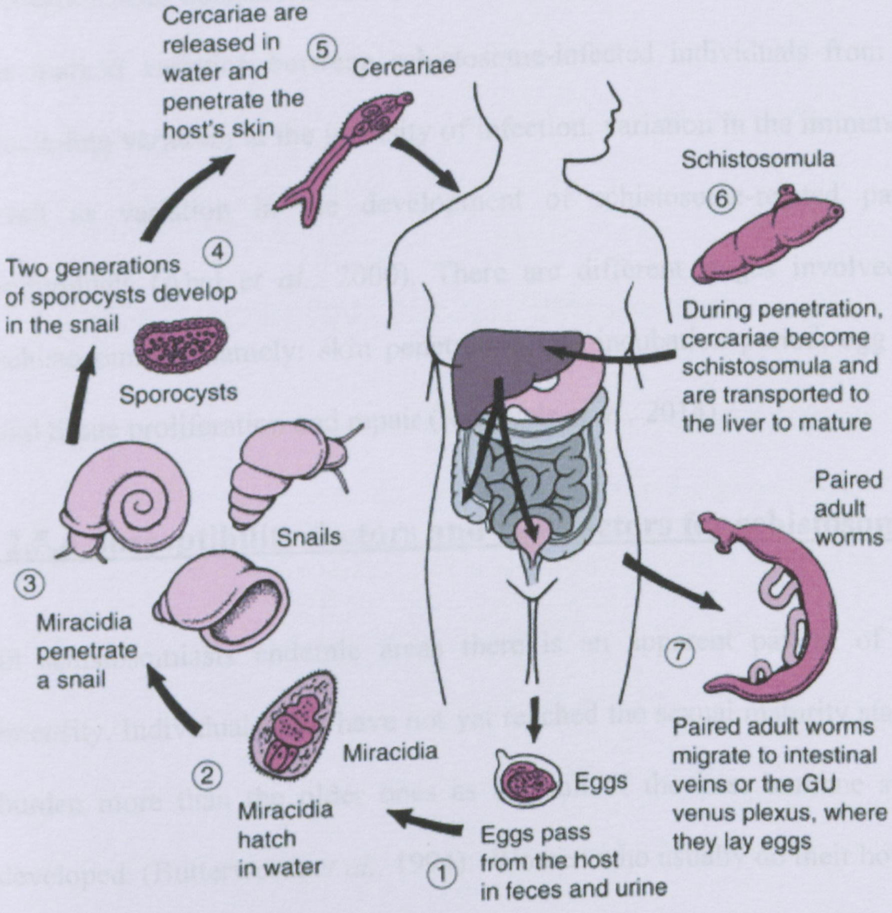


Figure 2.4: Life cycle of schistosomiasis (http://www.malevolentdesign.org/schistosomiasis/)

2.5.3 Pathogenesis of schistosomiasis

Schistosomiasis is atypical amongst the helminthic infections due to the fact that most of the pathogenesis is due to the eggs rather than the adult worms and most of its pathology is caused by the host immune response (Mowafy and Abdel-Hafeez 2015). Therefore, the intensity and duration of infection, may in turn determine the amount of antigen released from the eggs and the severity of chronic fibro-obstructive disease (Burke *et al.,* 2009). The complication of the infection with Schistosomiasis is evident at the level of the phenotype (Abel *et al.,* 2000). There is marked variation between schistosome-infected individuals from the same endemic area, including variation in the intensity of infection, variation in the immune response to infection, as well as variation in the development of schistosome-related pathology among infected individuals (Abel *et al.,* 2000). There are different stages involved in the pathogenesis of schistosomiasis namely: skin penetration and incubation period, egg deposition and extrusion and tissue proliferation and repair (Kenguele *et al.,* 2014)

2.5.4 Susceptibility factors and risk factors for schistosomiasis

In schistosomiasis endemic areas there is an apparent pattern of age dependent infection intensity. Individuals who have not yet reached the sexual maturity stage carry the largest worm burden more than the older ones as a result of the host immune system which is not fully developed (Butterworth *et al.,* 1994). Women who usually do their household chores in infected water are at risk of the infection. In endemic areas, the infection is usually acquired as a child (Kjetland *et al.,* 2012). Poggensee *et al.,* (2000) have hypothesized that other sexually transmitted infections have the ability to increase the susceptibility of women to HIV infection. This could be the results of breaches in the integrity of the mucosal barrier of the vagina and the cervix. Similarly, female genital schistosomiasis has also been hypothesized to increase HIV susceptibility of women (Kenguele *et al.,* 2014).

The symptomatology of female genital schistosomiasis include pelvic pain (PID) which can lead to ectopic pregnancies, postcoital bleeding, inter-menstrual bleeding, itching on genitals and abnormal vaginal discharge (Mbabazi *et al.,* 2011). Female genital schistosomiasis may therefore easily be mistaken for a sexually transmitted infection like chlamydia and generally women tend to be reluctant in seeking medical attention because some of the early manifestations seems to be normal among females. In Zimbabwe and Tanzania, cross sectional studies indicated that women with female genital schistosomiasis had a higher HIV prevalence than women without female genital schistosomiasis (Mbabazi, 2011).

2.5.5 Host Immune response to schistosomiasis

Pathogen-triggered immune responses that are not regulated appropriately can cause significant damage to host tissues when they persist, it can pose a major difficulty for the host (Hesse *et al.,* 2004). As stated in other studies, male and female worms of *Schistosoma* lives in the mesenteric venules where they feed and produce several hundred eggs per day (Secor, 2005). However some of the eggs go through the portal system and impact in the presinusoidal capillaries of the liver, while others can be shunted into the lungs (Barsoun *et al.,* 2013). Schistosomes have evolved several immune evasion strategies with the aim of down-regulating the host's immune response to promote their own survival (Jenkins *et al.,* 2005). Helminthic infections induce chronic immune activation, shifting from a T-helper cell type 1 (Th1) to type 2 (Th2) immune response (Bentwich *et al.,* 1995). Th2-lymphocytes down-regulate cytotoxic effects of $CD8^+$ T-lymphocytes, leading to an altered cytokine profile with increased viral replication.

2.5.6 Treatment, prevention and control of schistosomiasis

Given that schistosomiasis is a public health problem, in countries where other neglected tropical diseases are also prevalent, control programs and preventative measures can be integrated with goal of maximizing the use of resources in curtailing the infection (Inobaya *et al*., 2014). Praziquantel, a pyrazinosoquinolone derivative, is an anthelminthic drug that targets a broad range of parasitic infections, it is therefore advocated by the World Health Organization (WHO) for population-based mass chemotherapy. Praziquantel is well tolerated in human and has been shown to be effective against various species of *Schistosoma* and is therefore considered as the drug of choice for the infection with schistosomiasis (Reich *et al.,* 2013).

The basic means of preventing schistosomiasis is to avoid any contact with fresh water infected with *Schistosome* parasites, although contact with infected water cannot always be avoided. Aquatic activities such as swimming, wading, fishing and household chores in the water bodies infected with schistosomiasis exposes the skin to cercariae penetration. In countries where schistosomiasis causes significant disease, control efforts usually focuses on reduction in the number of infections in people and eliminating the snails that are required to maintain the parasite's life cycle (CDC, 2012). The control of schistosomiasis is based on large-scale treatment of at-risk population groups, access to safe water, improved sanitation, hygiene education, snail control or chemotherapy (Inobaya *et al*., 2014). Furthermore, discovery of new drugs such as nitrimidazoles and possible vaccine targets has refocused the attention of control strategies on chemotherapy.

2.6 General introduction to Chlamydia

Chlamydia is the most common sexually transmitted bacterial pathogen caused by an obligate intracellular bacterium called *Chlamydia trachomatis*. It is also the etiologic agent of blinding trachoma (Massari *et al*., 2013). It is an intracellular parasite that survives within targets cells of mucous membrane. Chlamydia is currently being diagnosed in over 90 million people each year. Although the infection usually resolves without long-term consequences, its impact on public health is considerable because of increasing incidence rates, especially among the youth (Starnbach and Roan, 2008). *Chlamydia trachomatis* has a dangerous and widespread pathogenicity factor because it has no sensitivity to conventional drugs and has no obvious symptoms (Snavely *et al*., 2014). Genetic variation, particularly single nucleotide polymorphisms in immunoregulatory genes such as TLR genes, has been shown to affect host susceptibility to numerous pathogens (Schroder and Schumann 2005). Chlamydial components like lipopolysaccharide (LPS) and heat shock protein 60 (HSP60) are recognized by TLR4, the intact organisms stimulates the innate immune cells through TLR2, which also plays an important role as a PRR for chlamydia (Rizzo et al., 2015).

2.6.1 History and discovery of Chlamydia

Chlamydia, from the Greek word meaning cloak, was falsely given its name due to its ability to cloak the nucleus of infected cells (Byrne, 2003).The earliest known descriptions of chlamydia like diseases, resembling the symptoms of trachoma, date back from ancient Chinese and Egyptian writings. Halberstädter and von Prowazek were the first to discover the organism after observing it in conjuctivital scrapings in 1907 (Budai, 2007). It was first isolated in China in the year 1957 by T'ang and colleagues within embryonated eggs which ultimately confirmed its existence as a bacteria. In 1958, inoculation of human volunteers provided evidence that it indeed caused disease in humans (Budai, 2007). Because of chlamydia's unique developmental cycle, it was taxonomically classified in a separate order. It can thus be found with the other well-known intracellular parasites, rickettsiae, in diagnostic manuals (Barnes and Toomey, 1990). *C. trachomatis* was the first chlamydia species to be discovered and has been divided into subgroups based on antigenic variation in the major outer membrane proteins (MOMP), serovars and on clinical expression (Rours *et al.,* 2010).

2.6.2 Life cycle of Chlamydia Trachomatis

Chlamydiales are endosymbiotic bacteria with a very unique life-cycle, the life cycle alternates between a non-replicating, infectious elementary body (EB) and a replicating, non-infectious reticulate body (RB) as shown in figure 2.5, it is therefore considered as a biphasic developmental cycle (Schachter, 1999). It takes place inside a parasitophorous vacuole called an inclusion (Knowlton *et al.,* 2011). Intracellular replication necessitates an intimate association between the bacterium and the host cell that likely involves altering host cell functions in order to establish and maintain an environment conducive for replication within the inclusion (Loomis and Starnbach, 2002). There are four stages involved in the development of chlamydia (Baron, 1996). The first stage is called the dormant phase where the Elementary bodies have no metabolic activity. The second stage involves the initiation of the metabolism of the elementary bodies, this is where the cycle begins when the infectious elementary bodies actually attaches to receptors on the surface of a suitable host cell and induce their own entry through receptor-mediated endocytosis (Brendan, 2000). Once it has entered the cell, the surrounding plasma membrane becomes inclusion membranes. The EB's subsequently converts to replicative reticulate bodies (RB) and replication begins by binary fission. The chlamydial inclusion which neither fuses with lysosomes nor acidifies, then traffics to the Golgi region, where it can intercept spingolipid containing vesicles (Hackstadt *et al.*, 1997). The RB's then grows and divide within the specialized cellular inclusion. Late in the infectious process, RB's asynchronously begin to differentiate back to EB's. Eventually the specialized unique inclusion ruptures and the infectious particles are released. The entire cycle then repeats itself. Depending on the serovars the whole chlamydia life cycle takes approximately 44 -48hours.

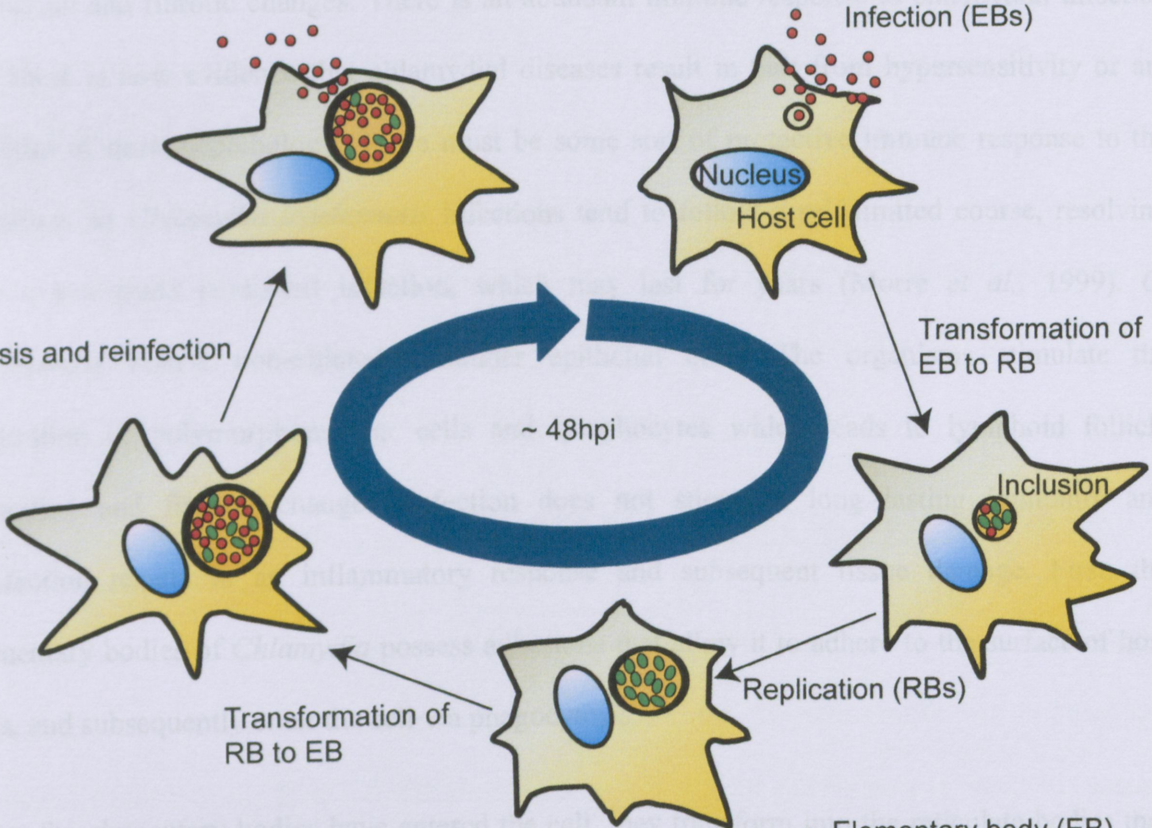


Figure 2.5: An illustration of the life cycle of *Chlamydia trachomatis* (Loomis and Starnbach, 2002)

2.6.3 Pathogenesis of *Chlamydia Trachomatis*

In the infected individuals chlamydial agent causes tissue damage and induction of interleukin-1α, interleukin-1ß, and TNFα, which are cytokines involved in the inflammation process (Becker, 1966). The disease process and clinical manifestations represent the combined effects of tissue damage from chlamydial replication and inflammatory responses to chlamydiae and the necrotic material from destroyed host cells (Morre *et al.,* 1999). The organisms stimulate the infiltration of polymorphonuclear cells and lymphocytes which leads to lymphoid follicle formation and fibrotic changes. There is an abundant immune response to chlamydial infection and there is now evidence that chlamydial diseases result in part from hypersensitivity or are diseases of immunopathology. There must be some sort of protective immune response to the organism, as *Chlamydia trachomatis* infections tend to follow a self-limited course, resolving into a low-grade persistent infection, which may last for years (Morre *et al.,* 1999). *C. trachomatis* infects non-ciliated columnar epithelial cells. The organisms stimulate the infiltration of polymorphonuclear cells and lymphocytes which leads to lymphoid follicle formation and fibrotic changes. Infection does not stimulate long lasting immunity and reinfection results in an inflammatory response and subsequent tissue damage. First, the elementary bodies of *Chlamydia* possess adhesions that allow it to adhere to the surface of host cells, and subsequently enter the cell via phagocytosis.

After the elementary bodies have entered the cell, they transform into the reticulate bodies that are able to carry out replication. A key component to this bacteria's ability to evade the host immune system is the ability of the bacteria to prevent the fusion of the phagosome with the lysosome. Without destruction by the phagolysosomes, the organism is able to multiply and eventually rupture the host cell (Kaiser and Gary, 2005). *Chlamydia* as gram-negative bacterium lacks peptidoglycan and enables the bacteria to detect antibodies that are formed against it (Rugpao *et al.,* 2010).

Chlamydia undergoes antigenic variation to out-manoeuvre the host's immune system. The major outer membrane protein is considered to play an important role in its ability to alter its surface proteins (Fan *et al.,* 1997). It also relies on several debilitating enzymes that allow it to evade the host immune system. It releases an enzyme called chlamydial proteasome/protease-like activity factor (CPAF) that enables the organism to degrade the host DNA. This allows it to evade the host immune system because it hinders the activation of the gene responsible for the production of its major histocompatibility complex (Darville and Hiltke, 2010). It is apparent that the frequency of complications in chlamydial infection is insufficiently known, which is intricate when considering the need and cost-effectiveness of screening. Chlamydial infection is thought to be responsible for 12 –65% of cases of pelvic inflammatory diseases (PID), a third of tubal infertility and the majority of ectopic pregnancies.

2.6.4 Geographical distribution and epidemiology of *Chlamydia trachomatis*

Most epidemiologic data on chlamydia is from industrialized nations. However, it is important to characterize the disease from resource limited regions where most people are infected and unaware (Vidwan *et al.,* 2012). In the United States, *Chlamydia trachomatis* is the most common sexually transmitted pathogen, with an estimated 4.5 million new cases reported each year (Malhotra *et al.,* 2013). In England, the National Study of Sexual Attitudes and Lifestyle (Natsal-2) estimated a prevalence among 18–24 year olds to be 3.0% in women and 2.7% in men.

More than two-thirds of chlamydia cases occur in the developing world, where diagnostic and treatment facilities are almost non-existent (Nwankwo and Sadiq, 2014). Owing to varied characteristics of the study populations and different methods used for chlamydia detection, there is a wide variation in prevalence rates of chlamydia infection (Verkoyeen *et al.,* 2002). A study conducted in Nigeria found a prevalence of 29.4% which was determined by ELISA and also indicated a high incidence of asymptomatic *Chlamydia trachomatis* (Ikeme *et al.,* 2011). In Rwanda, a study conducted among fertile and subfertile women found a relatively low prevalence (3.3 versus 3.8%), which was determined by PCR as compared to the 86.4% versus 90.9% as determined by ELISA method (Muvunyi *et al.,* 2011). In Kwa-Zulu Natal province, South Africa a prevalence of 13% for Chlamydia Trachomatis was reported by Naidoo *et al.,* (2014). In Limpopo Province, an overall prevalence of 15% was reported in the Vhembe District (Mafokwane and Samie, 2016).

2.6.5 Host Immune response to Chlamydia trachomatis

As with most infections, the immune system plays an elementary role in the body's attempts to eradicate the infection (Redgrove *et al.,* 2014). The initial and most important immune response to Chlamydia infection is local, where the immune cells such as leukocytes are recruited to the site of infections, and eventually secrete pro-inflammatory cytokines such as IL-16 and tumour necrosis factor and chemokines such as interferon gamma (IFNγ) and IL-8. The secretion of Il-8 by infected cells can recruit classical innate immunity cells such as natural killer (NK) cells and DCs, which are abundant in the genital mucosa (Buchholz and Stephens, 2006).

Chlamydia infection usually occurs in the lower genital tract and attracts different types of immune cells such as lymphocytes, macrophages and dendritic cells to infiltrate the epithelium. They stimulate responses from the blood vessels, connective tissue and lymphocyte infiltration (Redgrove *et al.,* 2014). Infections with *Chlamydia* induce IgG, IgM, IgE and IgG antibodies, however, these antibodies do not prevent the host from reinfection (Becker, 1966). Upon infection, antigen presenting cells are sequestered to the site of infection and start to release pro-inflammatory cytokines. At this point of infection there is a strong inflammatory reaction brought about mainly by CD4+ T cells with a Th1 phenotype to clear the infection (Loomis & Starnbach, 2002). CD4+ T cells go on to form either T-helper 1 (Th1) or T-helper 2 type (Th2) T cells (Gondek *et al.,* 2012). Th1 cell interact with B cells through the T cell receptor (TCR) and the major histocompatibility complex (MHC) with the aim of producing antibodies against chlamydial infection (Redgrove and McLaughlin, 2014). Figure 2.6 shows the illustration of the process.

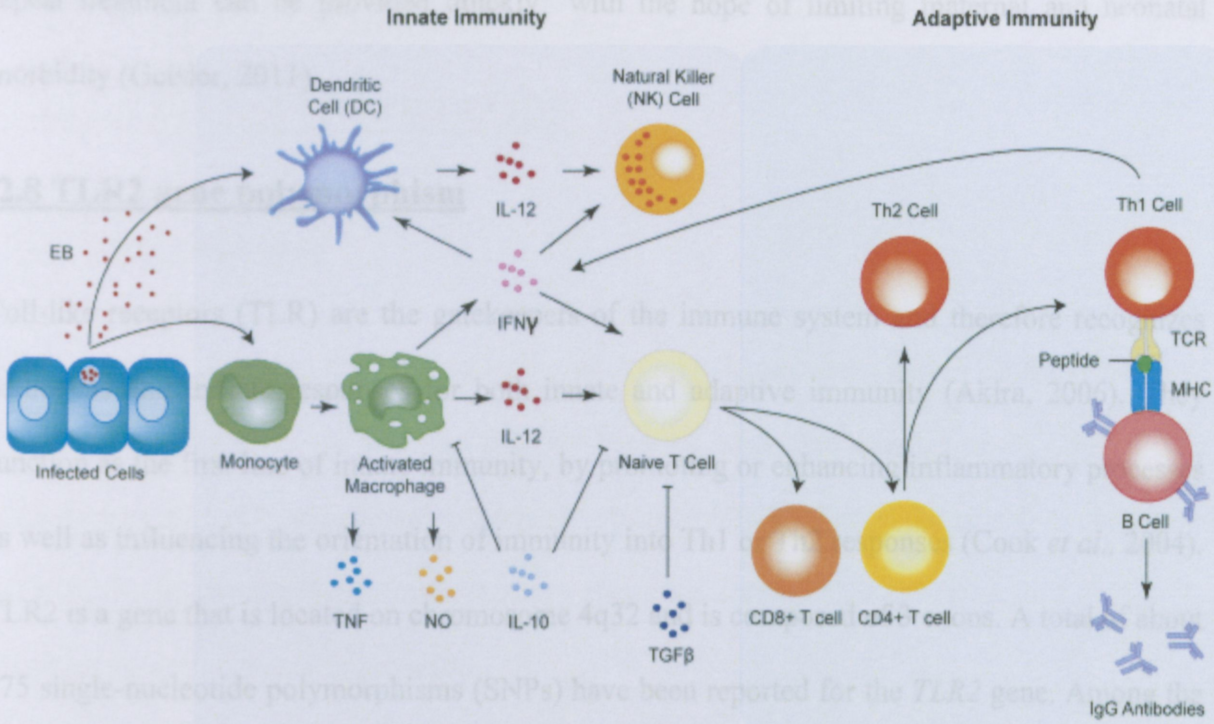


Figure 2.6: an illustration of the immune response against Chlamydia Trachomatis (Redgrove and McLaughlin, 2014).

2.7 Prevention and control of *Chlamydia trachomatis*

To prevent recurrent transmission within partnerships, it is important that management of chlamydial infection is based on simultaneous treatment of infected people with their sexual partners (Geisler, 2004). The approach to the better management of uncomplicated *C. trachomatis* infection in adolescents and adults includes routine chlamydia screening as recommended by the CDC, using more sensitive tests (Barrow *et al.,* 2008). Other approaches include treatment with recommended therapy to reduce complications and prevent transmission to others, treatment of sexual partners to prevent reinfection of patients and complications in patients and partners; risk-reduction by counselling, repeated chlamydia testing a few months after treatment to identify recurrent infection; and a test of cure in pregnant women at a minimum of 3 weeks after treatment to identify treatment failure or early recurrence so that repeat treatment can be provided quickly with the hope of limiting maternal and neonatal morbidity (Geisler, 2011).

2.8 TLR2 gene polymorphism

Toll-like receptors (TLR) are the gatekeepers of the immune system and therefore recognizes pathogens and initiate responses for both innate and adaptive immunity (Akira, 2006). They function as the first line of innate immunity, by promoting or enhancing inflammatory processes as well as influencing the orientation of immunity into Th1 or Th2 responses (Cook *et al.,* 2004). TLR2 is a gene that is located on chromosome 4q32 and is composed of 3 exons. A total of about 175 single-nucleotide polymorphisms (SNPs) have been reported for the *TLR2* gene. Among the SNP's on the TLR2 gene, some polymorphisms particularly Arg753Gln and Arg677Trp have been associated with impaired recognition of the pathogen associated molecular patterns (Merx *et al.,* 2007). Another polymorphism which is found on the promoter region of the gene causes a 22-bp nucleotide deletion at positions − 196 to − 174 of the untranslated 5′-region has been shown to be associated with reduced transcriptional activity (Noguchi *et al.,* 2004). These are the most studied SNP's and has been associated with susceptibility to various infectious and inflammatory diseases such as leprosy, increased risk of gram negative sepsis, asthma, malaria, recurrent bacterial infections as well as colorectal cancer (Kang *et al.,* 2001, Schröder *et al*., 2003).

CHAPTER THREE:

MATERIALS AND METHODS

3.1 Ethical clearance

The study was approved by the University of Venda Health and Ethics Committee. Ethical clearance was also obtained from the different hospitals where the samples were collected. The objectives of the study were clearly explained to the patients and those who agreed to participate in the research were requested to sign consent forms and complete the questionnaires in order to obtain socio-demographic information.

3.2 Study site

The study was undertaken in different clinics and hospitals around three major Districts of Limpopo Province namely: Waterberg, Sekhukhune and Capricorn Districts. These are shown in figure 3.1.

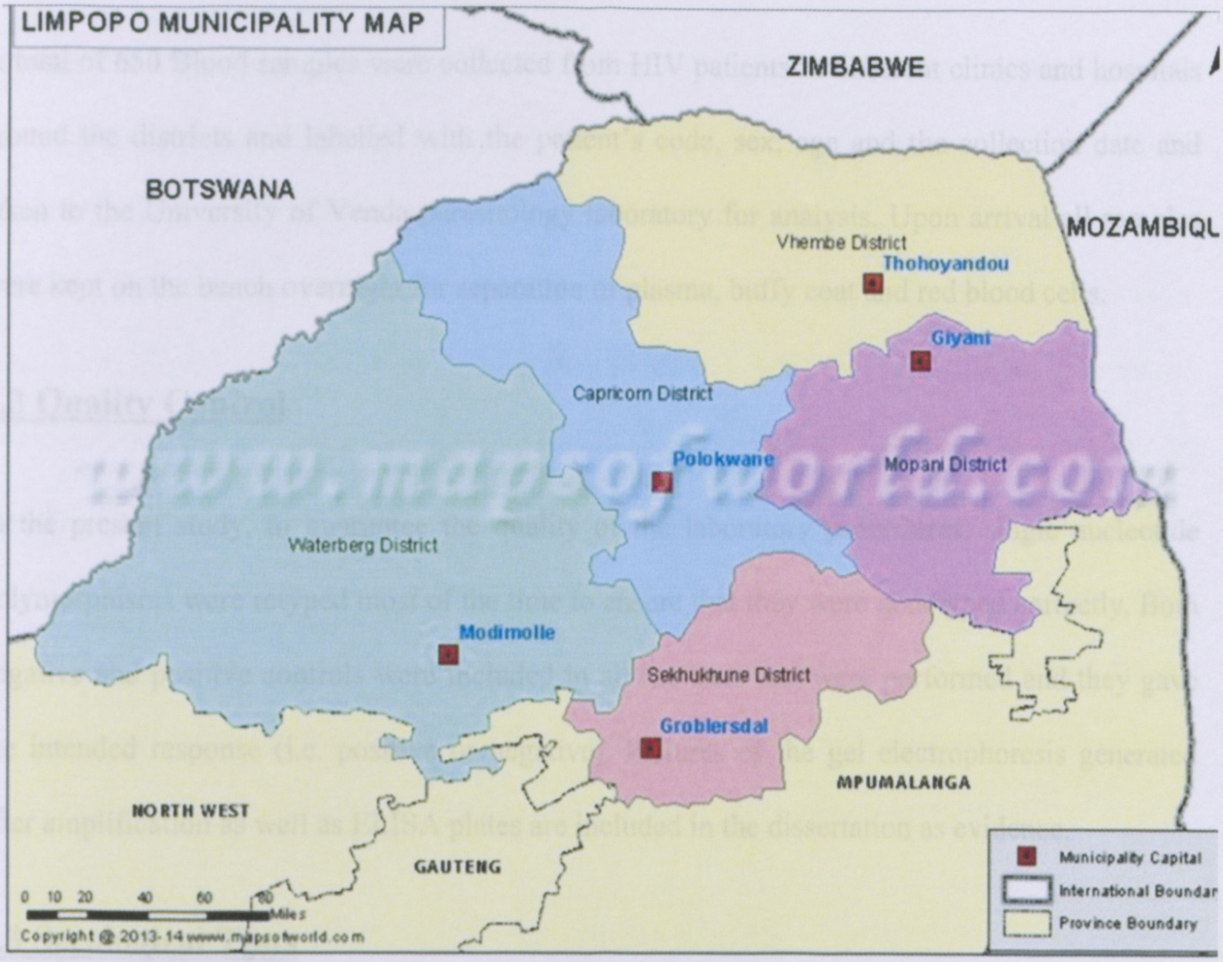


Figure 3.1: Map showing areas and districts of Limpopo Province (www.mapAfrica.com)

3.2 Sample Collection and processing

A total of 650 Blood samples were collected from HIV patients in different clinics and hospitals around the districts and labelled with the patient's code, sex, age and the collection date and taken to the University of Venda parasitology laboratory for analysis. Upon arrival all samples were kept on the bench overnight for separation of plasma, buffy coat and red blood cells.

3.3 Quality Control

In the present study, to guarantee the quality of the laboratory procedures, single nucleotide polymorphisms were retyped most of the time to ensure that they were genotyped correctly. Both negative and positive controls were included in all the tests that were performed and they gave the intended response (i.e. positive or negative). Pictures of the gel electrophoresis generated after amplification as well as ELISA plates are included in the dissertation as evidence.

3.3 Serological Tests

Following manufacturers' instructions, serological assays using enzyme-linked immunosorbent assays (ELISA's) were performed to detect antibodies against *Schistosoma* and *Chlamydia* (IgM and IgG) in serum. The IL-4 serum levels were measured with a specific enzyme-linked immunosorbent assay (ELISA) and a standard curve was drawn and used as reference to quantify the levels.

3.3.1 ELISA for detection of IgG antibodies against Chlamydia

AccuDiag™ *Chlamydia trachomatis* ELISA (Cortez Diagnostics, Inc CA,US) was used for detection of IgG antibodies against Chlamydia, where 5µl of each test samples, negative and positive controls as well as the calibrator were added to 200µl of the sample diluent and mixed well. The diluted sera, calibrator and controls were dispensed in appropriate wells as well as the blank (100 µl/well). The test samples were then incubated for 30 minutes at room temperature. The liquid was removed from the wells and washed three times with a diluted washing buffer. Enzyme conjugate was added (100 µl/well) to each well and incubated for 30 minutes at room temperature. The enzyme was removed from the wells and washing was repeated. TMB chromogenic substrate was dispensed into each well (100 µl/well) and incubated for 15 minutes at room temperature, stop solution was then added to the wells and the plate was placed in a micro well plate reader and the OD values were read at 450nm (VersaMax™, USA).

3.3.2 ELISA for detection of IgM antibodies against Chlamydia

AccuDiag™ *Chlamydia trachomatis* ELISA (Cortez Diagnostics, Inc CA, US) was used for detection of antibodies (IgM) against Chlamydia. Serum samples were diluted (1:40) by adding 10µl of the serum to 390µl of the dilution buffer. Negative and positive controls and the diluted test samples were added to the appropriate tubes then 40µl of the absorbent was added to the tubes, mixed well and incubated at room temperature for 10 minutes. From the mixture, 140µl was transferred to the appropriate wells and incubated at room temperature for 10 minutes. Enzyme conjugate was added to each well (100 µl/well) and incubated for 10minutes at room temperature and the samples were washed. After washing 100µl of the chromogen was added to each well and incubated at room temperature for 10 minutes. To stop the reaction, 100µl of stop solution was added to each well and the plates were read at 450nm in an ELISA micro plate reader (VersaMax™, USA).

3.3.3 ELISA for detection of IgG antibodies against schistosomiasis

DRG Schistosoma IgG ELISA (DRG International Inc., USA) was used for detection of IgG antibodies against schistosomiasis. Negative, positive control and test samples were diluted 1:40 and added to the appropriate wells. The test samples were incubated at room temperature for 10 minutes. The contents were then shaken out and the plates were washed three times with a wash buffer. The enzyme conjugate was added to the wells and incubated at room temperature for 10 minutes. The contents were shaken out and the plates were washed again three times with a diluted wash buffer. Drops of Chromogen (2/well) were added to each well and incubated for 5 minutes at room temperature then the stop solution was added to each well. The wells were then placed in an ELISA micro plate reader and read at 450nm (VersaMax™, USA).

3.3.4 ELISA for detection of human IL4

The IL-4 serum levels were determined using human interleukin 4 ELISA kit from MABTECH (Australia, Pty, Ltd). In brief, a high protein binding ELISA plate was coated with Monoclonal antibodies which were diluted to 0.5µg/ml with PBS at pH 7.4. One hundred µl of the antibody was added into each well and the plates were incubated overnight at 4-8°C after which the plate was washed twice with PBS. IL-4 standards were prepared by reconstitution in 1ml PBS to a concentration of 1µg/ml. Each standard and samples were added to incubation buffer and transferred to the wells in a plate. The plate was incubated for 2 hours at room temperature. After incubation the plates were washed 5 times with PBS containing 0.05% tween. Biotin diluted in incubation buffer was added to the wells and incubated for an hour at room temperature. It was then rinsed 5 times with PBS containing 0.05% tween. Streptavidin–HRP diluted 1:1000 was added in incubation buffer, transferred to each well and incubated for an hour at room temperature. The plate was washed 5 times with PBS containing 0.05% Tween 20. The substrate solution was added and the plates were read in an ELISA micro plate reader at 450nm.

3.4 DNA Extraction

DNA was extracted from blood buffy coat using Gen-Elute^{TM} blood genomic kit from Sigma Aldrich following instructions from the manufacturer. Briefly 20µl of proteinase K solution was placed into 1.5ml micro-centrifuge tubes where 200µl of blood buffy coat was added. Two hundred µl of lysis solution C was added to the samples and was vortexed for 15 seconds. The sample mixture was then incubated at 55^0 C for 10 minutes. The samples were prepared for binding by adding 200µl of ethanol to the mixture and vortexed again for 10 seconds. The entire mixture was transferred to a column which was first prepared by addition of 500µl of column preparation solution and centrifuged at 12000 rpm for 1 minute. The columns were then washed twice with the addition of wash solutions, and centrifuged for 3 minutes at maximum speed. The DNA was then eluted by pipetting a 200µl of elution solution into the center of the column directly and centrifuged for 1 minute at maximum speed. The DNA was then stored in the freezer at -20^0c until further analysis.

3.5 TLR genotyping

The stored DNA was used to investigate the non-synonymous SNP at TLR2 -196-174 deletion using allele specific PCR (Newton et al 1989). In this method, specific primers (shown on figure 3.1) for the target gene are designed to permit amplification by DNA polymerase only if the nucleotide at the 3'-end of the primer perfectly complements the base at the variant or wild-type sequences. After the PCR and electrophoresis, the patterns of specific PCR products permit the differentiation of the SNPs. The PCR conditions and parameters described previously by (Tahara *et al.*, 2007) were used. Briefly a 25µl reaction was carried out, which contained 5µl genomic DNA, 10 pmol each forward and reverse primers, 200ng each dNTP and 0.6 U Taq DNA polymerase.

Table 3.1: Primer sequences used for genotyping of TLR2 polymorphism by allele specific PCR

Gene	Sequence	Band size	Reference
-196 to -174 del polymorphism	F:5'-CACGGAGGCAGCGAGAAA-3' R:5'-CTGGGCCGTGCAAAGAAG-3'	264bp and 286bp	Tahara *et al.*,2007

3.6 Sequencing and phylogenetic analysis

A 20µl aliquot of the representative PCR product from the Genotyping which showed successful amplification was sent for sequencing at Inqaba biotechnologies (Inqaba, Pretoria, South Africa). This was done in order to confirm the different genotypes obtained during amplification. Sequences were then assembled using Staden package (Staden *et al.,* 1999). This software permits addition and removal of nucleotides present in the sequence during editing. During editing, foreign nucleotides that were present in the sequence were removed and replaced by appropriate nucleotides. Bio edit package (Hall, 1999) was then used to further edit the nucleotide sequences, which were then aligned using Clustural W multiple aligner. With the Clustural Multiple aligner, the nucleotides were aligned alongside a referral strain obtained from Gene-bank.

3.7 Statistical analysis

The results were entered on an excel spread sheet and edited appropriately and analyzed using Statistical Package for social sciences (SPSS version 17.0). Data was summarized using frequency tables and bar charts. Contingency tables were used and the strength of association was measured using the chi-square and its associated p-value. The results were considered to be statistically significant when the p-value obtained is less than 0.05.

CHAPTER FOUR:

RESULTS

4.1. Demographic characteristics of the study population

Most of the study participants were females (69.5%). Majority of our study participants were aged between 26 to 45 years (61%). About 43% of the study participants had a CD4 count between 5-349 cells/µL, while 34.5% of the participants had a CD4 count of more than 500cells/µL. Most of the study participants were from Capricorn District (49.7%). Sekhukhune District had 27.8% while Waterberg had the least participants (22.5%). Most of the samples received originated from clinics (47.1%) while only 22.5% were from hospital settings. The results are shown on table 4.1 below.

Table 4.1 General characteristics of the study participants

Characteristics		Frequency	Percentage (%)
Gender	Male	164	30.5
	Female	373	69.5
Age group	0-16 years	31	6
	16-25 years	68	13.1
	26-45 years	316	61
	>46 years	103	14.1
CD4 count	5-349	220	42.6
	350-499	118	22.9
	>500	178	34.5
District	Capricorn	254	49.7
	Sekhukhune	142	27.8
	Waterberg	115	22.5
Origin	Clinic	345	47.1
	Hospital	187	25.5

4.2 Enzyme Linked immunosorbent assay (ELISA) for detection chlamydia and schistosomiasis using IgG and IgM as immunological markers

A total of 422 serum samples were tested by ELISA to detect antibodies (IgG and IgM) against chlamydia and schistosomiasis as shown in Figure 4.1, figure 4.2 and figure 4.3. Well 1A and 1B contained the negative and positive controls, the remaining wells contained the test samples.

Out of the 422 samples tested for antibodies (IgM) against chlamydia, 29.9% were positive. For chlamydia IgG 59.2% were positive. For schistosoma IgG, 379 samples were tested and 60.7% of the samples were found to be positive. The results are illustrated on figure 4.4.

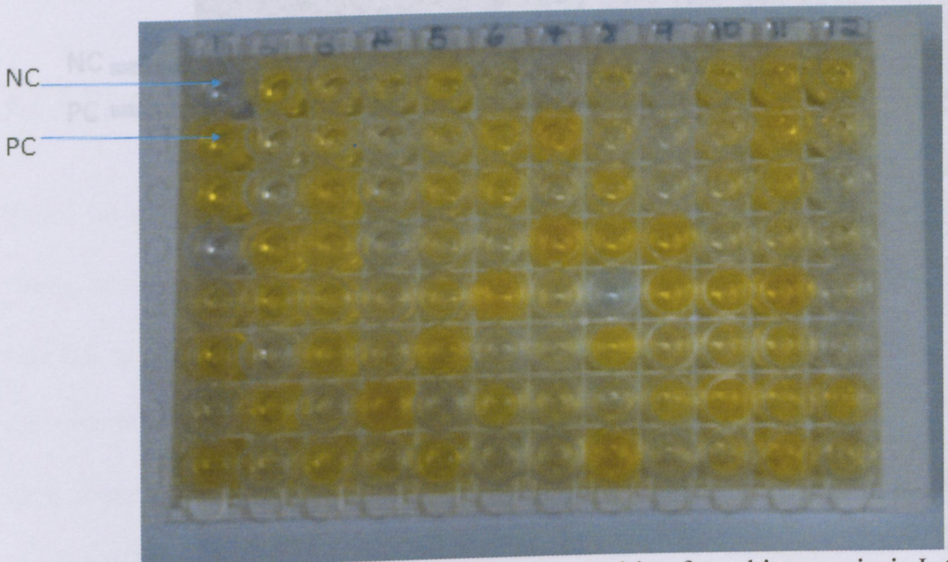


Figure 4.1. Micro-titer plate showing samples positive for schistosomiasis IgG (PC-positive control, NC-negative control)

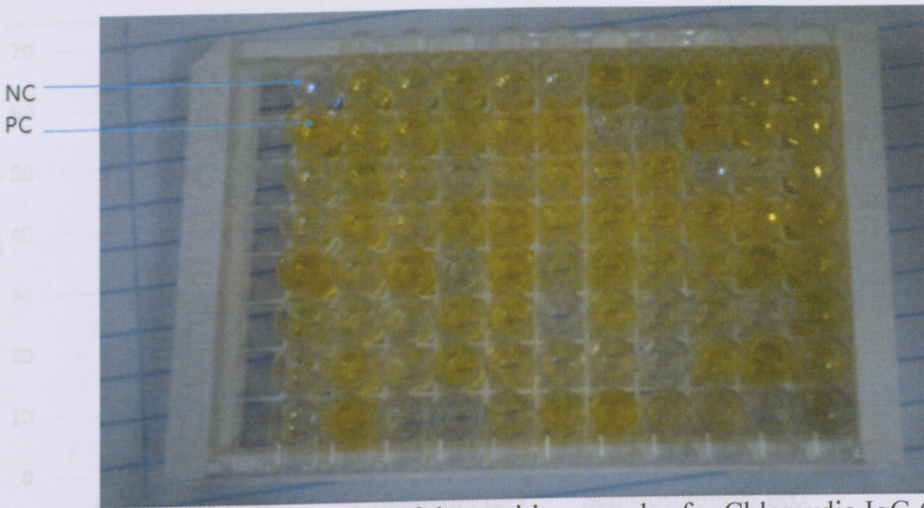


Figure 4.2 Elisa plate showing some of the positive samples for Chlamydia IgG (NC-negative control, PC-positive control)

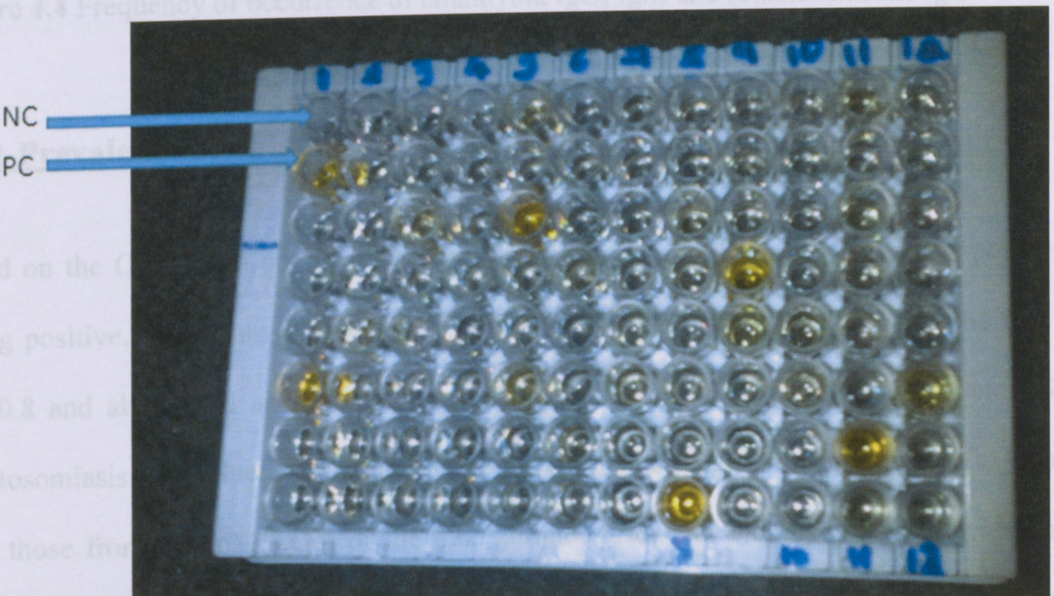


Figure 4.3. Micro-tire plate showing some of the positive samples for chlamydia IgM

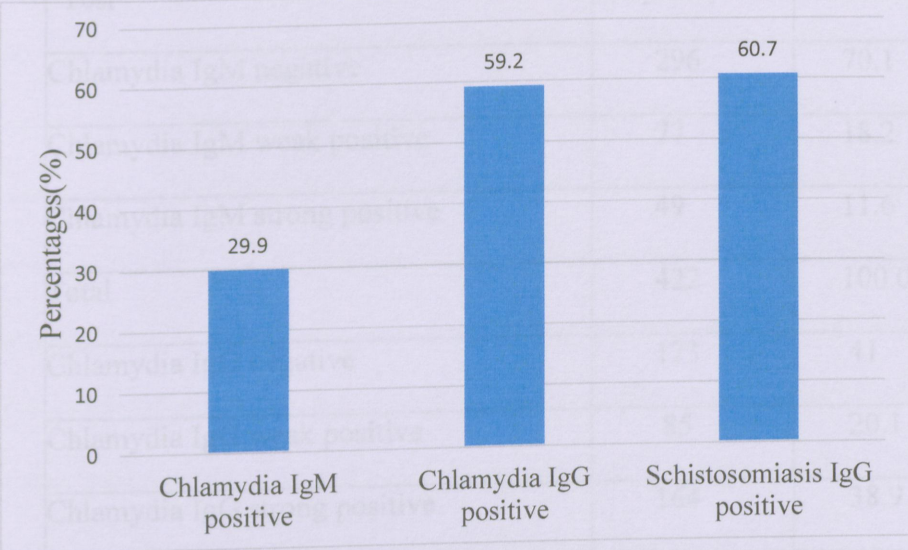


Figure 4.4 Frequency of occurrence of chlamydia IgG, IgM and schistosomiasis IgG

4.2.1 Prevalence of chlamydia and schistosomiasis in the tested samples

Based on the OD values obtained, the samples were classified as negative, weak positive and strong positive. For chlamydia, samples were classified as strong positive when the OD value was 0.8 and above and as weak positive when the OD value was between 0.4 and 0.7. For schistosomiasis OD values of 0.6 and above were considered as strong positive. Weak positive were those from 0.2 to 0.5.Out of the 422 samples tested for chlamydia IgM, 11.6% showed strong positivity. For Chlamydia IgG 38.9% showed to be strongly positive. For Schistosomiasis IgG only 19.9% showed to be strongly positive. This is illustrated on table 4.2

Table 4.2 Prevalence of chlamydia and schistosomiasis in the tested samples

Test	Frequency	Percentage (%)
Chlamydia IgM negative	296	70.1
Chlamydia IgM weak positive	77	18.2
Chlamydia IgM strong positive	49	11.6
Total	422	100.0
Chlamydia IgG negative	173	41
Chlamydia IgG weak positive	85	20.1
Chlamydia IgG strong positive	164	38.9
Total	422	100
Schistosomiasis IgG	147	39
Schistosomiasis IgG weak positive	155	41.1
Schistosomiasis IgG strong positive	75	19.9
Total	377	100

4.2.2 Distribution of chlamydia IgG based on gender, age and CD4 count

The results obtained are illustrated on table 4.3,which shows that females (62.2%) were mostly infected than males (54.5%) and the difference was statistically significant (p=0.009). The highest rate of infection was among participants who are 46 years and older (71.8%) and the lowest rate was amongst those who are 0-15 years old (44%), however the difference was not statistically significant (p=0.069). There was not much difference observed while looking at the CD4 count of the participants, those who had CD4 count between 350 to 499cells/μL were mostly infected (61.8%) than by those with a CD4 count between 5 and 349cells/μL with 60.1%, also the results were not statistically significant (p=0.868).

Table 4.3 Distribution of Chlamydia IgG based on gender, age and CD4 count

Characteristics		Chlamydia IgG positive	Total	Statistics
Gender	male	61 (54.5%)	112	**χ^2=9.365,p=0.009**
	Female	155 (62.2%)	249	
Age group	0 to 15 Years	8 (44.4%)	18	χ^2=7.088,p=0.069
	16 to 25 years	27 (60%)	45	
	26 to 45 years	121 (56.3%)	215	
	Above 46 years	51 (71.8%)	71	
Cd4 count	5-349	86 (60.1%)	143	χ^2=0.284,p=0.868
	350-499	55 (61.8%)	89	
	>500	71 (58.2%)	1222	

4.2.3 Distribution of Chlamydia IgM based on age, gender and CD4 count

For Chlamydia IgM females were more positive (36.5%) than males (20.5%) and the results were statistically significant (p=0.002). Based on the age group, participants who were between 26-45 years of age were mostly positive (34.4%), the least positive for IgM was observed among participants who were 0-15 years of age, however the difference was not statistically significant (p=0.326). Based on the CD4 count, not much difference was observed. Participants who had a CD4 count of 500 and more were mostly positive (34.4%), as compared to those with 350-499 cells/μL (30.3%) and those with 5-349 cells/μL (30.1%).The difference was not statistically significant (p=0.715) The results are illustrated on table 4.4

Table 4.4 Distribution of chlamydia IgM based on gender, age and CD4 count

Characteristics		Chlamydia IgM negative	Chlamydia IgM positive	Total	Statistics
Gender	Male	89(79.5%)	23(20.5%)	112	**χ^2=9.165,p=0.002**
	Female	158 (63.5%)	91(36.5%)	249	
Age group	0-15 years	15 (83.3%)	3 (16.7%)	18	χ^2=3.460,p=0.326
	16-25 years	30 (66.7%)	15 (33.3%)	45	
	26-45 years	141 (65.6%)	74(34.4%)	215	
	Above 46 years	52(73.2%)	19(26.8%)	71	
Cd4 count	5-349	100(69.9%)	43(30.1%)	143	χ^2=0.671,p=0.715
	350-499	62(69.7%)	27(30.3%)	89	
	>500	80 (65.6%)	42(34.4%)	122	

4.2.4. Distribution of chlamydia by origin

Table 4.5 shows the distribution of *Chlamydia* by origin, using IgG as the immunologic marker, Waterberg had the highest rate of infection (60.5%) more than the other districts. However, no statistical significance was observed (p=0.878). Most of the cases were found in clinics (62.2%) than in hospital settings (54.5%) and the results were also not statistically significant (p=0.154). For Chlamydia IgM the highest rate was observed in Sekhukhune district (34.7%) and no statistical significance was observed (p=0.532) as well. The infection was more common in Clinics (32.9%) than in Hospitals (30.3%).

Table 4.5 Distribution of Chlamydia by Origin

Characteristics		Chlamydia IgG positive	Statistics
District	Capricorn	99(59.3%)	χ^2=0.261,p=0.878
	Sekhukhune	54(56.8%)	
	Waterberg	49(60.5%)	
Origin	Clinic	140(62.2%)	χ^2=2.033,p=0.154
	Hospital	72(54.5%)	
		Chlamydia IgM positive	
District	Capricorn	55(32.9%)	χ^2=1.264,p=0.532
	Sekhukhune	33(34.7%)	
	Waterberg	22(27.2%)	
Origin	Clinic	74(32.9%)	χ^2=0.256,p=0.613
	Hospital	40(30.3%)	

4.2.5. Distribution of Schistosomiasis based on gender, age and CD4 count

Table 4.6 illustrates the distribution of schistosomiasis by gender, age and Cd4 count. There was not much difference in terms of gender. Based on the age groups the highest infection rate was found within the younger age group with 62.5% as compared to 58.5% in those who were 26 to 45 years. Participants with a CD4 count between 5-349 were mostly positive (60.9%).

Table 4.6 Distribution of Schistosomiasis based on gender, age and CD4 count

Characteristics		Positive for Schistosoma IgG	Statistics
Sex	Male	58(58%)	χ^2=0.001,p=0.970
	Female	131(58.2%)	
Age Group	0 to 16 Years	8(50%)	χ^2=0.739,p=0.864
	16 to 25 years	25(62.5%)	
	26 to 45 years	113(58.5%)	
	Above 46 years	38(58.5%)	
CD4 count	5-349	78(60.9%)	χ^2=0.700,p=0.705
	350-499	49(57%)	
	>500	58(55.8%)	

4.2.6. Distribution of Schistosoma by Origin of the samples

Table 4.7 shows the distribution of schistosomiasis by origin. Waterberg had the highest rate of infections (61.3%) more than Sekhukhune (56%) and Capricorn (55.4%), however the difference was not statistically significant (p=0.705). Participants who were from clinics had higher prevalence (62.4%) than those in hospitals (52.1%).

Table 4.7 Distribution of Schistosoma by Origin of the samples

Characteristics		**Positive for Schistosoma IgG**	**Statistics**
District	Capricorn	82(55.4%)	χ^2=0.700,P=0.705
	Sekhukhune	47(56%)	
	Waterberg	46(61.3%)	
Origin	Clinic	126(62.4%)	χ^2=0.766,P=0.682
	Hospital	62(52.1%)	

4.2.7 Association between chlamydia and schistosomiasis

Table 4.8 shows the association between chlamydia IgM, IgG and schistosomiasis IgG. The results shows that 57.4% of the participants who were positive for schistosomiasis IgG also had 69.3% of chlamydia IgM. 53.1% of patients with schistosomiasis had 67.3 % of chlamydia IgG, and the results were statistically significant (p=0.002).

Table 4.8 Association between chlamydia and schistosomiasis

		Negative for schistosomiasis	Positive for schistosomiasis	Statistics
Chlamydia IgM	Chlamydia IgM negative	115(42.6%)	155(57.4%)	χ^2=4.631,p=0.037
	Chlamydia IgM positive	31(30.7%)	70(69.3%)	
Chlamydia IgG	Chlamydia IgG negative	75(48.7%)	79(51.3%)	**χ^2=9.641,p=0.002**
	Chlamydia IgG positive	71(32.7%)	146(67.3%)	

4.3 Genotyping of TLR2 Single Nucleotide Polymorphisms (-196 to -174 deletion polymorphism)

Allele specific PCR was employed to genotype the TLR2 gene in detection of the TLR2 (-196 to -174 deletion polymorphism). Figure 4.5 below represents the agarose gel obtained after amplification

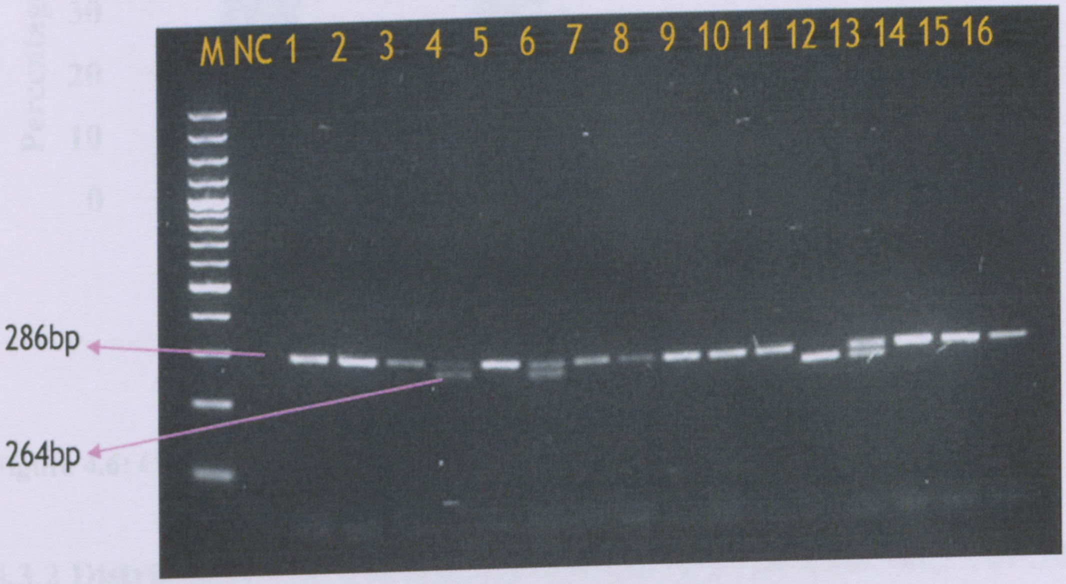


Figure 4.5: Electrophoretic pattern of fragments generated by allele specific polymerase chain reaction for the genotyping of TLR2 -196 to -174 deletion polymorphism.

M represents the molecular weight marker (100bp), NC-negative control, Lane 1-16 test samples. A single band at 286bp is indicative of a wild type (ins/ins) (lane 1, 2, 35, 7, 8, 9, 10, 1114, 15, 16). Double bands at both 286 and 264 indicates the heterozygote (ins/del) (Lane 4, 6 and 13) and lastly a single band at 264 indicates a homozygote (del/del) (lane 12).

4.3.1 Distribution of TLR2 genotypes in the study population

Distribution of the different TLR2 genotypes where (49.4%) ins/ins, 44.2% Ins/del, and 6.5% del/del showed on figure 4.6

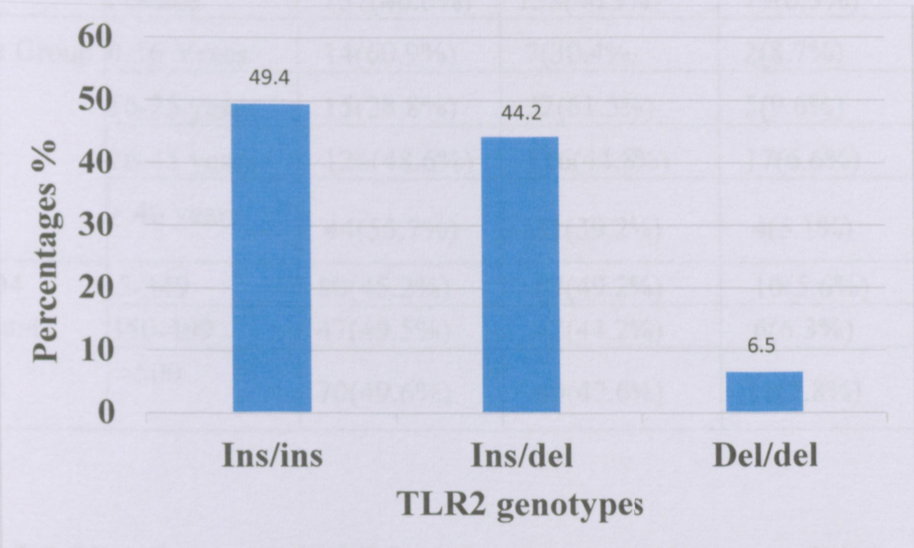


Figure 4.6: Overall distribution of TLR2 genotypes in the study population.

4.3.2 Distribution of TLR2 genotypes according to gender, age group and CD4 count of the study participants.

Table 4.9 shows the distribution of TLR2 deletion polymorphism according to gender, age group and CD4 count of the study participants. Most females harbored the ins/del genotype, 46.6% had ins/ins while only 6.5% had del/del genotype. Among males the most commonly occurring genotype was ins/ins (51.5%), Participants who were between the age group 0-16 years mostly had the ins/ins genotype (60.9%).

Table 4.9 Distribution of TLR2 genotypes based on age, gender and CD4 count of the study participants

Characteristics		TLR2 genotypes			Statistics
		ins/ins	ins/del	del/del	
Sex	Male	68(51.5%)	55(41.7%)	9(6.8%)	χ^2=1.034, p=0.596
	Female	137(46.6%)	138(46.9%)	19(6.5%)	
Age Group	0-16 Years	14(60.9%)	7(30.4%)	2(8.7%)	χ^2=11.669, p=0.070
	16-25 years	15(28.8%)	32(61.5%)	5(9.6%)	
	26-45 years	126(48.6%)	116(44.8%)	17(6.6%)	
	> 46 years	44(55.7%)	31(39.2%)	4(5.1%)	
CD4 Count	5-349	80(45.2%)	87(49.2%)	10(5.6%)	χ^2=1.784, p=0.775
	350-499	47(49.5%)	42(44.2%)	6(6.3%)	
	>500	70(49.6%)	60(42.6%)	11(7.8%)	

4.3.3 Distribution of TLR2 genotypes according to origin

Table 4.10 illustrates the distribution of TLR2 genotypes according to the origin of the samples. Capricorn district showed to have most of the Ins/Ins genotype (49.8%) more than Sekhukhune (47.7%) and Waterberg (45.6%). However Waterberg district had most of ins/del genotype (48.9%) compared to Sekhukhune (45.9%) and Capricorn Districts (43.3%). The del/del genotype was the least prevalent of all the observed genotypes with Capricorn at 7% followed by Sekhukhune (6.3%) and Waterberg with 5.6%, however no statistical significance was observed (p=0.391). In Clinics the most prevalent genotype was ins/ins (46.4%) as well as in hospitals (50%) but the difference was not statistically significant (p= 0.757).

Table 4.10 Distribution of TLR2 genotypes according to origin

Variables		TLR2 genotypes			Statistics
		Ins/ins	Ins/del	Del/del	
District	Capricorn	100(49.8%)	87(43.3%)	14(7%)	χ^2=1.189, p=0.391
	Sekhukhune	53(47.7%)	51(45.9%)	7(6.3%)	
	Waterberg	41(45.6%)	44(48.9%)	5(5.6%)	
Origin	Clinic	124(46.4%)	122(45.7%)	21(7.9%)	χ^2=1.183, p=0.757
	Hospital	77(50%)	70(45.5%)	7(4.5%)	

4.3.4 Association of chlamydia with TLR2 genotypes

The infections were associated with the different genotypes within TLR2 (-196 to 174 delete polymorphism). The samples which were positive for Chlamydia had most of the ins/del genotype (53%), ins/ins (40.2%) and del/del (6.8%) respectively although the results did not show any statistical significance (p=0.149). For participants who were positive for Chlamydia IgG the most commonly occurring genotype was found to be ins/ins (47.2%),ins/del (45.9%) and del/del(6.9%) respectively, however the difference was not statistically significant (p=0.531). The data has been shown on table 4 .11

A correlation between Chlamydia weak positive participants with the TLR2 polymorphism was investigated as well. Ins/del (p=0.006) and ins/ins (p=0.006) was significantly associated with chlamydia IgG weak infections. Results are shown on table 4.12

Table 4.11 Association of Chlamydia IgG and IgM with TLR2 genotypes

Variables		TLR2 genotypes			Statistics
		ins/ins	ins/del	del/del	
Chlamydia IgM	Chlamydia IgM negative	142(50%)	122(43.7%)	15(5.4%)	χ^2=3.812,p=0.149
	Chlamydia IgM positive	47(40.2%)	62(53%)	8(6.8%)	
Chlamydia IgG	Chlamydia IgG negative	80(48.5%)	78(47.3%)	7(4.2%)	χ^2=1.268,p=0.531
	Chlamydia IgG positive	109(47.2%)	106(45.9%)	16(6.9%)	

Table 4.12 Classification of chlamydia IgM and its association with TLR 2 genotype

TLR2 genotype	Chlamydia weak positive	Statistics
Ins/del	43 (61.4%)	**χ^2=7.654,p=0.006**
Ins/ins	23 (32.9%)	**χ^2=7.536,p=0.006**
Del/del	4(5.7%)	χ^2=0.001,p=0.971

4.3.5 Association of Schistosomiasis with TLR2 genotypes

Table 4.13 illustrates the association between schistosomiasis IgG and TLR2 genotype. Among the schistosomiasis positive samples the most frequently occurring genotype was ins/ins (50.2%)

Table 4.13 Association between schistosomiasis IgG and TLR2 genotypes

Variables		TLR2 genotypes			Statistics
		ins/ins	ins/del	del/del	
Schistosoma IgG	Schistosoma IgG Negative	63(43.2%)	75(51.4%)	8(5.5%)	χ^2=1.940,p=0.379
	Schistosoma IgG Positive	103(50.2%)	90(43.9%)	12(5.9%)	
Total		166(47.3%)	165(47%)	20(5.7%)	

4.13 Sequencing and phylogenetic analysis

4.13.1 Sequence analysis of the newly discovered TLR2 gene polymorphism

After amplification of the TLR2 gene polymorphism, the amplicons were sent to Inqaba (Inqaba Biotechnologies, Pretoria, South Africa) for sequencing. The sequences were analyzed using staden package and bioedit softwares. Figure 4.7 shows the chromatograms of the nucleotide sequences of TLR2 gene showing variations at position 175 (indicated by an arrow) (175 A/C, 175 C/A and 175 A/A) that were obtained during the editing process.

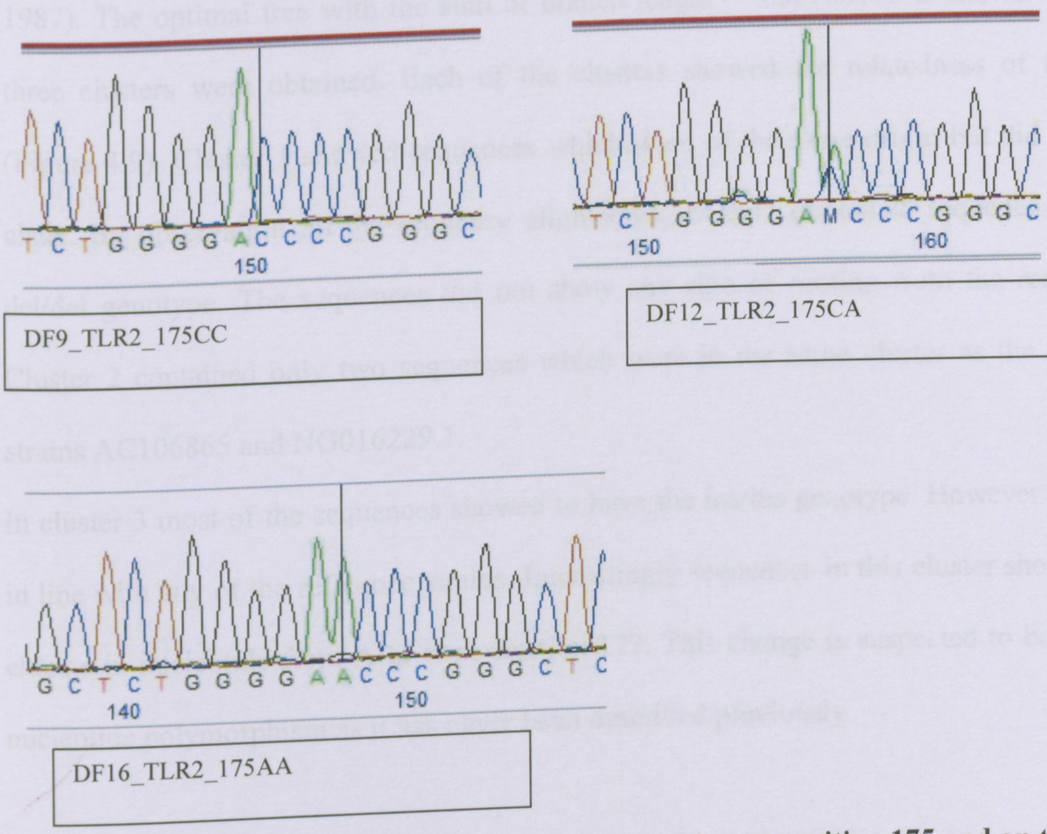


Figure 4.7: Chromatogram showing the polymorphism at position 175 and on the Toll like Receptor 2 gene.

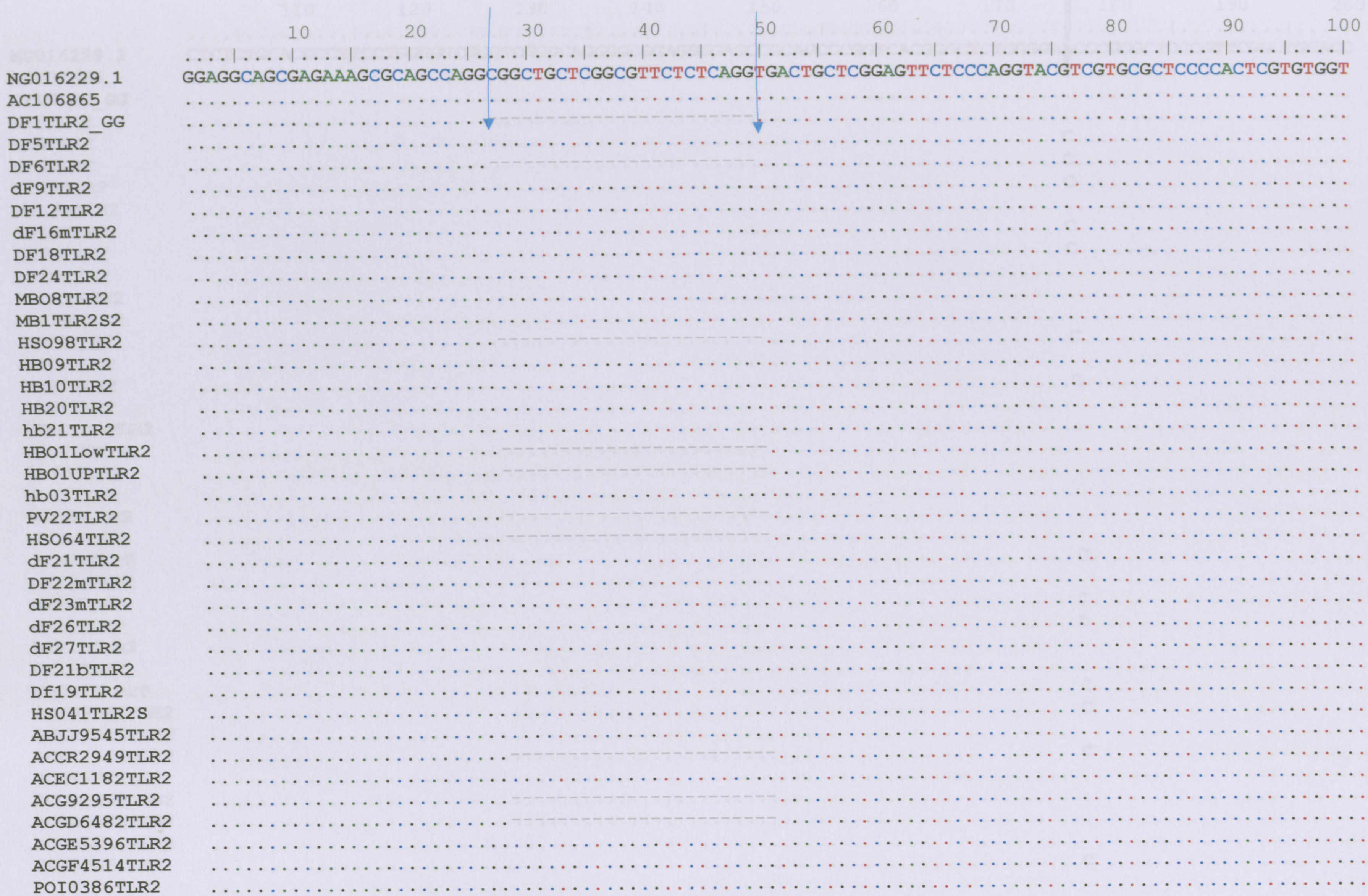
4.13.2 Sequence diversity of the TLR gene polymorphism

The nucleotide sequences of the TLR2 gene polymorphism were edited using Bioedit package (Hall, 1999), and aligned with two other sequences AC106865 and NG016229.1 obtained from Genebank. Using Clustaral W multiple aligner. Figure 4.8 shows the nucleotide sequence alignments of the TLR2 gene with the (-196 to -174 deletion) polymorphism (indicated by the arrows, from position 22 bp to 50 bp). Another change at position 175bp A/C was observed.

4.13.3 Phylogenetic tree analysis

Phylogenetic tree representing the evolutionary history of TLR2 (-196-174 delete polymorphism) sequences from South Africa was inferred using the Neighbor-Joining method (Saitou and Nei M 1987). The optimal tree with the sum of branch length = 8.30768110 is shown. From the tree three clusters were obtained. Each of the clusters showed the relatedness of the sequences (Figure 4.9). Cluster 1 showed sequences which were of the same origin but did not show any clade. By observation on the sequence alignments, cluster1 contained sequences that had the del/del genotype. The sequences did not show any sign of rooting from the reference strain. Cluster 2 contained only two sequences which were in the same cluster as the two reference strains AC106865 and NG016229.1.

In cluster 3 most of the sequences showed to have the ins/ins genotype. However they were not in line with any of the reference strains. Interestingly sequences in this cluster showed to have a change in nucleotide from A to C at position 177. This change is suspected to be a new single nucleotide polymorphism as it has never been described previously.


10
20
30
40
50
60
70
80
90
100
NG016229.1
GGAGGCAGCGAGAAAGCGCAGCCAGGCGGCTGCTCGGCGTTCTCTCAGGTGACTGCTCGGAGTTCTCCCAGGTACGTCGTGCGCTCCCCACTCGTGTGGT
AC106865
DF1TLR2_GG
DF5TLR2
DF6TLR2
dF9TLR2
DF12TLR2
dF16mTLR2
DF18TLR2
DF24TLR2
MBO8TLR2
MB1TLR2S2
HSO98TLR2
HB09TLR2
HB10TLR2
HB20TLR2
hb21TLR2
HBO1LowTLR2
HBO1UPTLR2
hb03TLR2
PV22TLR2
HSO64TLR2
dF21TLR2
DF22mTLR2
dF23mTLR2
dF26TLR2
dF27TLR2
DF21bTLR2
Df19TLR2
HS041TLR2S
ABJJ9545TLR2
ACCR2949TLR2
ACEC1182TLR2
ACG9295TLR2
ACGD6482TLR2
ACGE5396TLR2
ACGF4514TLR2
POI0386TLR2

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                       110       120       130       140       150       160       170       180       190       200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NG016229.1     CTCTCTGCACCCCTTCCTGGGGTCGGGTCGGGCAGGGGCGGAGGGGAGCCGCAGCCCGGTCACGGGCTCTGGGGAACCCGGGCTCCCGTTCGGCTGCACC
AC106865       ....................................................................................................
DF1TLR2_GG     ....................................................................................................
DF5TLR2        ....................................................................................................
DF6TLR2        ...........................................................................C........................
dF9TLR2        ...........................................................................C........................
DF12TLR2       ...........................................................................C........................
dF16mTLR2      ....................................................................................................
DF18TLR2       ...........................................................................C........................
DF24TLR2       ...........................................................................C........................
MBO8TLR2       ....................................................................................................
MB1TLR2S2      ....................................................................................................
HSO98TLR2      ....................................................................................................
HB09TLR2       ...........................................................................C........................
HB10TLR2       ....................................................................................................
HB20TLR2       ...........................................................................C........................
hb21TLR2       ....................................................................................................
HB01LowTLR2    ....................................................................................................
HB01UPTLR2     ....................................................................................................
hb03TLR2       ....................................................................................................
PV22TLR2       ....................................................................................................
HSO64TLR2      ....................................................................................................
dF21TLR2       ....................................................................................................
DF22mTLR2      ...........................................................................C........................
dF23mTLR2      ....................................................................................................
dF26TLR2       ...........................................................................C........................
dF27TLR2       ...........................................................................C........................
DF21bTLR2      ....................................................................................................
Df19TLR2       ....................................................................................................
HS041TLR2S     ...........................................................................C........................
ABJJ9545TLR2   ...........................................................................C........................
ACCR2949TLR2   ....................................................................................................
ACEC1182TLR2   ...........................................................................C........................
ACG9295TLR2    ....................................................................................................
ACGD6482TLR2   ....................................................................................................
ACGE5396TLR2   ....................................................................................................
ACGF4514TLR2   ....................................................................................................
POI0386TLR2    ...........................................................................C........................
```

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               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NG016229.1     TGGGCCCCTAGCTCCTGTCCGGGCGGGGATAGCGGGAAGCGCACCAGGCCCCCGGGACGCCGGTGCTTCTTTGCACGGCC
AC106865       ................................................................................
DF1TLR2_GG     ................................................................................
DF5TLR2        ................................................................................
DF6TLR2        ................................................................................
dF9TLR2        ................................................................................
DF12TLR2       ................................................................................
dF16mTLR2      ................................................................................
DF18TLR2       ................................................................................
DF24TLR2       ................................................................................
MBO8TLR2       ................................................................................
MB1TLR2S2      ................................................................................
HSO98TLR2      ................................................................................
HB09TLR2       ................................................................................
HB10TLR2       ................................................................................
HB20TLR2       ................................................................................
hb21TLR2       ................................................................................
HBO1LowTLR2    ................................................................................
HBO1UPTLR2     ................................................................................
hb03TLR2       ................................................................................
PV22TLR2       ................................................................................
HSO64TLR2      ................................................................................
dF21TLR2       ................................................................................
DF22mTLR2      ................................................................................
dF23mTLR2      ................................................................................
dF26TLR2       ................................................................................
dF27TLR2       ................................................................................
DF21bTLR2      ................................................................................
Df19TLR2       ................................................................................
HS041TLR2S     ................................................................................
ABJJ9545TLR2   ................................................................................
ACCR2949TLR2   ................................................................................
ACEC1182TLR2   ................................................................................
ACG9295TLR2    ................................................................................
ACGD6482TLR2   ................................................................................
ACGE5396TLR2   ................................................................................
ACGF4514TLR2   ................................................................................
POI0386TLR2    ................................................................................
```

Figure 4.8: nucleotide sequence alignments for TLR2 -196 – 174bp deletion polymorphism where NG_016229 and GU227616 represents reference strains obtained from gene bank.

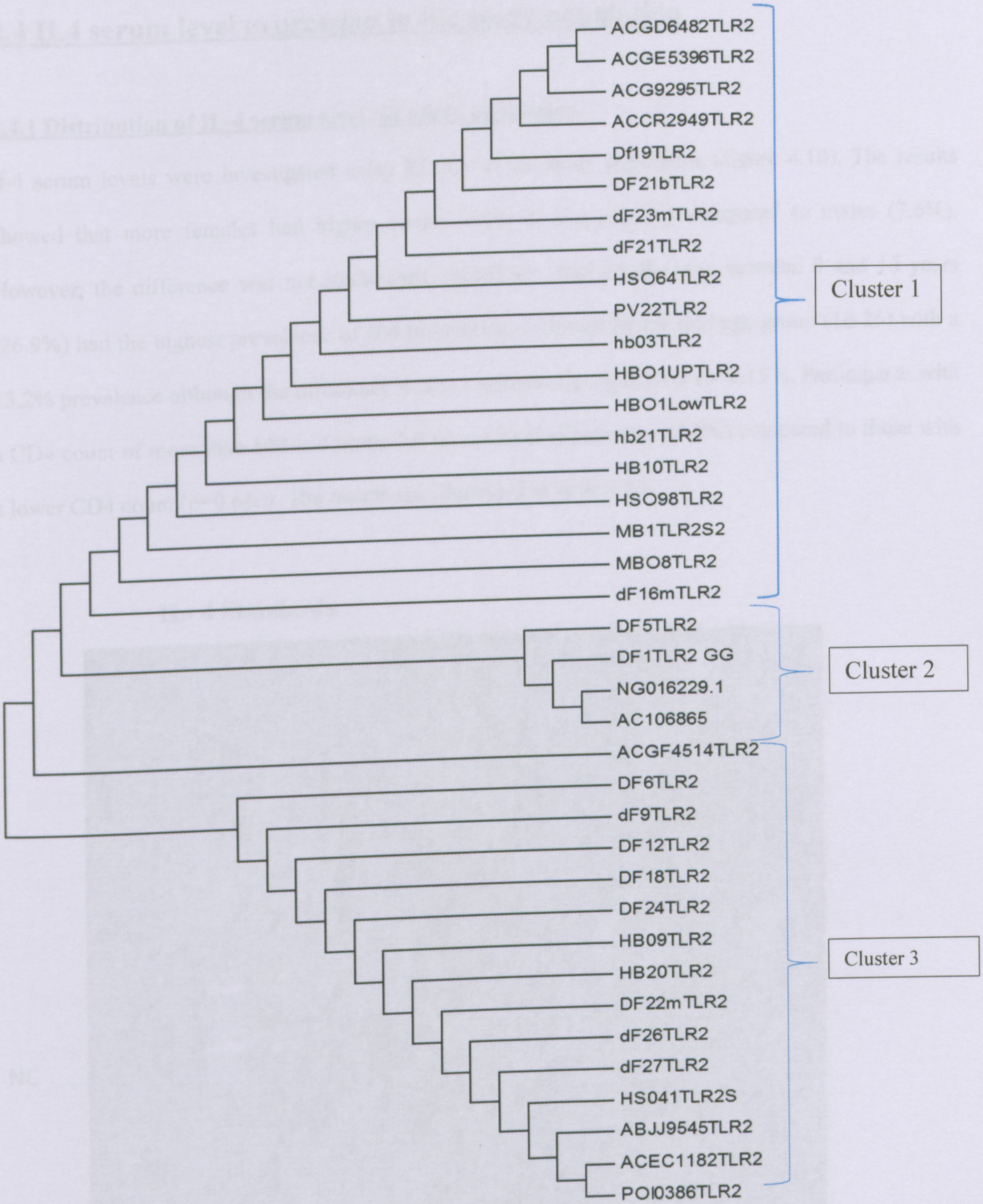
ACGD6482TLR2
ACGE5396TLR2
ACG9295TLR2
ACCR2949TLR2
Df19TLR2
DF21bTLR2
dF23mTLR2
dF21TLR2
HSO64TLR2
PV22TLR2
hb03TLR2
HBO1UPTLR2
HBO1LowTLR2
hb21TLR2
HB10TLR2
HSO98TLR2
MB1TLR2S2
MBO8TLR2
dF16mTLR2
DF5TLR2
DF1TLR2 GG
NG016229.1
AC106865
ACGF4514TLR2
DF6TLR2
dF9TLR2
DF12TLR2
DF18TLR2
DF24TLR2
HB09TLR2
HB20TLR2
DF22mTLR2
dF26TLR2
dF27TLR2
HS041TLR2S
ABJJ9545TLR2
ACEC1182TLR2
POI0386TLR2
Cluster 1
Cluster 2
Cluster 3

Figure 4.9 Neighbor-joining phylogenetic tree of the TLR2 gene sequences

4.4 IL4 serum level expression in the study population

4.4.1 Distribution of IL-4 serum level the study population

Il-4 serum levels were investigated using ELISA in the study population (figure 4.10). The results showed that more females had higher serum levels of IL4 (15.1%) compared to males (7.6%). However, the difference was not statistically significant. Individuals aged between 0 and 16 years (26.9%) had the highest prevalence of IL4 production, followed by the mid age group (16-25) with a 13.2% prevalence although the difference was not statistically significant (p=0.157). Participants with a CD4 count of more than 500 had more IL4 serum level expression (14.9%) compared to those with a lower CD4 count (p=0.682). The results are illustrated in table 4.14.

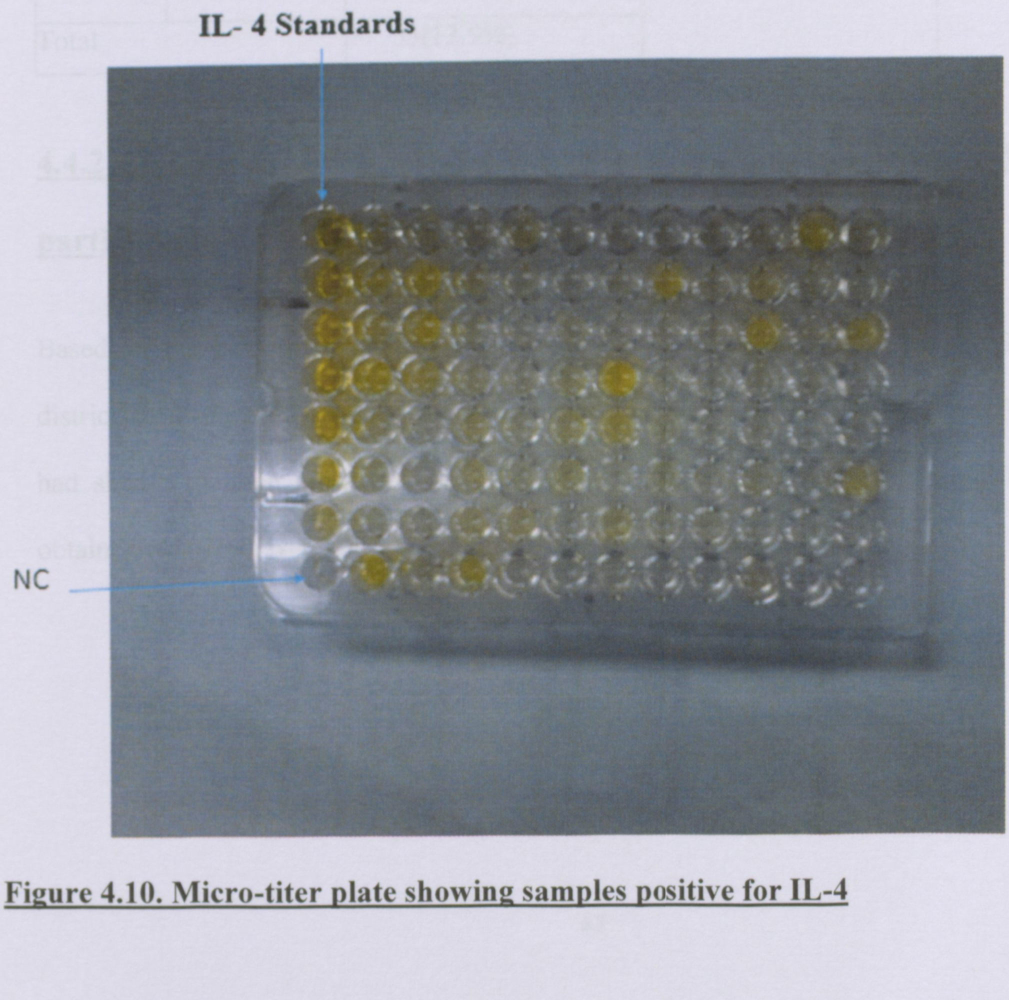

Figure 4.10. Micro-titer plate showing samples positive for IL-4

Table 4.14 Distribution of IL-4 serum level in the study population

Characteristics		Positive for IL-4	Statistics
Gender	Male	10(7.6%)	χ^2=4.544, p=0.033
	Female	47(15.1%)	
Total		57(12.9%)	
Age Group	0 - 15 Years	7(26.9%)	χ^2=5.217, p=0.157
	16- 25 years	6(10.5%)	
	26 - 45 years	34(13.2%)	
	> 46 years	9(10.5%)	
Total		56(13.1%)	
cd4count	5-349	23(12%)	χ^2=0.765, p=0.682
	350-499	11(11.7%)	
	>500	21(14.9%)	
Total		55(12.9%)	

4.4.2 IL4 serum level expression according to the origin of the Study participants

Based on the origin of the samples most of the patients who were from the Sekhukhune districts had more IL-4 serum expression and most of the participants who are from clinics had shown to have more IL-4 expression. In both cases, no statistical significance was obtained (p=0.760, p=0.790) the results are illustrated in table 4.15

Table 4.15 IL4 serum level according to the origin of the study participants

		Positive for IL-4	Statistics
District	Capricorn	25(12.5%)	$\chi2$=0.549,p=0.760
	Sekhukhune	18(15.4%)	
	Waterberg	13(13%)	
Clinic	Clinic	37(13.3%)	χ^2=0.071,p=0.790
	Hospital	20(12.4%)	

4.4.4 IL-4 serum levels in relation to the infections

IL4 serum levels were investigated and associated with the presence or absence of the infection with chlamydia and schistosomiasis. Participants who were negative for chlamydia had more percentage for IL4 (14.1%) as compared to those who were chlamydia IgM positive (8.7%). For Chlamydia IgG those who were negative also had most percentages (15.3%) for serum levels as compared to those who were positive (10.7%), however in all the results no statistical significance was reached. Schistosomiasis negative patients also had more il4 serum levels (14.8%) as compared to the participants who were positive for schistosomiasis (12.4%), these results are shown on table 4.16

Table 4.16 Association of IL-4 serum levels with the infections

Characteristic		Positive for IL-4	Statistics
Chlamydia IgM	Chlamydia IgM negative	32(14.1%)	χ^2=1.870,p=0.171
	Chlamydia IgM positive	9(8.7%)	
Chlamydia IgG	Chlamydia IgG negative	19(15.3%)	χ^2=1.534,p=0.216
	Chlamydia IgM positive	22(10.7%)	
Schistosomiasis	Negative for Schistosomiasis	17(14.8%)	χ^2=0.365,p=0.546
	Positive for Schistosomiasis	24 (12.4%)	

4.5 Correlations between TLR2 genotypes with chlamydia, schistosomiasis and the demographic Characteristics of the study participants

Correlation between TLR2 (-196 to -174 del polymorphism) with the demographic characteristics and the CD4 count.

Bivariate analysis was performed to measure the strength of association between the polymorphism and the demographic characteristic. The TLR2 -196 to -174 ins/ins was strongly associated with younger age group (p=0.003). The results are shown on Table 4.17

Weak chlamydia IgM was highly associated with Ins/ins genotype, (p=0.006) and Ins/Del genotype (p=0.006). The results are shown on Table 4.18. Ins/del genotype was significantly associated with high IL-4 concentration (p=0.044).The results are shown on Table 4.19

Table 4.17 Bivariate correlations between the TLR2 -196 to -176 deletion with demographic characteristics and CD4 count.

Characteristics		Ins/Ins	INS/DEL	DEL/DEL
Age Group	χ^2	0.062	-0.071	0.019
	P value	0.211	0.148	0.708
	N	413	413	413
0-15 years	χ^2	-0.147**	0.126*	0.043
	P value	**0.003**	**0.010**	**0.386**
	N	413	413	413
16-25 years	χ^2	0.012	-0.006	-0.011
	P value	0.807	0.896	0.821
	N	413	413	413
26-45 years	χ^2	0.073	-0.057	-0.033
	P value	0.138	0.251	0.501
	N	413	413	413
Sekhukhune	χ^2	-0.006	0.008	-0.004
	P value	0.900	0.868	0.935
	N	402	402	402
Capricorn	χ^2	0.030	-0.040	0.020
	P value	0.550	0.424	0.686
	N	402	402	402
Waterberg	χ^2	-0.029	0.039	-0.020
	P value	0.561	0.435	0.691
	N	402	402	402
Clinic/Hospital	χ^2	0.034	-0.002	-0.064
	P value	0.483	0.962	0.189
	N	421	421	421
CD4COUNT	χ^2	0.031	-0.029	-0.006
	P value	0.524	0.563	0.903
	N	413	413	413
Low CD4	χ^2	-0.043	0.059	-0.031
	P value	0.379	0.232	0.528
	N	413	413	413
Mid CD4	χ^2	0.019	-0.017	-0.005
	P value	0.694	0.730	0.921
	N	413	413	413
High CD4	χ^2	0.028	-0.046	0.037
	P value	0.570	0.347	0.456
	N	413	413	413

Table 4.18 Bivariate correlations between the TLR2 -196 to -176 deletion with different types of infections.

Variables	Statistics	INS/INS	INS/DEL	DEL/DEL
Chlamydia IgM	χ^2	-0.098	0.085	0.029
	P value	**0.051**	**0.092**	**0.572**
	N	396	396	396
WeakCT IgM	χ^2	-0.138^{**}	0.139^{**}	-0.002
	P value	**0.006**	**0.006**	**0.971**
	N	396	396	396
StrongCTIgM	χ^2	0.025	-0.044	0.042
	P value	0.627	0.378	0.400
	N	396	396	396
Chlamydia IgG	χ^2	-0.027	-0.011	0.083
	P value	0.587	0.820	0.099
	N	396	396	396
WeakCTIgG	χ^2	0.042	-0.022	-0.043
	P value	0.408	0.668	0.394
	N	396	396	396
StrongCTIgG	χ^2	-0.042	-0.002	0.094
	P value	0.401	0.973	0.062
	N	396	396	396
Shistosoma IgG	χ^2	0.070	-0.074	0.008
	P value	0.191	0.168	0.882
	N	351	351	351
Weak schisto	χ^2	0.082	-0.090	0.018
	P value	**0.127**	**0.092**	**0.733**
	N	349	349	349
Strong schisto	χ^2	0.082	-0.090	0.018
	P value	**0.127**	**0.092**	**0.733**
	N	349	349	349

Table 4.19. Bivariate correlations between the TLR2 -196 to -176 deletion with Interleukin 4.

Variables		Ins/Ins	INS/DEL	DEL/DEL
IL4 concentration	χ^2	0.062	-.076	0.029
	P value	0.218	.133	0.566
	N	392	392	392
FoldIL4	χ^2	0.064	-.098	0.074
	P value	.206	.053	0.146
	N	392	392	392
lowIL4	χ^2	-0.073	.046	0.062
	P value	0.149	.367	0.223
	N	392	392	392
HighIL4	χ^2	0.082	-.102*	0.040
	P value	**0.103**	**0.044**	**0.425**
	N	392	392	392

CHAPTER 5:

Discussions and Conclusions

5.1 Discussion

5.1.1 Seroprevalence of *Chlamydia Trachomatis* in the study population using IgG and IgM as immunologic markers

The prevalence of sexually transmitted infections is high in South Africa although it varies significantly between sentinel populations (Johnson *et al.,* 2005). It varies even when compared to other African countries. In the present study out of the 422 samples tested 126 were positive for Chlamydia IgM, making an overall seroprevalence of 29.9%, however the results obtained differ substantially from the results that were obtained from other countries. For example in a study by Ghazi *et al.,* (2006) in Saudi Arabia a lower prevalence of 1.5% was reported for chlamydia IgM. The present study also shows an overall percentage of 59.2% for chlamydia IgG. In Zimbabwe Gowritz *et al.,* (2013) reported a prevalence of 36% for chlamydia IgG among women presenting with incident *Chlamydia trachomatis.*

These results are in contrary with the lower prevalence reported by Ghazi in 2006 and Kamel, 2013, who reported a prevalence of 8.7% and 9.84% among women in Saudi Arabia. Another study by Kadama *et al.,* (2014) also reported a lower prevalence of 5.6 % by IgG and 1.3% by IgM among pregnant women attending maternity hospital in Saudi Arabia. Gowritz *et al.,* (2013) also reported a lower prevalence of 11.9% among women presenting with incident *Chlamydia trachomatis* infection in Zimbabwe.The high prevalence observed in the present study indicates a dire need for chlamydia screening programs in the region.

Some of the sociodemographic risk factors for chlamydial infection include female gender, young age, nulliparity, urban settings and low income (Macmillan *et al.,* 2000 and Tobin *et al*., 2002). In the present study females were mostly infected (62.2%) than males (54.5%) for IgG and (36.5% versus 23.5%) for IgM. The difference was statistically significant (p=0.009) for IgG and for IgM (p=0.002). Ghazi *et al* (2006), Naidoo *et al.,* (2014) and Crichton *et al.,* (2014) also reported a high prevalence among females compared to males. This is due to high susceptibility factors such as cervical ectropion in females than males as well as the asymptomatic nature of STI's particularly in women.

In many countries which have chlamydia screening programs (for example Sweden and Canada), age is used as a primary selective determinant. In the present study, the highest prevalence was observed among participants aged between of 16 to 25 years. This is a trend that has been observed in many other studies around the globe (Gazhi *et al.,* 2006, Kamel, 2013, Naidoo *et al.,* 2014). This is explained by the higher rate of sexual activity among young women as well as their anatomy because during young adulthood, women's columnar epithelial cells (which are more sensitive to invasion by sexually transmitted pathogens) extends out over the vaginal surface of the cervix where they are unprotected by the cervical mucus, but recede to a more protected location as women age.

The present study also attempted to find a correlation between the CD4 counts of the study participants with the reported infections. The highest prevalence for chlamydia IgG was observed among individuals whose CD4 count was between 5-349cell/µl, but for IgM, participants whose CD4 count was greater than 500 cell/µl had most of the infections. In both cases no statistical significance was observed (p=0.868, p=0.326), therefore no correlation was found between CD4 count and the infection. Similar outcomes were observed in a study conducted in India by Bhattar *et al.,* (2014) and Magnus *et al.,* (2003). Tositti *et al.,* 2005 also reported an inverse relation between Chlamydia and CD4 count in a study on influence of Sex hormones, HIV Status, and concomitant sexually transmitted Infection on cervicovaginal inflammation. In contrary Ghosh *et al.,* (2011) found a significant correlation between CD4 cell count and the detection of *C. trachomatis* in HIV infected individuals.

5.1.2 Seroprevalence of schistosomiasis in the study population

Schistosomiasis is the second most prevalent tropical infection after Malaria, but is first among the neglected tropical diseases (NTDs) (Adenowoa et al., 2015). It mainly affects populations living in poverty without adequate sanitation and in close contact with infectious vectors and domestic animals as well as livestock. Prevalence of schistosomiasis, at present, is still high in sub-Saharan Africa (Simon, 2016). In South Africa however, most of the research on schistosomiasis have been conducted in Mpumalanga, Kwa-Zulu Natal and the Eastern Cape (Appleton and Naidoo, 2012). In Limpopo Province schistosomiasis is considered as an endemic disease (Van Bogaert, 2010). However, its sero-prevalence has not been extensively studied.

In the present study, an overall prevalence of schistosomiasis exposure was 60.7%, the results are in accordance with the result obtained by Samie *et al.,* (2010) who reported that schistosomiasis in the Vhembe region of Limpopo Province occurs the whole year round with the prevalence varying between 43% and 92%. In Eastern Cape Province, South Africa, a pilot study was conducted among school children in Hobeni School west of Port St John where a prevalence of 73.2% was obtained. In 1990, Mqoqi *et al*., (1996) found 42% of school children to be infected with schistosomiasis in the Port St Johns area. The high prevalence observed in the study might be due to inadequate water supply in the districts as well as poor sanitation in some parts of the province.

In contrary, Dawaki *et al.,* (2015) reported a prevalence of 17.8% among rural communities in Nigeria while Botes *et al.,* (2013) also reported a lower prevalence of 10.2% in Mpumalanga Province South Africa. Sadlier *et al.,* 2013 also reported a prevalence of 10%

among HIV infected patients from endemic areas attending European infectious disease clinic. In China, a low prevalence of 3.17% was observed following serum examinations in infection-controlled regions in Anhui Province.

Prevalence of infection with Schistosoma species varies by age and gender. Population age profiles typically shows an increase in the prevalence and intensity of infection from the youngest age group to the early teenage but there is often a decline in the middle to late teen age (Bowie *et al.,* 2004). Infection with schistosomiasis has been shown to be more evident and intense in males than in females (Zalata *et al.,* 2005, Bowie *et al.,* 2004), but it has more damaging consequences to females as it can cause reproductive health complications. In the present study, there was not much difference observed between females and males, although females showed to have slightly more percentages of schistosomiasis IgG 58.2% than males 58% but the difference was not statistically significant (p=0.970). This may be an indication that both genders were being equally exposed to the infection. Previous reports also indicated that both genders had almost similar prevalence for example in Nigeria, *Schistosoma haematobium* was shown to be non-gender specific (Useh and Ejezie, 1999, Agbolade *et al.,* 2004).

However other studies showed conflicting results, a non-significant difference was observed by Samie *et al.,* (2010) among university students where females were more prone to the infection with *Schistosoma haematobium* than males. In Nigeria Tohon *et al.,* (2008) also found similar outcomes. In Brazil, Parraga and colleagues (1996) also showed a higher prevalence in boys than girls. In contrary Meents and Boyles (2010) in Eastern Cape Province, South Africa, indicated that the infection rate in boys was 76.9% more than the 69.8% found in girls. Furthermore, a study by Ossai *et al.* (2012) in Nigeria found that the infection rate was higher in males (37%) than in females (31.2%) but the difference was not statistically significant (p=0.08).

The highest prevalence was observed in participants between ages of 16-25 years, but there was no statistical significance (p=0.864). Samie *et al.,* (2010) found the highest prevalence among females between 21-25 years. In Nigeria, Ossai *et al*., (2014) reported a highly significant prevalence among children aged between 13-15 years (52.8%) similarly, Dada, (2015) also found the highest prevalence in children between ages of 13-16 years. Disparities of schistosomiasis being non-gender and age specific has been reported, but the highest prevalence obtained among participants between 16-25 years may be attributed to the reason by Samie *et al,*. (2010) that individuals are often more involved in water related household chores like cleaning, washing clothes and dishes fetching water from the rivers and probably fishing during the age of 15 to 25 years.

Schistosomiasis has been suggested to be a widespread cause of immune suppression in Africa (Kellestrup, 2005). In the present study most infections were among participants who had a CD4 count of 5-349. Our study extends to a study by Elliott *et al.,* (2003) that compared baseline characteristics of schistosome-infected and uninfected HIV-positive patients, where schistosome-infected patients had higher baseline CD4 counts and CD4:CD8 ratios but comparable viral loads.

5.1.3 Association between chlamydia and schistosomiasis

Many studies have shown an overlap in the epidemiology of sexually transmitted infections (STIs) and urogenital schistosomiasis among young women living in areas where schistosomiasis is endemic (Yirenya –Tawiah *et al.,* 2014). In the present study although this outcome did not form part of the overall objective, a more significant correlation between Chlamydia IgG and Schistosoma IgG was found (p=0.002). A study by Haberberger and colleagues in 1993 also reported a highly significant association between patients with active *schistosoma* infection and those with urine and urethral specimens positive for chlamydia (p =0.01).

5.1.4 Effects of TLR2 genetic polymorphism in the study population

Host genetic factors such as polymorphisms in inflammatory and immune response genes, are mainly related to the recognition of the bacteria by the immune system and the variation in the level of cytokine response (Garza-González *et al*., 2005). This polymorphism is located on chromosome 4 and it causes a 22-bp nucleotide deletion that alters the promoter activity of the gene. The del/del genotype is reported to show decreased transcriptional activity of the TLR2 gene as compared to the ins/ins genotype.

In our study population, a higher percentage of ins/ins (49.4%) genotype and 44.2% of the ins/del genotype was obtained. The lowest prevalence was found for the genotype del/del with 6.5%. Nischalke *et al.,* (2012) found a percentage of 60.8% ins/ins, 33.3% ins/del and 5.9% del/del in patients with chronic HCV. However, de Oliviera *et al.,* (2012) found a significantly higher prevalence of *TLR2* ins/del and del/del among people who had gastric cancer compared to the healthy individuals. The ins/del (53%) and del/del (6.8%) genotypes were overexpressed in participants who were chlamydia IgM positive as compared to those who were negative but the results did not reach any statistical significance (p=0.149). These results show similarity with those obtained in a pilot study by Kalinderi *et al.,* (2013) while investigating TLR9 -1237 T/C and TLR2 -196 to -174 del polymorphisms on the risk of Parkinson's disease in the Greek population. However, a different case was observed in the present study where the ins/del and del/del genotypes were overexpressed in patients who were positive for chlamydia IgG than in chlamydia IgM (45.9% and 6.9%), but the difference was also not statistically significant (p=0.149). Based on the seropositivity of the samples, chlamydia weak positive showed a significant correlation with Ins/Ins (p=0.006) and Ins/del (p=0.006). This indicates a positive association between TLR2 Ins/Ins genotype with weak chlamydia IgM and could be suggestive of the protective role of the Ins/Ins genotype.

For schistosomiasis IgG, participants who were negative carried mostly the ins/del (51.4%) and ins/ins (5.5%) genotype compared to those who were positive (45.9% and 5.9%) and the results did not reach statistical significance. The failure of the present study to demonstrate the association between the TLR2 -196 to -174 deletion polymorphism with schistosomiasis is suggestive of a possibility that the polymorphisms does not have any effect on the infections. Moreover, the lack of enough participants who had the del allele for the polymorphism has prevented us from assessing its function and association between the diseases.

5.1.5 IL-4 Serum Levels In The Study Population.

According to existing information, cytokines participates actively in the pathogenesis of infection with Chlamydia. Shavlakadze and Gorgoshidze, (2010) showed that as the levels of IL-12 were elevated in patients who were chlamydia positive, IL-4 levels seemed to be low to normal. In our study, the highest percentage of IL-4 was observed in patients who were negative for both chlamydia IgG and IgM as compared to those who were positive, but in both cases no statistical significance was reached. Jha *et al.,* (2009) reported that Il-4 serum levels together with IL-8, IL-13 and ICAM-1 were enhanced following an infection with *Chlamydia pneumonia*.

Acquired immunity to schistosomiasis infection has been reported to correlate with elevated serum levels of antigen specific IgE (Medhat *et al.,* 1998). The mechanisms by which IL-4 production participates in the elimination of the parasite remain uncertain. In the present study, participants who were negative for the parasite IgG showed more expression of IL-4 levels, this can be an indication of the protective role of interleukin 4 in the infection with schistosomiasis. However, it has been suggested that the role of interleukin 4 in protection might be more complex because IL-4 stimulates immunoglobulin E and Immunoglobulin G4 production in human helminthic infection (King *et al.,* 1993). However, during schistosomiasis the absence of IL-4 is sufficient enough to inhibit the development of an antigen- specific type 2 response (Cheever *et al.,* 1994).

5.2 Limitations of the study

Genetic association studies usually requires a larger set of samples, so one of the limitations in the present study is the sample size which was smaller and could not be used as a representative of the whole population in the district covered in the study population.

ELISA used for the detection of antibody against schistosomiasis only one antibody (IgG) was used which can only detect the past encounter but not the current infection.

Since the study was focusing on sexually transmitted infections and helminthes, the species studied for schistosomiasis should have also been specified for example *Schistosoma haematobium* which shows to have almost the same adverse effects as chlamydia.

5.3 Conclusions

The study highlighted an alarming high prevalence of the infections with chlamydia and schistosomiasis. Chlamydia was found to be much common in females than in males. Schistosomiasis was common in our study population and its occurrence was not gender specific. Therefore there was not much significant difference between both males and females. There was a significant correlation observed between chlamydia and schistosomiasis. Our study showed that chlamydia and schistosomiasis should be prioritised for health programs in the Limpopo Province. This will aid in assuring that there are less ectopic pregnancies among women and reduced bladder and stomach cancer in the future as a result of the sequelae presented by the burden of these two infections.

As the family of TLR is comprised of multiple members participating in complex TLR signalling pathways more studies examining the genetic role of different polymorphisms in different TLR genes are needed in order to clarify the role of TLR genes in the pathogenesis of infectious diseases. The 22 base pair deletion polymorphism at TLR2 has shown an association with weak chlamydia, suggesting that the polymorphism might not have an impact on the occurrence of schistosomiasis, however more larger, better designed studies need to be carried out using appropriate molecular and statistical methods to further analyse the associations.

The deletion polymorphism on TLR2 interestingly had a change from A to C, which was suspected to be a new SNP. Therefore, the newly identified SNP should be evaluated using a larger sample size, more sensitive methods of genotyping as well as sequencing for example next generation sequencing should be used to look into the new SNP in detail as well as to validate the findings of the present study.

5.4 Recommendations

Looking at the high prevalence of schistosomiasis in the present study, it is clear that the disease does not get enough attention it deserves therefore regular education and treatment campaigns must be undertaken to teach people about the infections and rid them of the consequences due to the disease.

Considering the outcomes of the present study, more surveys on schistosomiasis are needed in Limpopo Province at large in order to properly design and execute control programs that can help eradicate the infection and get people in rural and semi urban areas more informed of the risk factors, consequences as well as the burden that occurs as a result of the infection.

There should be an improvement in sanitation and access to safe water supply in regions in order to minimize the risk of people getting infected.

People in the general population, both young and old should be educated on the negative implications of voiding their fecal waste as well as urine into the water body, which will ultimately lead to reduction of transmission of not only schistosomiasis but other water transmitted diseases as well.

Chlamydia infection has also shown a higher prevalence among women, which calls for an urgent need for females to be screened for STI's and get the necessary treatment.

Chlamydia screening programs should also be prioritized following possibility of lack of information concerning the infection and its devastating long term consequences.

Vaccination should also be prioritized so that the community can be protected against this debilitating burden of disease, therefore candidate molecules that can be used as vaccine targets should be urgently identified, studied and tested.

CHAPTER SIX:

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