GENETIC AND PHENOTYPIC VARIATIONS OF

TRICHOMONAS SPP IN HUMANS

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This thesis is presented for the degree of Doctor of Philosophy, 2018
DECLARATION

I solemnly declare that this thesis is my own account of my research and contain as its main content work which has not previously been submitted for a degree at any tertiary educational institution

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Daniel Sai Squire
Population genetic studies of *Trichomonas vaginalis* have detected high genetic diversity associated with phenotypic differences in clinical presentations. In this study, microscopy and multi-locus sequence typing (MLST) were used to identify and genetically characterise *T. vaginalis* isolates from patients in Australia and Ghana. Epidemiological data were also obtained from the Ghanaian population. Of 479 women recruited from Ghana, 63 (13.2%; 95% CI 9.5-17.6) and 78 (16.8%; 95% CI 12.2-21.1) had *T. vaginalis* based on microscopy and PCR respectively. Regional prevalence of *T. vaginalis* infection was 21.7% (95% CI 17.1-26.9%) and 12.8% (95% CI 8.6-18.3%) for Volta and Greater Accra regions respectively. There was a significant difference in prevalence between the two regions (Fisher exact test, *P* =0.013). *Trichomonas vaginalis* infection was significantly associated with vaginal itch (OR = 1.38, *P* = 0.04), a prior history of stillbirth (OR = 3.62, *P* = 0.04) and participation in oral sex (OR = 1.10, *P* = 0.04). There were no significant associations between genotypes at any locus and clinical presentation score. Seventy-one polymorphic nucleotide sites, 36 different alleles, 48 sequence types, 24 of which were novel, were identified among 178 isolates, revealing a genetically diverse *T. vaginalis* population. Polymorphism was found at most loci among both Australian and Ghanaian isolates, although there was some variation between countries. The number of alleles for each locus ranged from two to nine. Study results confirmed geographic expansion and diversity of the *T. vaginalis* population. Two-type population were identified in this study. Linkage disequilibrium was observed, suggesting that the *T. vaginalis* population is highly clonal. Multilocus disequilibrium was observed even when analysing clades separately, as well as widespread clonal genotypes, suggesting that there is no evidence of recent recombination. The potential impact of genetic diversity on varied pathology observed among *T. vaginalis*
infected individuals requires further research. Ethical clearance for this study was granted by
the Ethics and Review Committee of Murdoch University and the Human Ethics Committee
of the Ghana Health Service.
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List of Abbreviations

16SrDNA  16 Small-unit ribosomal DNA
AFLP  Amplified fragment length polymorphism
AICc  Akaike information criterion, corrected
AP  Adhesion protein
C3  Complement 3
CDF  Cell- detaching factors
COX  Cyclooxygenase
CP  Cellular proteins
CPI-GC  Ceramide phospho-inositol glycan core
DNA  Deoxyribonucleic acid
EcoRI  Restriction endonuclease enzyme from Escherichia coli
ELISA  Enzyme-linked immunosorbent assay
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GLM  Generalised linear model
HIV  Human Immunodeficiency virus
HSP  Heat-shock protein
IgA  Immunoglobulins A
IgG  Immunoglobulins G
IL  Interleukin
iNOS  Nitric oxide synthase
ITS  Internal transcribed spacer
ITS  Internal transcribed spacer analysis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LnP(D)</td>
<td>Log probability of the data</td>
</tr>
<tr>
<td>LPG</td>
<td>Lipophosphoglycan</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>mPCR</td>
<td>Multiplex PCR</td>
</tr>
<tr>
<td>NAAT</td>
<td>Nucleic acid amplification techniques</td>
</tr>
<tr>
<td>NJ</td>
<td>Neighbour joining</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RSH</td>
<td>Repetitive sequence hybridization</td>
</tr>
<tr>
<td>SABC</td>
<td>State Agriculture Biotechnology Centre</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOPs</td>
<td>Standard operating procedures</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-strand conformation polymorphism</td>
</tr>
<tr>
<td>SSR</td>
<td>Single sequence repeats</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>START</td>
<td>Sequence type analysis and recombinational tests</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infection</td>
</tr>
<tr>
<td>TARC</td>
<td><em>Trichomonas</em>-associated reproductive complications</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TVV</td>
<td><em>Trichomonas vaginalis</em> virus</td>
</tr>
<tr>
<td>TYM</td>
<td>Trypticase-yeast extract medium</td>
</tr>
<tr>
<td>ZOTU</td>
<td>Zero operational taxonomic unit</td>
</tr>
</tbody>
</table>
PUBLICATIONS


CONFERENCE ABSTRACTS


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Special thanks go to the Almighty God for His help and strength throughout this journey of my study.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 TRICHOMONADS

Trichomonads are anaerobic flagellated protozoa inhabiting the genitourinary and digestive tracts of humans and other vertebrates (Maritz et al., 2014). *Trichomonas vaginalis* and *Tririchomonas foetus* are the aetiological agents of human and bovine genitourinary trichomoniasis respectively. Vaginal trichomoniasis is considered the most common non-viral sexually transmitted disease of humans, affecting an estimated 170 million people worldwide every year (Ginocchio et al., 2012). Infection is concentrated mainly in developing countries as well as in socio-economically disadvantaged groups (Sutton et al., 2007) and has major medical, social and economic implications.

*Trichomonas vaginalis* is known to elicit a broad range of clinical symptoms (Petrin et al., 1998). In women, the presentation of the infection ranges from totally asymptomatic to a severe vaginitis characterized by foul-smelling discharge, itching and occasionally pain. In pregnant women, *T. vaginalis* infection has been linked to significant morbidity. It is noted for its role in the causation of a number of *Trichomonas*-associated reproductive complications, including premature membrane rupture, preterm labour and low birth weight, as well as vaginitis, cervicitis, urethritis, and pelvic
inflammatory disease (Cotch et al., 1997; Nanda, 2006). In recent times, the parasite has also been linked with urethritis and prostate cancer in men (Stark et al., 2009; Tsai & Li, 2013; Menezes et al., 2016), and has been found to increase the risk of infection with human immunodeficiency virus (HIV) (McClelland et al., 2007; Shafir et al., 2009; Kissinger & Adamski, 2013).

The parasite inhabits the mucosal surfaces of the genitourinary tract of humans (Fichorova, 2009). Despite being extracellular in nature, *T. vaginalis*, through a variety of mechanisms, can cause damage to the mucosal membrane. Some of these mechanisms depend on epithelial cell contact (e.g. adherence-based cytotoxicity) while others do not (e.g. release of soluble factors such as proteinases) (Fichorova, 2009). *Trichomonas vaginalis* infection is also known in most cases to excite the host immune response, resulting in inflammation and further mucosal damage (Fichorova et al., 2006; Fichorova, 2009).

Despite being one of the most common non-viral sexually transmitted infections, the epidemiology, genetic variation and pathogenicity of the *T. vaginalis* remains poorly studied. In particular, there is a paucity of information on the extent to which genetic variation in *T. vaginalis* impacts on the clinical presentation of the infection. Also, previous studies on the phenotypic characteristics of *T. vaginalis*, have often been limited to small sample size, and not considered clinical phenotypes.

Parabasalia are single-celled flagellated eukaryotes that belongs to the supergroup Excavata and subgroup Metamonada (Simpson, 2003; Adl et al., 2005; Hampl et al., 2009), including parasites and commensals of vertebrate hosts, commensals and endosymbionts of invertebrates, and a few described free-living species. Among the parasitic parabasalids, several are important agents of human urogenital, subgingival, oral, bronchial and gastrointestinal infections (Malik et al., 2011).
Morphological and molecular phylogenetic analysis in recent years have led to the identification of six parabasal groups; Trichomonadea, Tritrichomonadea, Hypotrichmonadea, Cristamonadea, Spirotrichonympha and Trichonympha (Ohkuma et al., 2009; Carpenter et al., 2010; Cepicka et al., 2010; Noda et al., 2012), different from previous identification of two groups based on morphological characters observed, using light microscopy: large (~200 µm) multiflagellated forms typically found in termite and cockroach hindguts are commonly referred to as “hypermastigotes” and smaller (~10–20 µm) flagellates, found in both vertebrate and invertebrate hosts, are called “trichomonads” (Malik et al., 2011). The use of morphological characteristics has resulted in the description of over 80 genera and 400 parabasalid species. Eleven genera have been identified in the family Trichomonadidae; Tetratrichomonas, Pseudotrypanosoma, Pentatrichomonas, Pentatrichomonoides, Lacusteria, Cochlosoma, Trichomitopsis, Tritrichomonas, Histomonas, Dientamoeba and Trichomonas. Several species of these genera have been reportedly found harbouring different sites in a wide range of vertebrate hosts (Gould et al., 2014). About 36 species of the genus Trichomonas have so far been identified (https://www.ncbi.nlm.nih.gov/Taxonomy).

Trichomonads are anaerobic protists with modified mitochondria. They have one or more flagella and are mostly symbionts or commensals. Trichomonads generally have between three to five anterior flagella and an undulating membrane which is a recurrent flagellum running along the surface of the cell in a wave. They also have an axostyle, which runs centrally through the body, protruding at the posterior end, except for Dientamoeba fragilis and Pentatrichomonoides spp., which have a peripheral axostyle that tapers and protrudes posteriorly. While some trichomonads are free-living, a number of parasitic species exist (Table 1.1). Among the parasitic group are those of human importance, harbouring the urogenital, subgingival, oral, bronchial and gastrointestinal tracts (Malik et al., 2011). Four species have been identified; Dientamoeba fragilis, Pentatrichomonas hominis, Trichomonas vaginalis and Trichomonas tenax. Trichomonas vaginalis
and *T. tenax* are human-specific, while *D. fragilis* and *P. hominis* have also been isolated from domestic and farm animals, indicating a varied host range and the possibility of zoonotic transmission (Maritz *et al.*, 2014). *Pentatrichomonas hominis* and *T. tenax* are morphologically identical with *Trichomonas vaginalis* but inhabit different sites: *T. vaginalis* in the genitourinary tract, *P. hominis* in the gut and *T. tenax* in the oral cavity. Epidemiological and clinical studies have suggested the possibility of human trichomonads other than *T. vaginalis* being involved in the causation of vaginal trichomoniasis. For example, *P. hominis* has been isolated from vaginal swab specimens using nucleic acid amplification techniques, although this may arise from specimen contamination during sampling or handling, and has also been attributed to short ano-vaginal distances which could contribute to microbial colonisation of the vagina (Crucitti *et al.*, 2004).
<table>
<thead>
<tr>
<th>Host</th>
<th>Parasite species</th>
<th>Site of infection</th>
<th>Pathogenicity</th>
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<td>Human</td>
<td><em>Trichomonas tenax</em></td>
<td>mouth</td>
<td>commensal</td>
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<td></td>
<td><em>Trichomonas vaginalis</em></td>
<td>genitourinary tract</td>
<td>trichomoniasis</td>
</tr>
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<td></td>
<td><em>Pentatrichomonas hominis</em></td>
<td>caecum &amp; colon</td>
<td>commensal</td>
</tr>
<tr>
<td></td>
<td><em>Trichomonas foetus</em></td>
<td>lungs</td>
<td>pulmonary trichomoniasis</td>
</tr>
<tr>
<td></td>
<td><em>Dientamoeba fragilis</em></td>
<td>gastrointestinal tract</td>
<td>commensal</td>
</tr>
<tr>
<td>Other mammals</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Rhesus monkey</td>
<td><em>Trichomonas macacovaginiae</em></td>
<td>vagina/ urethra</td>
<td>vaginal trichomoniasis</td>
</tr>
<tr>
<td>Pig</td>
<td><em>Trichomonas foetus</em></td>
<td>genital tract</td>
<td>vaginal trichomoniasis</td>
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<td><em>Trichomonas ovis</em></td>
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<td>intestinal tract</td>
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<td></td>
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<td>diarrhoea</td>
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<td><em>Trichomonas equibuccalis</em></td>
<td>mouth</td>
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<td><em>Trichomonas butreyi</em></td>
<td>caecum &amp; colon</td>
<td>diarrhoea</td>
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<td>(Cattle)</td>
<td><em>Trichomonas rotunda</em></td>
<td>caecum &amp; colon</td>
<td>non-pathogenic</td>
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<td></td>
<td><em>Trichomonas suis</em></td>
<td>intestine, nasal passage</td>
<td>diarrhoea</td>
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<tr>
<td></td>
<td><em>Trichomonas foetus</em></td>
<td>intestine</td>
<td>diarrhoea</td>
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<tr>
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<td><em>Trichomonas pavlovi</em></td>
<td>large intestine</td>
<td>diarrhoea</td>
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<td><em>Trichomonas enteris</em></td>
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<td>Rodents</td>
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<td>caecum</td>
<td>commensal</td>
</tr>
<tr>
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<td><em>Trichomonas venoni</em></td>
<td>caecum &amp; colon</td>
<td>not known</td>
</tr>
<tr>
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<td><em>Trichomonas minuta</em></td>
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<td>not known</td>
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<td></td>
<td><em>Trichomonas microti</em></td>
<td>caecum</td>
<td>not known</td>
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<td>Guinea pig</td>
<td><em>Trichomonas caviae</em></td>
<td>caecum</td>
<td>non-pathogenic</td>
</tr>
<tr>
<td>Birds</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Duck</td>
<td><em>Trichomonas anatis</em></td>
<td>intestine</td>
<td>not known</td>
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<tr>
<td>Domestic fowl</td>
<td><em>Trichomonas eberthi</em></td>
<td>caecum</td>
<td>not known</td>
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<td>Pigeon</td>
<td><em>Trichomonas gallinae</em></td>
<td>upper digestive tract</td>
<td>metastatic lesions</td>
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<td><em>Trichomonas gallinarum</em></td>
<td>caecum &amp; liver</td>
<td>hepatic lesions</td>
</tr>
<tr>
<td>Birds</td>
<td><em>Histomonas melagrindis</em></td>
<td>liver and caecum</td>
<td>enterohepatitis, histomonas</td>
</tr>
<tr>
<td>Duck and Turkey</td>
<td><em>Cochlosoma anatis</em></td>
<td>intestines</td>
<td>enteritis</td>
</tr>
</tbody>
</table>
1.2 REPRODUCTION AND LIFE CYCLE

Although the general outline of the life cycle of *T. vaginalis* (Figure 1.1) is well known, the details are poorly understood. The parasite is found in the urogenital tract of both male and female hosts where it exists only in the trophozoite form with no cystic stage (Petrin *et al.*, 1998). The trophozoites reproduce asexually by mitosis, followed by longitudinal binary fission occurring every 8-12 hours under favourable conditions. A previous study, however, indicated that there may be preceding developmental stages before the appearance of the mononuclear flagellates (Abonyi, 1995). These round forms are believed to be morphologically different from the smaller, flagellated forms and are thought to undergo amitotic budding instead of mitotic division (Abonyi, 1995; Petrin *et al.*, 1998). These round forms of the parasite have not been found in the human host, however, but only in dividing growth phase culture. They include aflagellate forms, flagellate forms with dividing nuclei, and flagellate forms without dividing nuclei, and are presumed to occur only under unfavourable conditions, such a high level of *Lactobacillus acidophilus* during menstruation or very high vaginal pH (Petrin *et al.*, 1998). This is yet to be studied extensively in the human host and remains speculative. To date, no direct evidence of meiosis and sexual reproduction has been observed in *T. vaginalis* or other trichomonads, although recombination events have been inferred from population genetic data (Ramesh *et al.*, 2005; Malik *et al.*, 2008).
1.3 CLINICAL FEATURES OF INFECTION

The clinical impact of *T. vaginalis* infection is largely restricted to women and is often associated with a well outlined clinical syndrome and considerable morbidity, with regimented diagnostic procedures for detection of infection. This is often not the case in men, where the infection is associated with poorly defined clinical signs and symptoms, uncertain morbidity and no clearly structured diagnostic procedures.

In women, clinical features may range from asymptomatic, subclinical, to overt vaginitis (Shafir *et al.*, 2009; Van Der Pol, 2016). What accounts for this is not yet clear, but could be attributed to
differences in the virulence of individual trichomonad strains as well as host susceptibility. Vaginal discharges and inflammatory responses are common clinical features of *T. vaginalis* infection. The vaginal discharges may be of any colour or characteristic (Swygard *et al*., 2004) and range from whitish to a yellowish colouration, which may be malodorous with pH >6.0 (Garber, 2005) and purulent. This is often accompanied by erythema of the vagina and vulva and abnormal vaginal odour (Hainer & Gibson, 2011). Pruritus, dysuria, lower abdominal pain, irritation of the vulva and inflammation have all been associated with the infection in women (Hussein *et al*., 2015), as well as colpitis macularis (strawberry cervix, believed to result from microscopic, punctate haemorrhages of the cervix) and cervical friability (Seña *et al*., 2007; Van Der Pol, 2016). Complications in women have been frequently described, particularly pelvic inflammatory disease in those patients co-infected with HIV, cervical intraepithelial neoplasia and reproductive health sequelae including premature rupture of membranes, preterm delivery and low-birth-weight infants (Cotch *et al*., 1997; Viikki *et al*., 2000; Moodley *et al*., 2002).

In males, infection is largely asymptomatic, although it has been implicated as a cause of urethritis, dysuria, and prostatitis (Krieger, 1990; Abdolrasouli *et al*., 2007). Spontaneous resolution of male trichomonal urethritis may occur (Kanno & Sobel, 2003), although this is not consistent with earlier findings (Watt & Jennison, 1960). Epididymitis and infertility have also been reported as complications (Krieger, 1995; Seña *et al*., 2007), although in most cases the infection is self-limiting (Valadkhani *et al*., 2008).

### 1.4 Diagnosis of Infection

Most persons infected with *T. vaginalis* show no symptoms therefore over-reliance on clinical manifestations to commence treatment is not recommended. The poor predictive value of the signs
and symptoms of infection makes it imperative for an efficient laboratory diagnostic technique to be adopted to ensure early therapy and the avoidance of transmission to other sexual partners. There are, however, controversies regarding which diagnostic method is most appropriate for detection of this parasite.

1.4.1 Wet mount microscopy

Wet mount microscopy is the most cost effective laboratory diagnostic method for the detection of *T. vaginalis*. This technique, also called saline wet mount, involves the examination of vaginal or cervical secretions for the characteristic motility and morphological features of the protozoon.

There is reported variation in the sensitivity of wet mount microscopy, ranging from 38%-82% (Leli *et al.*, 2016). This variation can be attributed, at least in part, to different reference culture media (Patel, 2000). Other reasons for reduced sensitivity include delayed examination of wet mount specimens resulting in reduced parasite motility (Kingston, 2003), the presence of non-motile trichomonads amongst copious epithelial cells and polymorphonuclear leucocytes in the vaginal and cervical exudates, impairing the visualization of the parasite (Garber, 2005) and low protozoan numbers in exudates (Fouts & Kraus, 1980).

These shortcomings notwithstanding, the wet mount technique generally has a high specificity and considered in most health facilities to be the most acceptable routine method for diagnosis. Recent studies have reported a specificity rate of 100% for this diagnostic technique (Leli *et al.*, 2016; Nabweyambo *et al.*, 2017).
1.4.2 Culture

Cultivation of *T. vaginalis* is considered a more sensitive diagnostic method than wet mount preparation, as it permits low numbers of the protozoan present in exudates to be amplified and identified (Garber, 2005; Patil *et al.*, 2012). Although this technique can be more sensitive, it is time consuming and labour intensive. A swabbed sample is immersed in culture broth contained in a pouch and is incubated at 37°C. Observation of the culture is performed daily until the seventh day for the presence of trichomonads. Different culture media exist for this purpose. The constituents are often buffered salt solution, protein hydrolysates, liver digests or yeast extracts, reducing agents and serum. Antibiotics and antifungals are added to eliminate bacterial and fungal growth which may serve as contaminants. Penicillin, streptomycin, gentamycin, neomycin, and chloramphenicol are some antibiotics that may be included in the media. Antifungals such as nystatin, amphotericin and miconazole may also be used.

A modification of the standard culturing technique is the in-pouch system, which is a two-chambered bag which allows immediate wet mount microscopy and culture to be performed through the bag without the traditional daily sampling (Petrin *et al.*, 1998). This method has been found to perform equally well as culturing and daily sampling using the modified tryppticase-yeast extract medium with a sensitivity of 88.2% as compared to 91.12% for culturing (Adu-Sarkodie, 2005).

1.4.3 Serology

*Trichomonas vaginalis* is reported to have eight serotypes (Garber *et al.*, 1986). Previous studies have demonstrated the use of wide range serological techniques for the detection of circulating *T. vaginalis* antibodies in infected persons, including complement fixation, haemagglutination, gel
diffusion, fluorescent antibody and ELISA. (Jaakmees et al., 1966; Teras et al., 1966; Mason, 1979; Mathews & Healy, 1983; Sibau et al., 1987). Most of these studies have demonstrated the presence of Immunoglobulin (Ig) G in the sera of the infected persons. The antibody response to the pathogen has been attributed to several factors; the live or inactivated form, inoculum size, and the frequency and the length of exposure (Bhatt et al., 1996). ELISA has successfully been used to detect the presence of circulating antibodies, using whole cell and aqueous protein extracts as antigens (Alderete & Garza, 1984). This study also detected antibodies in persons with no current or previous infection history. This could be attributed to the presence of natural antibodies. Nevertheless, compared to haemagglutination and fluorescence antibody, ELISA has been shown to better correlate with past and current infection, suggesting its use as an alternative method of diagnosis (Cogne et al., 1985).

1.4.4 Molecular techniques

Nucleic acid amplification tests, including PCR and transcription-mediated amplification, have previously been developed and validated for the detection of T. vaginalis (Crucitti et al., 2003; Lee et al., 2012). A number of different primer sequences have been developed for this purpose (Table 1.2).

The high sensitivity of this technique makes it highly susceptible to contamination with minute quantities of exogenous DNA. This can be amplified together with the target template. A common type of contamination is carry-over contamination. This occurs usually when previous amplicons are introduced into new reaction mixture yet to be amplified. This can, however, be reduced when strict adherence to standard operating procedures is observed during PCR. Using aerosol barrier pipette tips, designing separate working areas for DNA extraction, PCR sample set-up and post
amplification analysis, assigning different pipettes for each working area and changing of gloves before preparing new samples are some of the procedures that can be adhered to. Adopting the use of premixed reaction components also minimizes contamination. Setting up of negative controls alongside the test reaction also helps to check for contamination.
<table>
<thead>
<tr>
<th>Primer used</th>
<th>Target region of parasite</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV16S</td>
<td>18S rRNA gene</td>
<td>(Simpson et al., 2007)</td>
</tr>
<tr>
<td>BTUB9/2</td>
<td>3β-tubulin gene</td>
<td>(Wendel et al., 2002)</td>
</tr>
<tr>
<td>TV8/9</td>
<td>actin gene</td>
<td>(Spotin et al., 2016)</td>
</tr>
<tr>
<td>TV10/11</td>
<td>actin gene</td>
<td>(Spotin et al., 2016)</td>
</tr>
<tr>
<td>TFR1/2</td>
<td>5.8S rRNA &amp; ITS regions</td>
<td>(Rivera et al., 2009)</td>
</tr>
<tr>
<td>TV18S</td>
<td>first half of 18S rRNA region</td>
<td>(Mao &amp; Liu, 2015)</td>
</tr>
<tr>
<td>TVK3/7</td>
<td>unspecified</td>
<td>(dos Anjos Gatti et al., 2017)</td>
</tr>
<tr>
<td>TVA5/6</td>
<td>A6p target sequence conserved region</td>
<td>(Riley et al., 1992)</td>
</tr>
<tr>
<td>TricV-O/TricV-I</td>
<td>not specified</td>
<td>(Lawing et al., 2000; Naing et al., 2010)</td>
</tr>
<tr>
<td>Actin-As1/Actin-S1</td>
<td>actin gene</td>
<td>(Matini et al., 2014)</td>
</tr>
<tr>
<td>Actin-As2/Actn-O</td>
<td>actin gene</td>
<td>(Matini et al., 2014)</td>
</tr>
<tr>
<td>TVA 5/6</td>
<td>A6p genome of <em>T. vaginalis</em>**</td>
<td>(Shaio et al., 1997; Madico et al., 1998)</td>
</tr>
<tr>
<td>AP65 A/B</td>
<td>conserved region in the ap65 adhesin gene</td>
<td>(Alderete et al., 1995a)</td>
</tr>
<tr>
<td>Hydrogenase A</td>
<td>entire hydrogenosome gene</td>
<td>(Bui &amp; Johnson, 1996)</td>
</tr>
<tr>
<td>α-succinyl CoA</td>
<td>entire α subunit of the hydrogenosomal synthetase</td>
<td>(Lahti et al., 1994)</td>
</tr>
<tr>
<td>TV1/2</td>
<td>18S rRNA region ***</td>
<td>(Mayta et al., 2000)</td>
</tr>
<tr>
<td>TVK3/4/7</td>
<td></td>
<td>(Kengne et al., 1994)</td>
</tr>
</tbody>
</table>

*Although the use of the B-tubulin has successfully been used to identify the parasite, it fails to detect some strains due to strain variations.

**The A6p region is found to be highly selective for a broad range of *T. vaginalis* isolates. This presents a relatively high potential for its use in diagnosis of the parasite.

*** The 18S rRNA region is the most unconserved region and this makes it suitable for genetic variation and population studies.
1.5 INFECTION AND PATHOGENESIS

Despite the severe outcomes that can occur in *T. vaginalis* infection, the mechanisms underlying pathogenesis are poorly understood, even less so in the male than the female host. The following discussion therefore focuses on processes of infection and pathogenesis in women.

1.5.1 Invasion and adhesion

The usual mode of transmission of *T. vaginalis* is through sexual contact. The parasite is believed to undergo an incubation period of 4-28 days (Harp & Chowdhury, 2011) when it enters the female host, with the vaginal mucus layer being the first point of contact. The mucus layer is rich in mucin, which presents a fortified barrier to the parasite. Adhesion to the vaginal epithelial cells is paramount in the pathogenesis of the parasite as the parasite’s ability to colonize the host depends mainly on the outcome of this interaction with the host’s mucus layer (Alderete & Pearlman, 1984; Alderete & Garza, 1985). *Trichomonas vaginalis* has between 11 and 23 different cellular proteins, most of which are lysosomal in their activities (Arroyo & Alderete, 1989; Garber & Lemchuk-Favel, 1994). Beside these proteins playing a major role in adhesion of the parasite to the mucus layer, they also have the ability to degrade host immunoglobulins G and A (IgG and IgA) (Provenzano & Alderete, 1995).

After adhering to the vaginal wall, the parasite assumes an amoeboid form with increasing cellular surface contact interaction. This leads to the formation of cytoplasmic projections that interdigitate with host target cells (Harp & Chowdhury, 2011). The cellular surface of *T. vaginalis* plays a vital role in this, as well as in host-parasite interaction and nutrient acquisition (Sommer *et al.*, 2005). Mucin secretion follows adhesion and this results in the
dissolution of the mucosal matrix of the host, thus detaching the parasite from the mucosal layer (Lehker & Sweeney, 1999). The parasite, aided by its flagellar movement, penetrates the dissolved matrix and colonizes the underlying epithelial cells (Lehker & Sweeney, 1999). As well as the cell-to-cell interaction, several other mechanisms are believed to contribute to the establishment of infection. These include haemolysis (Dailey et al., 1990; Fiori et al., 1993) and the excretion of soluble factors such as proteinases (Garber & Lemchuk-Favel, 1994; Mallinson et al., 1994) and cell-detaching factors (CDF) (Pindak et al., 1986).

During colonization of the vaginal microenvironment, constant changes occur. There is a drastic change during menstruation with the influx of erythrocytes, host molecules and serum constituents, as well as significant pH change (Petrin et al., 1998). The influx of erythrocytes provides nutrients for the parasite, as well as iron required for gene regulation (Petrin et al., 1998). The influx of the iron and its utilization by the parasite in the vaginal host results in oxidative stress, which in turn leads to upregulation of diverse heat shock proteins and various adhesins, immunogens, and C3-degrading proteinases (Sorvillo & Kerndt, 1998; Ryu et al., 2004). These are known to be cardinal in the parasite’s ability to deal with stresses in its host environment and persist in causing infection (Petrin et al., 1998).

### 1.5.2 Erythrocytic lysis

Lipids and iron are major nutritional requirements for *T. vaginalis*, but the parasite lacks the ability to synthesize them. To meet these nutritional needs, *T. vaginalis* depends solely on the vaginal mucosal erythrocytes through lysis, which is related to virulence (Lehker, 1990). This process is optimal at the normal vaginal pH of 4.5 (Fiori et al., 1993). Erythrocytic lysis is mediated by protein receptors found on the surfaces of both the parasite cell and erythrocytes.
in the vaginal mucosa (Fiori et al., 1993). Five adhesion proteins are known to be involved in this process; AP65, AP51, AP33, and AP23, all of which act as ligand receptors (Petrin et al., 1998). There is release of porphyrin-like proteins following the adhesion of the parasite to the vaginal mucosal erythrocytes and this creates pores in the erythrocyte membrane (Petrin et al., 1998). Cell lysis occurs when the parasite detaches itself from the cell.

1.5.3 Host Response to Infection

The attachment of \textit{T. vaginalis} to the vaginal cell surface results in leucocyte activation and secretion of interleukin-8 (IL-8), parasite-specific IgG, IgA, Th1 cytokines, leukotrienes, reactive nitrogen intermediates and macrophage inflammatory protein-3a, which induces nitric oxide synthase (iNOS), primes helper T cells, and promotes transmigration of neutrophils across the endothelium (Fichorova et al., 2006). While the overall response by the host to \textit{T. vaginalis} invasion is not clearly understood, it is believed that the activities of these complexes are the primary means by which the host responds to infection (Shaio, 1994, 1995).

Recent studies into the structural composition of \textit{T. vaginalis} suggest the parasite lipophosphoglycan (LPG) molecule, which plays a major role in parasite adherence, also has defined sites (an outer branch saccharide and ceramide phospho-inositol glycan core (CPI-GC)) which possess pro-inflammatory properties, activating nucleus factor kappa B, extracellular signal-regulated kinase 1/2 and mitogen-activated protein kinase kinase1/2 in the host macrophages during infection (Harp & Chowdhury, 2011). The LPG CPI-GC contains terminal poly-n-acetylactosamine repeats that represent the ligand for the animal lectins called galectins (Singh, 2009). During \textit{T. vaginalis} infection, the cervical and vaginal epithelial cells are thought to release galectin-1 and galectin-3. This results in a change in the
inflammatory responses in opposite mode; while galectin-1 suppresses inflammation, galectin-3 enhances leucocyte response to inflammation (Than, 2008; Fichorova, 2009). In-depth knowledge and understanding of the mechanisms involved are, however, lacking. Also, *T. vaginalis* is known to induce COX-2 expression, and up-regulates and activates toll-like receptors 2, 4, and 9 via the p38 mitogen-activated protein kinase pathway (Fichorova, 2009).

1.5.4 **Immune system evasion**

Although attempts have been made to explain the virulence factors of *T. vaginalis* responsible for evasion of the host immune system and the severity of the pathologic inflammatory reaction, in-depth understanding is still limited (Singh, 2009). Upon entering the host, resistance to complement-mediated destruction, molecular mimicry and coating with host plasma proteins are mechanisms adopted by *T. vaginalis* to elude the host’s immune system (Harp & Chowdhury, 2011). The complex carbohydrates on the surface of the parasite, which are involved in host invasion, are also believed to play a critical role in the evasion of the host’s immune system (Fichorova *et al.*, 2006).

1.5.5 **Resistance to complement mediated destruction**

Complement is deficient in the cervical mucus (Demes *et al.*, 1988b; Alderete *et al.*, 1995b) and the only source in the vagina is the menstrual blood. Although complement activity in menstrual blood is less than half that of venous blood (Demes *et al.*, 1988b; Alderete *et al.*, 1995b), there is appreciable complement-mediated cytotoxic activity toward *T. vaginalis* in menstrual blood, leading to a depletion in the parasite population during menses (Demes *et al.*, 1988a).
Demes et al. (1988a) found that fresh isolates of *T. vaginalis* differed in their susceptibility or resistance to complement-mediated lysis in serum and that complement-resistant fresh isolates became susceptible to complement after lengthy cultivation in vitro. Iron has been reported as a contributing factor in the resistance of *T. vaginalis* to complement (Alderete et al., 1995b). Cysteine proteinase expression, found to degrade the C3 portion of complement, appears to be upregulated by iron, allowing the parasite to evade complement-mediated destruction (Alderete et al., 1995b).

### 1.5.6 Molecular mimicry

In *T. vaginalis*, as in other parasitic protozoans, phenotypic variation and mimicry is a key factor in immune evasion. Two classes of markers expressed alternately on the parasite surface have been found (Alderete, 1992): the highly immunogenic glycoproteins (P270) and the adhesins (AP65, AP51, AP33, and AP23) (Alderete, 1992). Whereas P270 is synthesized by all *T. vaginalis* strains, there is variation in the cytoplasmic and cell surface expression of the glycoproteins, leading to A1 B2 (P270 positive) and A2 B1 (P270 negative) phenotypes (Alderete, 1988). Unlike P270 negative phenotype organisms, P270 phenotype positive organisms lack adhesins and thus cannot cytoadhere to host cells (Alderete, 1992). The P270 negative phenotype organisms lack cell surface expressions. This coupled with the presence of adhesins (which are immunorecessive in nature) enable the negative-phenotype organisms to avoid host antibody attack (Alderete, 1987a) by mimicking the host glycoproteins. The parasite may also evade the host’s immune response through the presence of P230, a high molecular weight immunogen on its surface. This immunogen also undergoes conformational change (epitope phenotypic variation), mimicking the host glycoproteins, thus, allowing it to evade the immune system (Alderete, 1987b).
1.5.7 Surface coating

Another mechanism of immune system evasion is surface coating and degradation of IgG, IgM, and IgA by cysteine proteins secreted by *T. vaginalis* (Provenzano & Alderete, 1995). Secretion of highly immunogenic soluble antigens by the parasite neutralizes antibody or cytotoxic T lymphocytes thereby short-circuiting specific anti-*T. vaginalis* defence mechanisms (Alderete & Garza, 1984). The parasite also coats itself with host plasma protein, escaping recognition by the host immune system (Peterson & Alderete, 1982). Consequently, antigen processing and presentation and complement-mediated lysis do not occur.

1.6 EPIDEMIOLOGY

1.6.1 Prevalence

Humans are a natural host for *T. vaginalis*. The parasite is cosmopolitan, and found across all racial and socioeconomic groupings. The term socioeconomic used here is in relation to both the behaviours of people, including the ways they interact with one another and their income and financial status. An estimated 190 million cases are recorded annually (Poole & McClelland, 2013) with most of these cases occurring in developing countries and low socioeconomic groups. Over the years, different studies have reported varied estimates of prevalence (Table 1.3). Prevalence estimates are affected by a number of factors, including: the geographic locality, socioeconomic status, size and age profile of the study population, the sampling strategy (e.g. whether community or clinic-based); and the diagnostic method used. Prevalence appears to be lower in men than in women, although there are a paucity of data on *T. vaginalis* infection in men (Bowden, 2000).
<table>
<thead>
<tr>
<th>Region</th>
<th>Country</th>
<th>Year</th>
<th>Sample size</th>
<th>Prevalence (%)</th>
<th>Diagnostic method</th>
<th>Sex</th>
<th>Group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>USA</td>
<td>1998</td>
<td>213</td>
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<td>New York city inmates</td>
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<tr>
<td></td>
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<td>454</td>
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<td>(Joyner et al., 2000)</td>
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<td>(Crosby, 2002)</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>2005</td>
<td>12449</td>
<td>1.7</td>
<td>Urine: PCR</td>
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<td>Overall US population</td>
<td>(Miller, 2005)</td>
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<td></td>
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<td>454</td>
<td>2.8</td>
<td>Urine: culture</td>
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<td>Native Americans</td>
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<tr>
<td>Region</td>
<td>Country</td>
<td>Year</td>
<td>Sample size</td>
<td>Prevalence (%)</td>
<td>Diagnostic method</td>
<td>Sex</td>
<td>Group</td>
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<td></td>
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<td></td>
<td>Urine &amp; Urethral swab: microscopy and PCR</td>
<td>Male</td>
<td>Mwanza</td>
<td>(Watson-Jones, 2000)</td>
</tr>
<tr>
<td></td>
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<td>1440</td>
<td>10.7</td>
<td>Urine: PCR</td>
<td>Females</td>
<td>Moshi Urban District</td>
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<td></td>
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<td>2006</td>
<td>688</td>
<td>6.3</td>
<td>Urine: PCR</td>
<td>Male partners</td>
<td>Moshi Urban District</td>
<td>(Klinger, 2006)</td>
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<td>Urine: PCR</td>
<td>Females</td>
<td>Moshi Urban District</td>
<td>(Klinger, 2006)</td>
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<td>Urine: PCR</td>
<td>Male partners</td>
<td>Moshi Urban District</td>
<td>(Klinger, 2006)</td>
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<tr>
<td>Africa</td>
<td>Madagascar</td>
<td>2005</td>
<td>643</td>
<td>7.4/23.4</td>
<td>Urethral swab/vaginal swab: Culture</td>
<td>Male/Female</td>
<td>Rural Madagascar</td>
<td>(Leutscher, 2005)</td>
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<td>1993</td>
<td>68</td>
<td>1.9</td>
<td>Vaginal swab</td>
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<td>Kinshasa</td>
<td>(Laga et al., 1993b)</td>
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<td>2011</td>
<td>460</td>
<td>27.1</td>
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<td>Kumasi</td>
<td>(Adu-Sarkodie, 2004)</td>
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<td>Vaginal swab: latex agglutination</td>
<td>Female</td>
<td>Kumasi</td>
<td>(Adu-Sarkodie, 2004)</td>
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<td></td>
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<td></td>
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<td>Female</td>
<td>Accra</td>
<td>(Apea-Kubi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2005</td>
<td>517</td>
<td>2.7</td>
<td>Vaginal swab: microscopy</td>
<td>Female</td>
<td>Port Harcourt</td>
<td>(Oluwayemisi, 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2010</td>
<td>220</td>
<td>4.5</td>
<td>Vaginal swab: microscopy</td>
<td>Female</td>
<td>Abakaliki</td>
<td>(Alo et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2010</td>
<td>1500</td>
<td>23.7</td>
<td>Vaginal swab: microscopy and culture</td>
<td>Female</td>
<td>Maidugri</td>
<td>(Mairiga et al., 2011)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>vaginal swab: culture</td>
<td>Female</td>
<td>Aba state</td>
<td>(Kanu, 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vaginal swab: microscopy</td>
<td>Female</td>
<td>Nasawara state</td>
<td>(Abioye, 2014)</td>
</tr>
</tbody>
</table>

Aggl: Agglutination; PCR: Polymerase Chain Reaction. Data represents prevalence from selected key countries across the globe.
1.6.2 Transmission

The usual mode of transmission of *T. vaginalis* is through sexual contact (Figure 1.1). Transmission frequency differs between men and women. While 70%-77% of female sexual partners of infected males become infected with *T. vaginalis* (Seña *et al.*, 2007; Meites *et al.*, 2015), only 14%-70% of male sexual partners of infected females have been found to be infected (Seña *et al.*, 2007; Hoots *et al.*, 2013; Kissinger, 2015; Meites *et al.*, 2015). This may be partly due to inefficient transmission from females to males, but can also be explained by spontaneous resolution of the infection and inefficient *T. vaginalis* diagnostics in men. It could also be deduced that the proportion of male sex partners who can be infected at a given time will be dependent on the frequency of sex within the partnership, the likelihood of transmission per sex act, and the duration of infection in men.

Although transmission of the parasite is usually by sexual intercourse, there have been reports of non-sexual transmission, through towels, soaps and toilet seats, occurring among households and school-children (Adu-Sarkodie *et al.*, 2007; Crucitti *et al.*, 2011). The frequency of non-sexual transmission of *T. vaginalis* is still controversial, however, and requires more detailed studies to eliminate the possibility of cross-contamination during sampling or specimen handling, establish infection routes and infer causative risk factors.

Humans are the only natural host for *T. vaginalis*. *Trichomonas vaginalis* transmission from mother to Newborn babies during birth has also been reported (Smith *et al.*, 2002). Transmission between same sexes via vulva contact among women practicing same-sex is also increasingly occurring (Bhunu & Mushayabasa, 2011). It is however believed that these women once thought to be straight, were likely to be harbouring the infection before becoming lesbians.
T. vaginalis transmission is influenced by several causal factors. These include sociodemographic, behavioural, age and racial/ethnicity (Shuter et al., 1998; Sorvillo et al., 1998; Sutton et al., 2007; Helms et al., 2008; Silva et al., 2013). The transmission of T. vaginalis is reported to be prevalent in low socio-economic and demographic regions globally (Ryder et al., 2012; de Waaij et al., 2017; dos Anjos Gatti et al., 2017). Although not clearly understood how these directly impact on the transmission rate of the infection, behavioural patterns such as sex for money, use of recreational drugs, poor sexual practices and hygiene, douching, etc., commonly identified among persons dwelling in these regions, have been identified as predisposing factors for T. vaginalis infection (Cotch, 1991; Leon et al., 2009; Eshete, 2013; Miranda et al., 2014; Rogers et al., 2014).

Another factor influencing transmission dynamics of T. vaginalis is age (Miller et al., 2008; Silva et al., 2013; Kissinger, 2015). Predictors of infection among adolescents and older age groups are not clearly identified. Vaginal microbiota and glycogen in vaginal fluids, which is high among young adolescents act as protective barriers, inhibiting transmission of T. vaginalis and other sexually transmitted infections (Eschenbach et al., 2000; Petrova et al., 2013; Cone, 2014; Mirmonsef et al., 2014). However, high transmission among younger age groups has been reported (Van Der Pol et al., 2005; Crucitti et al., 2010; Swartzendruber et al., 2014; Kissinger, 2015). This could be attributed to poor and unsafe sexual practices and hygiene (i.e. having multiple sexual partners and having unsteady sexual partners, douching, etc.). High transmission of T. vaginalis has been attributed to vaginal epithelial and microbiota changes which occur with aging—epithelial thinning, glycogen depletion and vaginal flora depletion increase transmission of T. vaginalis among older women (Brotman et al., 2007; Brotman et al., 2010; Brotman et al., 2012). This phenomenon is not clearly
understood to date and an extensive study in this regard would provide increased knowledge on this.

Racial patterns in *T. vaginalis* transmission are yet to be clearly understood. Globally, the incidence of *T. vaginalis* is reportedly high in sub-Saharan Africa with Europe and the USA following (Kenyon *et al.*, 2014). This study is however, based on estimates and future study for accurate incidence rates is important. This phenomenon in ethnic/racial transmission disparity has been reported in several studies as well (Sorvillo *et al.*, 2001; Shafir *et al.*, 2009; Meites *et al.*, 2013). For instance, in the USA, the infection is high among blacks and non-Hispanic-blacks than whites (Ginocchio *et al.*, 2012; Nijhawan *et al.*, 2012). This trend is consistent with several other studies in the USA where high incidence of *T. vaginalis* is reported among black-dominated population than other ethnicity/race (Sorvillo *et al.*, 2001; Boyer *et al.*, 2006; Sutton *et al.*, 2007). Several factors have been highlighted as possible causes for this trend, including decreased use of condoms for barrier protection, increased practice of douching among black women (Annang *et al.*, 2006; Cottrell & Close, 2008), which has been identified as a causal factor for increased risk for other STIs (Scholes *et al.*, 1998). Disparities in education, the ability to consider accessing healthcare for what might be perceived as minor medical ailments, access to health care, access to good accurate diagnostic services and thereby access to appropriate and affordable treatment are some possible factors. Another possible factor, which could contribute to the ethnic/racial heightened disparity in *T. vaginalis* transmission, is genetic. This has however, not yet been studied and a future study in this area is worth considering. Strain differences in *T. vaginalis* could also be a possible attribute. For instance, a more virulent drug resistant *T. vaginalis* strain if found to infect blacks than whites, could prolong infection, contributing to increased transmission among
blacks. This is worth considering and a study to verify this would immensely contribute to understanding the existing racial trend of the infection.

1.6.3 Risk factors for infection

The prevalence of *T. vaginalis* infection is enhanced by a variety of risk factors, including geographic, socio-demographic, and socioeconomic as well as behavioural variables (Abdallah et al., 2017; de Waaij et al., 2017). Prevalence is relatively high in resource-poor countries (Gregson et al., 2001; Mgone et al., 2002; Wangnapi et al., 2015). In both developing and developed countries, women of lower socio-economic groups are reported to be at highest risk (Bowden, 2000), with up to 50% found among in women in minority groups (Bowden, 2000).

Educational level has also been identified as a risk factor for *T. vaginalis* infection (Gregson et al., 2001; Mgone et al., 2002; McClelland et al., 2007; Madhivanan et al., 2009; Eshete, 2013; Wangnapi et al., 2015). Although there is currently no reported study on how educational level directly influences high rates of *T. vaginalis* infection, some studies have linked high risk sexual behaviour, which is a common behavioural pattern among persons with lower education, as the major causal factor (Eshete, 2013). Low income, commonly associated with lower levels of education, has also been reported as an associated factor for *T. vaginalis* infection (Swartzendruber et al., 2014).

Other studies have suggested multiple sexual partners, which could be common among unpartnered women; concurrent infection with other STIs and older age as risk factors for *T. vaginalis* infection (Madhivanan et al., 2009; Goo et al., 2016; dos Anjos Gatti et al., 2017), although there has been no explanations as to how each of these factors might directly
influence the infection rate. The need for an in-depth study on risk factors and how each of 
these is directly associated with *T. vaginalis* infection in a larger and more diverse study 
population cannot be over-emphasized.

1.7 MOLECULAR EPIDEMIOLOGY

In the last two decades, molecular epidemiological studies have provided epidemiologists and 
researchers not only opportunities and insights into diseases, their prevention and treatment, 
but also provided information that can be used to enhance the health of populations (Slattery, 
2002). Molecular epidemiological studies have provided important information on parasite 
phylogeny and classification; population genetic processes, such as mode of reproduction, 
breeding system, selection, isolation and genetic drift, and gene flow; transmission dynamics; 
and markers for traits such as virulence, drug resistance and antigenicity (Huyse *et al.*, 2005; 

1.7.1 Molecular tools

Studies on genetic variation of *T. vaginalis* have been limited until recently because of the 
lack of appropriate molecular tools. However, there are now many examples of the 
development and application of molecular methods to better understand the epidemiology of 
trichomoniasis in people (Table 1.4).

Choosing an appropriate marker for molecular epidemiological studies requires consideration 
of the required level of resolution for the study, technical convenience and the precision of 
the genetic data collected (Lymbery & Thompson, 2012). Genetic variation is influenced by
evolutionary processes operating now and throughout the evolutionary history of a lineage, so choosing markers with a rate of genetic change appropriate to the level of question being posed (e.g. taxonomic assignment, species diagnosis or isolate tracking) is an essential first step. For example, a useful tool for the specific detection and identification of *Trichomonas* spp. is the amplification of the 5.8S rRNA gene. (Kikuta *et al.*, 1997; Walker *et al.*, 2003). The advantage of using this region is its presence in multiple copies in the parasite genome and the fact that although it is conserved in some regions, it is variable in others, even between closely related species.

There is also typically a trade-off between technical convenience and precision when choosing a marker. For example, markers such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) do not require specific sequence information and hence can be utilised for well-studied species, but their outcomes are often less discriminatory (Nybom *et al.*, 2014), making it impossible to distinguish between alternative alleles at a locus. This limits the cope and strength of analysis, which can be generated from the data. It also reduces its comparability with other studies thus, limiting its usefulness.

These include random amplified polymorphic DNA (RAPD) successfully used for molecular typing of *T. vaginalis* from clinical isolates (Kaul *et al.*, 2004; Fraga *et al.*, 2015; Oliaee *et al.*, 2017), with emphasis on the genetic polymorphism and clinical phenotype of *T. vaginalis*. Even though the level of correlation between the RAPD data and the *in vivo* and *in vitro* properties of the parasite, geographic origin, and clinical presentation has successfully
indicated, there are some limitations using RAPD for phenotypic studies (e.g. reproducibility, homology inferences and artefact fragments).

Multi-locus sequence typing (MLST) is an appropriate marker to study the relationships among strains. This is important because clones diversify with age as a result of mutation and recombination, and typing a single loci could result in error (Belén et al., 2009). It exploits nucleotide sequence data to characterize microbes, quantifying genetic relationships between isolates and output data from this analysis is transferrable between laboratories, making it suitable for global epidemiological studies (Cornelius et al., 2012). The utilisation of alleles based on nucleotide sequence determination of internal fragments from multiple housekeeping genes is clearly expressed and distinguishes more alleles per locus. This permits high discrimination between isolates (Belén et al., 2009). The use of MLST has enabled successful study on the population genetics and the genetic polymorphism of *T. vaginalis*. (Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015).
Table 1.4 Some molecular tools and their uses in the characterisation of genetic diversity of Trichomonads

<table>
<thead>
<tr>
<th>Molecular tool</th>
<th>Level of resolution</th>
<th>Purpose</th>
<th>Limitations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD</td>
<td>Intraspecific</td>
<td>Genetic polymorphism/clinical phenotype</td>
<td>Reproducibility, homology</td>
<td>(Kaul et al., 2004; Fraga et al., 2015; Oliae et al., 2017)</td>
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<tr>
<td></td>
<td></td>
<td>Reproducibility, homology</td>
<td>Interferences and artefact</td>
<td>(Vanacova et al., 1997)</td>
</tr>
<tr>
<td>MLST/SNP</td>
<td>Intraspecific</td>
<td>Population genetics/identification of multiple genotypes</td>
<td>Labour intensive, Cost</td>
<td>(Cornelius et al., 2012; Hawksworth et al., 2015)</td>
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<td></td>
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<td></td>
<td></td>
<td>(Conrad et al., 2012)</td>
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<td></td>
<td></td>
<td></td>
<td>(Matini et al., 2014; Masha et al., 2017)</td>
</tr>
<tr>
<td>mPCR</td>
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<td>Systematics/diagnosis</td>
<td>Prone to false positives</td>
<td>(McKechnie et al., 2009)</td>
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<tr>
<td>Isoenzyme analysis/RFLP/ PFGE/RSH</td>
<td>Species assignment/ Intraspecific</td>
<td>Systematics/genetic variation population structure sequence polymorphism</td>
<td>Cost/ labour intensive</td>
<td>(Soliman et al., 1982; Salem et al., 1992)</td>
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<td></td>
<td>(Upcroft et al., 2006; Crucitti, 2008)</td>
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<td></td>
<td>(Paces et al., 1992)</td>
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<tr>
<td>EcoRI-RFLP</td>
<td>Intraspecific</td>
<td>Phylogenetic analysis/molecular epidemiology</td>
<td>Cost</td>
<td>(Meade et al., 2009)</td>
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<tr>
<td>Microsatellite/ 18S rRNA</td>
<td>Intraspecific</td>
<td>Genetic variation, molecular epidemiology, diagnosis</td>
<td>Allelic band profiles difficult to interpret</td>
<td>(Conrad et al., 2011; Mao &amp; Liu, 2015)</td>
</tr>
<tr>
<td>PCR-SSCP/PCR-RFLP</td>
<td>Intraspecific</td>
<td>molecular epidemiology-tracking transmission, course of infection, competitive interaction,</td>
<td>Cost, labour intensive</td>
<td>(Matini et al., 2012; Matini et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotyping, drug susceptibility, genetic diversity. Phenotypic variations</td>
<td></td>
<td>(Masha et al., 2017)</td>
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</table>

Abbreviations: AFLP – amplified fragment length polymorphism; ITS – internal transcribed spacer; mPCR – multiplex PCR; PCR – polymerase chain reaction; PCR-RFLP – PCR-coupled restriction fragment length polymorphism; PFGE – pulse field gel electrophoresis; RAPD – random amplified polymorphic DNA; SSCP – single-strand conformation polymorphism; RSH – repetitive sequence hybridization; MLST – multi-locus sequence typing; HSP – heat-shock protein; SNP – single nucleotide polymorphism
1.7.2 Phylogeny and classification

Trichomonads belong to the primitive group of protists called Parabasalids (Viscogliosi et al., 1999), which comprises of a monophyletic but complex assemblage of diverse species of protists characterised by the presence of parabasal body, which is a Golgi complex associated with striated fibres (Noda et al., 2012). The previous evolutionary relationships of parabasalids were based on light-microscopic details of living, fixed or stained material, dividing them into two classes - Trichomonadida and Hypermastigia (Brugerolle & Lee, 2001). However, recent classifications based on molecular phylogenetic analyses have identified six parabasal groups: Trichomonadea, Tritrichomonadea, Hypotrichomonadea, Cristamonadea, Spirotrichonympha and Trichonympha (Ohkuma et al., 2007; Noda et al., 2009; Ohkuma et al., 2009; Carpenter et al., 2010; Cepicka et al., 2010), although their relationships have not yet been resolved. The identified clades within the parabasalids include genera within the family Monocercomonadidae (Monotrichomonas, Ditrichomonas and Pseudotrichomonas) and almost all species from the subfamily Trichomonadinae (Trichomonas vaginalis, Trichomonas tenax, Tetratrichomonas gallinarum, PentaTrichomonas hominis, Trichomitus trypanoides and Pseudotrypanosoma giganteum) (Viscogliosi et al., 1999).

Initial molecular studies inferred parabasalid taxonomic relationships using ribosomal DNA sequences (Gunderson et al., 1995; Silberman et al., 1996). Additionally, evolutionary relationships have been inferred for taxonomic representation of the six parabasal groups, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Viscogliosi & Muller, 1998; Noda et al., 2012), malate dehydrogenase (MDH) (Wu et al., 1999), enolase (Keeling, 2004), α- and β-tubulin (Gerbod et al., 2004; Cepicka et al., 2010).
The use of molecular phylogenetic analyses has changed many traditional groupings within the parabasalids. For example *Trichomitus barachorium*, which was previously classified in the Trichomonadinae family based on morphological characteristics, no longer belong to this cluster (Viscogliosi *et al*., 1999). Additionally, the phylogenetic relationship between trichomonads *Tritrichomonas foetus, Monoceromonas spp.* and *Dientamoeba fragilis*, which remained unresolved previously, has been resolved (Brugerolle, 1976; Viscogliosi *et al*., 1999); *Tritrichomonas* and *Dientamoeba* have been classified in the same group, while *Monoceromonas spp.* grouped independently (Viscogliosi *et al*., 1999).

### 1.7.3 Population genetic studies

The analysis of genetic variation within and among the populations of *T. vaginalis*, has detected high levels of genetic diversity, inferred a two-type population structure, identified clinically relevant differences between these two groups, and uncovered evidence of genetic exchange in an organism once believed to be clonal (Hawksworth *et al*., 2015).

*Trichomonas vaginalis* strain heterogeneity has previously been reported (Felleisen, 1997; Vanacova *et al*., 1997). More recently, high levels of genetic diversity of *T. vaginalis* have been demonstrated using a panel of twenty-one polymorphic microsatellite markers on populations of the parasite in the USA, Mexico, Chile, Italy, South Africa, Mozambique, Australia, Papua New Guinea (PNG) and India; examination of 231 isolates from these nine countries identified only four pair of isolates from different geographical regions sharing same haplotypes (Conrad *et al*., 2012). A Bristol study involving 23 isolates identified 19 unique genotypes (Hawksworth *et al*., 2015). Similarly, a previous study involving 68 isolates from the USA, the United Kingdom and Austria, identified 60 unique genotypes in these
isolates, revealing a diverse *T. vaginalis* population (Cornelius *et al.*, 2012). These studies, coupled with the findings by Conrad *et al.* (2012), indicate a globally diverse *T. vaginalis* population.

Phylogenetic analyses of *T. vaginalis* isolates has identified two major groups; group I consisting of three subgroups (A, B, and C) and group II consisting of subgroups (D and E). Each of these groups and subgroups are characterized by deeply diverging branches containing individual *T. vaginalis* isolates (Meade *et al.*, 2009). The two-type population structure identified by Meade *et al.* (2009), has been corroborated in other studies (Conrad *et al.*, 2012; Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015). Even though population types have been identified globally, their regional distribution has varied in some cases. For example, Conrad *et al.* (2012), identified nearly equal frequencies of both population types among all the geographical locations studied, with two exceptions observed; isolates from Southern Africa and Mexico were significantly biased towards Type 1 and Type 2, respectively. Hawksworth *et al.* (2015), contrary to findings by Cornelius *et al.* (2012), found amongst the Bristol isolates predominantly Type 2. The genetic diversity existing among isolates *T. vaginalis* has also been associated with metronidazole resistance. (Vanacova *et al.*, 1997).

The use of molecular markers in molecular epidemiological studies could help to examine evolutionary patterns and processes, mode of reproduction, breeding system, mutation, migration and selection with much emphasis on the use of appropriate analysis to evaluate the characterisation and genetic diversity of pathogens at different specificity levels. *Trichomonas vaginalis* is thought to be a clonal organism which reproduces strictly by
mitosis (Tibayrenc et al., 1990), thus, the genetic stability of the *T. vaginalis* parasite, over time, which is a unique feature of clonally reproducing populations, cannot be over emphasized (Tibayrenc et al., 1990; Tibayrenc & Ayala, 2012). Recent studies involving the whole genome sequence of the parasite, however, have revealed components of meiotic recombination elements present, suggesting the capacity for homologous recombination and genetic exchange (Malik et al., 2011). Further evidence on the occurrence of sexual recombination has been suggested in other studies (Conrad et al., 2012; Cornelius et al., 2012); no evidence for multi-locus linkage disequilibrium particularly among Type 1 isolates was observed, suggesting the likelihood of recombination. Furthermore, there also appears to be a much less frequent occurrence of recombination between Type 1 and Type 2 parasites, which suggests the existence of biological or special barriers to recombination between types (Conrad et al., 2012; Cornelius et al., 2012).

### 1.7.4 Transmission dynamics

Subtypes of *T. vaginalis* have been found in the general population and among sexual partners using HSP70 restriction fragment length polymorphism (Stiles et al., 2000). The presence of these multiple subtypes in persons within the same population makes it ideal as an epidemiologic tool for the study of sexual networks and the transmission dynamics of the infection. Multi-locus sequencing (MLST) can be utilized to study multiple locations of chromosome of an isolate or multiple isolates using a number of different housekeeping genes. Application of this tool is effective in epidemiological analysis and surveillance of pathogens (Belén et al., 2009) and can be used for the study of sexual networks and the transmission dynamics of the infection. Also, the use of Antigenic characterization, isoenzyme analysis, repetitive sequence hybridization, random amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis,
and sequence polymorphism in ribosomal RNA genes and intergenic regions of *T. vaginalis* have contributed to finding correlations between *T. vaginalis* genotypes and biologically relevant phenotypes or geographical distribution (Vanacova *et al.*, 1997; Snipes *et al.*, 2000; Hampl *et al.*, 2001; Rojas *et al.*, 2004; Upcroft *et al.*, 2006; Crucitti *et al.*, 2008), aiding in the epidemiological study and disease surveillance and producing information for a number of different phenotypes including metronidazole resistance and geographical distribution, as well as the clinical manifestation of infection and virulence of *T. vaginalis* (Vanacova *et al.*, 1997; Snipes *et al.*, 2000; Hampl *et al.*, 2001; Rojas *et al.*, 2004).

### 1.7.5 Molecular markers for traits of clinical/epidemiological importance

Molecular markers are specific segments of DNA, representative of the variation at the genome level that correlate with the expression of a phenotypic trait. The choice for an ideal molecular marker of a phenotypic trait should be based on the following criteria: 1) being polymorphic and evenly distributed throughout the genome, 2) providing adequate resolution of genetic differences, 3) generating multiple, independent and reliable markers, 4) being simple, quick and inexpensive, 5) requiring small amounts of tissue and DNA samples, 6) linkage to unique phenotypes and 7) need no prior information about the genome of the organism of interest (Botstein *et al.*, 1980).

Genetic diversity of *T. vaginalis* and the impact it has on clinical presentations has been reported in several studies across the globe, albeit with limited sample sizes. Eight different genotypes of the parasite have been identified using the actin gene in restriction fragment length polymorphism (RFLP) analysis in Zambia and Kinshasa. (Crucitti, 2008). The study however did not clearly indicate whether the variability observed impacted on the clinical
presentation. Two distinct clusters of *T. vaginalis* isolates from asymptomatic and symptomatic women in India have been identified and linked to the clinical presentation of the infection (Kaul *et al.*, 2004). Although these findings have provided some useful information on the genetic population in these regions, the limited sample size used in each region means further work would be required with a larger regional sample size to make a definitive statistical inference.

The two widely distributed clonal lineages of *T. vaginalis* may be differentially associated with varied clinical outcomes and health sequelae accompanying *T. vaginalis* (Cornelius *et al.*, 2012). Strains or group of isolates of microbial organism exhibit differing clinical properties, suggesting the potential for the manifestation of different clinical symptoms and infection outcomes by the different population types (Meade & Carlton, 2013). The correlation between *T. vaginalis* genotypes and clinical presentation, as well as susceptibility to metronidazole has been demonstrated in previous studies (Vanacova *et al.*, 1997; Snipes *et al.*, 2000; Hampl *et al.*, 2001; Rojas *et al.*, 2004; Fraga *et al.*, 2011). However, the evidence of association between genotypes and presenting clinical symptoms is not yet definitive. While some studies observed clustering of isolates based on their severity of clinical symptoms in a phylogenetic analysis, others showed no association (Vanacova *et al.*, 1997; Hampl *et al.*, 2001; Rojas *et al.*, 2004; Simoes-Barbosa *et al.*, 2005; Fraga *et al.*, 2011).
1.8 AIMS AND OBJECTIVES OF STUDY

This study aimed to improve our knowledge of the genotypic and phenotypic variation that exists in isolates of *Trichomonas vaginalis* infecting people in two geographic regions; Western Australia and Ghana. Specifically, the study aimed to:

1) Determine the level of genetic diversity of *T. vaginalis* in each region.

2) Examine risk factors for infection by recording demographic data and (for Southern Ghana) behavioural and clinical information.

3) Assess how the genetic characteristics of isolates impact on the clinical presentations of *T. vaginalis* infection.

The research questions addressed in this study were:

1) Are there different species/genotypes of *T. vaginalis* circulating within the same geographical location?

2) Are there different species/ genotypes of *T. vaginalis* circulating in the different regions?

3) Does the genetic variation existing among *T. vaginalis* isolates in Southern Ghana have any impact on the clinical presentations of the infection?
CHAPTER 2

TRICHOMONAS VAGINALIS INFECTION IN SOUTHERN GHANA: RISK FACTORS AND CLINICAL SIGNS ASSOCIATED WITH THE INFECTION

2.0 INTRODUCTION

*Trichomonas vaginalis* is responsible for most non-viral sexually transmitted infections worldwide. Globally, an estimated 190 million new cases occur annually (Poole & McClelland, 2013), mostly in developing countries and socioeconomically disadvantaged groups. The infection is associated with a wide range of clinical signs but none are definitive enough for its determination, with the commonest being vaginal discharge, vulval irritation, painful urination, strawberry punctate cervix, green-yellowish frothy vaginal discharge, and pain during coitus (Wølner-Hanssen *et al.*, 1989). *Trichomonas vaginalis* infection is also associated with adverse pregnancy and birth outcomes such as miscarriage, pre-term labour, stillbirth and low birth weight, and has been linked with increased HIV susceptibility (Minkoff *et al.*, 1984; Laga *et al.*, 1993b; Cotch *et al.*, 1997). Premature membrane rupture has also been reported in some cases (Draper *et al.*, 1995; Sangkomkamhang *et al.*, 2008).

Increasing epidemiological evidence has also indicated the potential role of *T. vaginalis* in both acquisition (Johnston & Mabey, 2008) and transmission (McClelland *et al.*, 2007) of HIV, and has been estimated to have facilitated 6% - 30% of all new HIV infection cases among African-American women in the United States (Chesson *et al.*, 2004). In Sub-Saharan Africa, an estimated 30 million new cases of *T. vaginalis* occur each year (WHO., 2001). Recent studies among general population in Zimbabwean and Uganda indicated as high as 3-fold HIV infection risk among women infection with *T. vaginalis* (Van Der Pol *et al.*, 2008). Similarly,
an increased risk of HIV among *T. vaginalis* infected women has been reported in Kenya and Zaire (Laga *et al.*, 1993a; McClelland *et al.*, 2007).

In Ghana, the National HIV Prevalence amongst pregnant women attending antenatal clinic in 2016 was 2.4%. Among the young population (15-24 years), a proxy for new infections was 1.1%. The prevalence by age group 45-49 was highest at 5.6%, followed by 35-39 at 3.5%, with 15-19 being the lowest at 0.6%. The regional HIV prevalence ranged from 2.7% in the Volta and Brong Ahafo regions as the regions with the highest prevalence to 0.7% in the Northern region, the lowest. The prevalence in the Greater Accra Region (GAR) was 2.4%. The national HIV prevalence amongst STI clients was 5.4% (Ghana AIDS Commission sentinel report, 2016).

Despite the high incidence of *T. vaginalis* worldwide, there is a paucity of data on regional prevalence in most parts of the world. In Ghana, for example, only two previous studies have reported prevalence of *T. vaginalis* infection, with estimates of 5.4% (Adu-Sarkodie, 2004) and 2.7% (Apea-Kubi *et al.*, 2006). Furthermore, prevalence estimates that are available are influenced by the method of diagnosis, age bracket of study subjects, and the sample size (Fouts & Kraus, 1980; McLellan *et al.*, 1982). The principal mode of diagnosis for *T. vaginalis* infection is wet preparation microscopy of vaginal fluid specimens, but this technique has a sensitivity as low as 50% (Wendel *et al.*, 2002). Although wet preparation microscopy is considered the gold standard, a recent study has reported a higher sensitivity for PCR techniques (Nabweyambo *et al.*, 2017). Problems with diagnostic sensitivity are compounded by the fact that almost 50% or more of women infected with *T. vaginalis* show few symptoms.
or are asymptomatic (Fouts & Kraus, 1980; Spence et al., 1980; McLellan et al., 1982), thereby resulting in most cases going undetected.

A number of studies have reported risk factors for *T. vaginalis* infection in women in various parts of the world (Mairiga et al., 2011; Ambrozio et al., 2016; Maufi et al., 2016; dos Anjos Gatti et al., 2017). These studies found that age, marital status, education and/or occupation may be associated with an altered risk of *T. vaginalis* infection. There has been no previous investigation of risk factors for *T. vaginalis* infections in Ghana and this presents a major setback in the management and treatment of the infection in public hospitals and health centres. The aim of this study was to estimate the prevalence of *T. vaginalis* in women attending health care clinics in Southern Ghana using two different techniques (microscopy and PCR), and to assess the clinical signs, sociodemographic and behavioural risk factors, and pregnancy outcomes associated with *T. vaginalis* infection in this region.

### 2.1 METHODOLOGY

#### 2.1.1 Study design

A cross-sectional study approach was adopted and carried out in four stages. It involved women attending gynaecological clinics in some selected hospitals in Southern Ghana. Cases of *T. vaginalis* were identified by screening the women who attended the gynaecological clinics. This helped to establish the association between infection and various socio-demographic, genital hygiene and sexual behavioural factors as well as assessing the association between the infection and various clinical parameters. The study also looked at the
genetic strains in circulation, how these strains differ from each other among and within the study sites, and how the genetic variation impacts on the virulence of the protozoon.

2.1.2 Study sites

The study sites were Southern Ghana, which comprised of the Amasaman Municipal Hospital, and Mamprobi polyclinic in Greater Accra Region; the South Tongu district hospital and Comboni hospital in the Volta Region of Ghana. Fairly high prevalence rate for *T. vaginalis* has been recorded from a pilot study carried out in the South Tongu district in 2013 (unpublished data). The Greater Region of Ghana on the other hand, has been reported to have an estimated prevalence of 2.7% (Apea-Kubi *et al*., 2006). The clinical set up in these study sites made it possible for the clinical and behavioural data to be obtained from the African populations. The selected health centres in the Greater Accra region serve as primary health centres and receive clients from the entire Southern Ghana. Study findings from these sites are therefore expected to provide an accurate and detailed data on the age, sex, demography, clinical presentations, sexual behavioural patterns and history of previous infection. This should help the study answer questions on prevalence, incidence of infection by age and suburb, clinical presentation and how behavioural risk and demographic factors are associated with *T. vaginalis* infection.

2.1.3 South Tongu district

South Tongu district is one of the twenty-five districts in the Volta Region and lies in the Southern part of Ghana, with Sogakope as its administrative capital (figure 2.1). The district lies within the semi- equatorial and dry equatorial climatic zones. The district shares boundaries with the Central Tongu District to the north and north- west, the Akatsi South
District to the north-east, Keta Municipal to the south and the Dangbe-East District to the west and south-west with an area of 448 km². The South Tongu district serves as a trade centre for all the adjoining districts hence, there is daily interaction of people with different behavioural practices. This also promotes compromised sexual activities for monetary gains. The 2010 population census in Ghana estimated the population of South Tongu at 87,950 inhabitants, with an annual growth rate of 2.5% which is slightly higher than that of the region (Ghana statistical service, 2013). The district has two hospitals, the Sogakope district hospital and Comboni hospital and nine health centres. They serve as secondary referral hospitals which serve the entire district and adjoining ones as well. Apart from the public health facilities, there are other health facilities in the private sector in the district. The study will be located in Sogakope where all gynaecological cases from the other towns in the district are referred to. There is also a research centre in this town where study into several protozoan diseases is ongoing.
2.1.4 Accra metropolis

Accra is the capital and the largest city of Ghana, with an estimated urban population of 2.27 million as reported in the 2010 national census report (Ghana statistical service, 2013). The Accra Metropolitan Area is the Greater Accra region’s administrative, educational, industrial and commercial centre, making it the major force for its population growth, with immigration contributing to over 35% of Accra’s population. Accra stretches along the Ghanaian Atlantic coast and extends north into Ghana’s interior. It serves as the Greater Accra region’s (the capital of Ghana) economic and administrative hub with a total area of 200 square kilometres (77 sq mm), (Figure 2.2). Accra is largely occupied by people from all tribes, culture, nationality and race, including African nationals and other nationals from all over the world.
Health services in Accra are organised around eleven sub-metro health teams, each with a hospital or more. The study was conducted at the Family and Sexual health clinics of Mamprobi polyclinic and Amasaman Municipal hospital. These are primary and secondary health facilities respectively. Apart from these public hospitals, there are other health facilities in the public and private sector in the metropolis.

Figure 2.2 Map of Accra Metropolis

2.1.5 Recruitment of study participants

This included women who attended the gynaecology clinic at the selected study sites. This population was chosen for convenience and ease to reach. The selected clinic and their staff
were chosen because of their daily handling of gynaecological and reproductive health cases, thus the ease to enrol them in the study.

A cross-sectional study approach was adopted and carried out in four stages. It involved women attending gynaecological clinics in some selected hospitals in Southern Ghana. This helped to establish the association between infection and various socio-demographic, genital hygiene and sexual behavioural factors as well as assessing the association between the infection and various clinical parameters. Recruitment of study participants and sample collection for the study sites in Ghana was from mid-February 2016 to April 2016, and October 2015 to May 2016 respectively. Inclusion criteria for the study in Ghana were:

i. Study subjects must be women and attending the gynaecological clinic of the relevant health facility.

ii. They should give their consent of participation independently.

iii. They should live within the selected study sites.

Exclusion criteria for the study were:

i. Women other than those attending the gynaecological clinic.

ii. Women attending gynaecological clinic but not at the relevant health facility.

iii. Women living outside the study sites.

iv. Women who refused to give their consent of participation.

Study interviewers (nurses/midwives) read out the study information sheet in the local language (or language best understood) to the potential study participants. Verbally witnessed consent was obtained from those who met the inclusion criteria and agreed to participate in the
study. Thumb printed consent was taken. Persons attending the gynaecological clinic were assured that non-participation would not affect the care they would receive at the clinic. Confidential interviews were conducted with a structured questionnaire (approved by the Murdoch University and the Ghana Health Service Human Ethics Committee) which sought information on socio-demographic factors, sexual practices and ano-genital hygiene practices (see Table 2.1). Participants were then passed on to other study staff for genital examination. Study interviewers informed participants on how the collection of vaginal samples will be done. They were also informed of being given 2 g metronidazole (single dose) therapy should their test prove positive for T. vaginalis.
Table 2.1 Socio-epidemiological variables collected in questionnaire

<table>
<thead>
<tr>
<th>Variable</th>
<th>Type</th>
<th>Range of values (Mean (± SD))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical presentation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal discharge</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Abnormal vaginal discharge</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Vaginal sores</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Vaginal itch</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Painful urination</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Vulva redness</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Strawberry cervix</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Discharge colour</td>
<td>Categorical</td>
<td>Clear/Yellowish-green</td>
</tr>
<tr>
<td>Discharge consistency</td>
<td>Categorical</td>
<td>Clumpy/Cheesy</td>
</tr>
<tr>
<td>Discharge frequency</td>
<td>Categorical</td>
<td>Normal/Moderate/Profuse</td>
</tr>
<tr>
<td>Discharge odour</td>
<td>Categorical</td>
<td>Normal/Abnormal</td>
</tr>
<tr>
<td><strong>Pregnancy outcome history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscarriage</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Still birth</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Continuous</td>
<td>(28.9±9.1)</td>
</tr>
<tr>
<td>Educational level</td>
<td>Continuous</td>
<td>&lt;HS/HS/Tertiary</td>
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<tr>
<td>Marital status</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Occupation</td>
<td>Categorical</td>
<td>Stud/trad/HW</td>
</tr>
<tr>
<td>Region of residence</td>
<td>Categorical</td>
<td>urban/rural</td>
</tr>
<tr>
<td>Pregnancy history</td>
<td>Categorical</td>
<td>Yes/NoTreatment mode for vaginal</td>
</tr>
<tr>
<td>Sore</td>
<td>Categorical</td>
<td>Orth/Herb/DouchingTreatment mode for vaginal</td>
</tr>
<tr>
<td>Discharge</td>
<td>Categorical</td>
<td>Orth/Herb/Douching</td>
</tr>
<tr>
<td>Cleaning pattern after visiting the toilet</td>
<td>Categorical</td>
<td>Back-Front/Front-Back</td>
</tr>
<tr>
<td>Cleaning material after toilet</td>
<td>Categorical</td>
<td>Toilet tissue, cloth, paper (other than toilet tissue)</td>
</tr>
<tr>
<td>Age at first sex</td>
<td>Continuous</td>
<td>(18.8±2.8)</td>
</tr>
<tr>
<td>Oral sex practice</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Saliva lubrication during sexual intercourse</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Multiple sexual partners</td>
<td>Categorical</td>
<td>Yes/No</td>
</tr>
</tbody>
</table>

HS-High school, Stud-Student, Trad-Trader, HW-Housewife, Orth-Orthodox, Herb-Herbal treatment
2.1.6 Vaginal examination and sampling

Vaginal sampling was done in selected gynaecology health facilities in Ghana by trained, experienced midwives. The vaginal examination and specimen collection procedure were thoroughly explained to participants. A total of two vaginal swab samples per participant were taken. Vaginal examination was undertaken under illumination and participants partially undressed.

The genitalia external was examined for any discharge, ulcer and/or vesicles and other pathologies. With a vaginal speculum carefully inserted, discharges and/or ulcers were observed in the vagina and cervix (especially punctate haemorrhage, characteristic of *T. vaginalis* infection). Two swabs from the posterior fornix were taken per participant and put in respective labelled tubes. All specimens were sent to the on-site laboratory immediately for examination. Examination findings were recorded on the Study Examination Form and forwarded to clinicians for treatment to commence. Abnormal discharge was defined as one with an abnormal odour and any colour other than clear. The quantity of discharge was described as normal (no noticeable discharge) or abnormal (copious discharge). A total of 1000 vaginal samples (two pair participants) were taken from all the study sites.

2.1.7 Metronidazole treatment

Oral metronidazole (2 g *stat*) was administered to all study participants in Ghana in whose specimen *T. vaginalis* was detected by wet preparation microscopy at the end of each day. Participants, as a precautionary step, were counselled to stay away from alcohol for 48 hours after ingestion of the drug and to also abstain from sexual intercourse for seven days.
2.1.8 Storage of swabbed specimen

One of each vaginal swabs collected from Ghana was kept in a dry sample tubes and stored at -20°C for wet preparation microscopy examination. The other vaginal swabs were stored in TYM media, incubated at 37°C and transported to the molecular laboratory of the School of Biomedical and Allied Health Sciences, Korle-bu, Accra, Ghana for DNA extraction. The DNA extracts were shipped subsequently on dry ice to Murdoch University in Perth, and used in nucleic acid amplification tests.

2.1.9 Laboratory analysis

2.1.9.1 Wet preparation examination

Sterile normal saline (0.9%) was added to one of the vaginal swabs obtained during vaginal examination and agitated. A drop of this was placed on a clean, dry grease-free slide and covered with a clean cover slide. This was initially scanned with the 10x objective light microscope for motile trichomonads, pus cells, epithelial cells and red blood cells, and then motility, flagella movement, and morphological features of *T. vaginalis* was confirmed with the 40x objective lens.

2.1.9.2 DNA extraction

Genomic DNA from 900 vaginal swabs for *T. vaginalis* isolate was extracted using QIAmp mini kit commercial kits (Qiagen, Australia), adhering to manufacturer’s instruction. Extraction was done under aseptic conditions and standard precautions strictly followed.
2.1.9.3 Initial Polymerase Chain Reaction (PCR) Screening

Five hundred DNA extracted samples from Ghana were taken through initial PCR screen at SABC to confirm the presence of *T. vaginalis*.

Amplification of DNA extract was performed in a programmed thermo-cycler using primer-Tryptophan(P1a)(5’CGTCAACATCGGTGGTTCA3’/3’GCGACAGCGACGACATTCAT5’) designed to amplify gene fragments of 489bp (protocol adopted from Cornelius et al (2012), and slightly modified). The PCR mixture consisted 1.25 U Taq DNA polymerase, ×1 PCR Buffer (MgCl2 free), 2 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate, 2 μL of DNA template, and 1 μM of each primer with PCR-grade water to make up a final volume of 25 μL. Negative controls consisting of PCR mix with primers but no DNA template and a positive control from a known *T. vaginalis* reference strain were included in all PCR runs. PCR was performed with 1 cycle of 95°C for 5 min, followed by 40 cycles 95°C for 1min, 45 s of annealing at 60°C, and 1.5min of extension at 72°C, followed by final extension step at 72°C for 10 min. Two microliter (2μl) of each reaction was visualized on a 1% agarose gel to verify amplification.

2.1.10 Data analysis

All analyses were performed using the *R* statistical package (*R* Development Core Team 2013). Infection prevalence (with 95% confidence intervals calculated assuming a binomial distribution) was estimated from both microscopy and PCR data, and proportion of infected women were compared between diagnostic methods using a Fisher exact test.

The relationship between infection with *T. vaginalis*, as determined by PCR (the more sensitive diagnostic technique; see Results), and epidemiological variables was investigated separately for clinical signs, previous childbirth history and risk factors for infection. First, for each set of
variables the association with infection status was examined by univariable tests (Fisher exact, Chi-squared or logistic regression) and an odds ratio calculated. For the clinical signs and risk factors variable set, those variables showing at least a moderate relationship with infection status (P < 0.3) were retained for a generalised linear model (GLM) analysis, with a binomial response variable (T. vaginalis infection present or absent) and a logit link function. Prior to GLM analysis, correlations among predictor variables were examined by Spearman’s Rho; and for any variables which were strongly correlated (ρ > 0.6), only one was retained for analysis. The significance of each predictor variable in the GLM was examined by a likelihood ratio test. In addition, I used a multimodel inference approach to determine the relative importance of predictor variables (Burnham & Anderson, 2002). A set of all possible models was generated from the full GLM using the R package MuMIn (Bartoñ, 2013). Models were then ranked by the Akaike Information Criterion, corrected for small sample size (AICc), and model averaging was performed using MuMIn across all models within three AICc values of the best model. The importance of each variable was determined by summing Akaike likelihood weights across all models within the top ranked set in which the variable occurred, providing the selection probability that a given variable will appear in the AIC best model (Burnham & Anderson, 2002).

2.2 RESULTS

2.2.1 Prevalence of T. vaginalis in women from southern Ghana

A total of 492 women were enrolled in the present study. Of these, 290 (58.9%) were from the Volta region and 202 (41.1%) were from the Greater Accra region. The mean age of the participants was 28.5±8.0 years for the Volta region and 29.8±9.3 years for the Greater Accra
region. Of the study subjects from the Volta region, 76.3% (213/279) have had less than 12 years of education (below senior high school education), while 23.7% (66/279) have had more than 21 years of education (senior high school and beyond). In the Greater Accra region, 67.8% (137/202) and 37.2% (65/202) of the subjects had history of education level below and above 12 years respectively. Among the Volta subjects, 63.1% (176/279) were married and 36.9% (103/279) were unmarried. The study subjects from the greater Accra region were made up of 25.3% (51/202) (19.8-31.7%) married and 74.8% (151/202) unmarried women.

The overall prevalence of \textit{T. vaginalis} infection was 13.2% (65/492) (95% CI 9.5-17.6%) by wet mount microscopy and 18.1% (89/492) (95% CI 12.2-21.1%) by PCR. There was a significant difference in positivity rate between the two techniques (Fisher exact test, \( P <0.001 \)). All samples which tested positive by wet mount microscopy were also positive by PCR, while an additional 13 samples which were negative by microscopy were positive by PCR.

The regional prevalence of \textit{T. vaginalis} infection by PCR was 21.7% (62/290) (95% CI 17.1-26.9%) for the Volta region and 12.8% (26/202) (95% CI 8.6-18.3%) for the Greater Accra region. There was no significant difference in prevalence between the two regions (Fisher exact test, \( P =0.09 \)).

\subsection*{2.2.2 Association with epidemiological factors}

Eleven subjects who provided inadequate sociodemographic data were excluded from analyses, leaving 481 (78 positive and 403 negative) samples. Univariate analyses showed no significant association between any of the presenting clinical signs with the presence of \textit{T. vaginalis}
Generalised linear model analysis of those predictor variables with $P < 0.30$ showed a residual deviance of 404.5 on 473 degrees of freedom, suggesting that no correction was necessary for overdispersion. The only significant effect was for vaginal itch (Table 2.3). Vaginal itch was also ranked highest in variable importance, occurring in 62% of the top eight AICc models; the only other variables to occur in the top set of models were discharge colour and discharge consistency (Table 2.3). Of the 89 infected participants in the study, 33 (37.1%) presented with vaginal itch, 61 (68.5%) presented with some sort of abnormal vaginal discharge, 45 (50.6%) with frothy malodorous discharge, and 22 (24.7%) with yellowish-green discharge.

*Trichomonas vaginalis* infection was associated with vaginal itch (OR = 1.71, $P = 0.04$) and history of engaging in oral sex (OR = 1.90, $P = 0.04$). There was a significant association between the presence of *T. vaginalis* and previous history of still birth (OR = 3.62; $\chi^2_1 = 4.39$, $P = 0.04$), but not with prior history of miscarriage ($\chi^2_1 = 0.15$, $P = 0.69$) (Table 2.2).

Univariate analyses found no significant associations between any of the sociodemographic and behavioural risk factors with the presence of *T. vaginalis* (Table 2.2). There was a strong correlation between treatment modes for vaginal sores and vaginal discharge, so only the vaginal sore treatment mode was included in the GLM analysis. The full GLM for those predictor variables with $P < 0.30$ in the univariate analysis had a residual deviance of 410.6 on 479 degrees of freedom, again indicating that the data were not overdispersed. The only variable with a significant effect was oral sex; people with a documented history of engaging in oral sex were more likely to be positive for *T. vaginalis* (Table 2.4). This was also ranked highest in variable importance (72%), with three other variables also occurring in the top
ranked set of models: cleaning material after toilet (66%), with using pieces of dirty cloth as cleaning material associated with a greater likelihood of infection; educational level (53%), with a lower level of education associated with a greater likelihood of infection; and treatment mode (38%), with douching associated with a greater likelihood of infection (Table 2.4).
Table 2.2 Odds ratios and significance of association of each epidemiological variable with *Trichomonas vaginalis* infection. Variables with significant association in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency</th>
<th>TV+ (%)</th>
<th>Odds ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical signs</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vulva redness</td>
<td>48</td>
<td>10 (20.6)</td>
<td>1.46</td>
<td>0.40</td>
</tr>
<tr>
<td>Yes</td>
<td>1.46</td>
<td>0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>433</td>
<td>68 (15.7)</td>
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<td></td>
</tr>
<tr>
<td>Painful urination</td>
<td>1.02</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>116</td>
<td>19 (16.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>365</td>
<td>59 (16.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present vaginal discharge</td>
<td>1.17</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>381</td>
<td>60 (15.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>100</td>
<td>18 (18.0)</td>
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<tr>
<td>Strawberry cervix</td>
<td>1.61</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>53</td>
<td>12 (22.6)</td>
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<td>No</td>
<td>428</td>
<td>66 (15.4)</td>
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<td>Vaginal sores</td>
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<tr>
<td>Yes</td>
<td>113</td>
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<tr>
<td>No</td>
<td>368</td>
<td>63 (17.1)</td>
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</tr>
<tr>
<td>Vaginal itch</td>
<td>1.71</td>
<td>0.04*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>238</td>
<td>30 (12.6)</td>
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</tr>
<tr>
<td>No</td>
<td>243</td>
<td>48 (19.8)</td>
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</tr>
<tr>
<td>Discharge colour</td>
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</tr>
<tr>
<td>Whitish</td>
<td>371</td>
<td>54 (14.6)</td>
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<tr>
<td>Greenish-yellow</td>
<td>110</td>
<td>24 (21.8)</td>
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</tr>
<tr>
<td>Vaginal discharge odour</td>
<td>1.36</td>
<td>0.27</td>
<td></td>
<td></td>
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<tr>
<td>Normal</td>
<td>234</td>
<td>33 (14.1)</td>
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</tr>
<tr>
<td>Frothy</td>
<td>247</td>
<td>45 (18.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discharge consistency</td>
<td>1.52</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clumpy</td>
<td>205</td>
<td>40 (19.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheesy</td>
<td>276</td>
<td>38 (13.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discharge frequency</td>
<td>1.67</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copious</td>
<td>131</td>
<td>15 (11.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>350</td>
<td>63 (18.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy outcome</td>
<td>1.12</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscarriage</td>
<td>121</td>
<td>21 (17.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>360</td>
<td>57 (15.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stillbirth</td>
<td>3.62</td>
<td>0.04*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behavioural and demographic factors</td>
<td>1.07</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 21 yrs</td>
<td>70</td>
<td>12 (17.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 21 yrs</td>
<td>411</td>
<td>66 (16.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education</td>
<td>1.45</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 12 yrs</td>
<td>348</td>
<td>61 (17.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 12 yrs</td>
<td>133</td>
<td>17 (12.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marital status</td>
<td>1.19</td>
<td>0.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>254</td>
<td>44 (17.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>227</td>
<td>34 (14.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>1.55</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greater Accra</td>
<td>202</td>
<td>26 (12.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volta region</td>
<td>279</td>
<td>52 (18.6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 (Cont’d) Odds ratios and significance of association of each epidemiological variable with *Trichomonas vaginalis* infection. Variables with significant association in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Frequency</th>
<th>TV+(%)</th>
<th>Odds ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saliva as lubricant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>75</td>
<td>11 (14.7)</td>
<td>1.15</td>
<td>0.73</td>
</tr>
<tr>
<td>No</td>
<td>406</td>
<td>67 (16.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age at first sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 18yrs</td>
<td>252</td>
<td>44 (17.5)</td>
<td>1.21</td>
<td>0.46</td>
</tr>
<tr>
<td>≥ 18yrs</td>
<td>229</td>
<td>34 (14.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Multiple sex partners</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>23</td>
<td>3 (13.0)</td>
<td>1.31</td>
<td>0.78</td>
</tr>
<tr>
<td>No</td>
<td>458</td>
<td>75 (16.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pregnancy history</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>365</td>
<td>64 (17.5)</td>
<td>1.55</td>
<td>0.19</td>
</tr>
<tr>
<td>No</td>
<td>116</td>
<td>14 (12.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Previous vaginal sore treatment</strong></td>
<td></td>
<td></td>
<td>0.52</td>
<td>0.15</td>
</tr>
<tr>
<td>Yes</td>
<td>62</td>
<td>6 (9.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>419</td>
<td>72 (17.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
<td></td>
<td>2.52</td>
<td>0.62</td>
</tr>
<tr>
<td>Student</td>
<td>49</td>
<td>9 (18.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trader</td>
<td>188</td>
<td>32 (17.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>House wife</td>
<td>68</td>
<td>14 (20.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farmer</td>
<td>44</td>
<td>5 (11.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Professional</td>
<td>132</td>
<td>18 (13.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oral sex</strong></td>
<td></td>
<td></td>
<td>1.90</td>
<td>0.04*</td>
</tr>
<tr>
<td>Yes</td>
<td>73</td>
<td>18 (24.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>408</td>
<td>60 (14.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vaginal discharge treatment mode</strong></td>
<td></td>
<td></td>
<td>0.90</td>
<td>0.71</td>
</tr>
<tr>
<td>Tablets</td>
<td>270</td>
<td>42 (15.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Douching</td>
<td>211</td>
<td>36 (17.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vaginal sore treatment mode</strong></td>
<td></td>
<td></td>
<td>3.22</td>
<td>0.06</td>
</tr>
<tr>
<td>Tablets</td>
<td>432</td>
<td>75 (17.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Douching</td>
<td>49</td>
<td>3 (6.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Toilet cleaning material</strong></td>
<td></td>
<td></td>
<td>1.82</td>
<td>0.12</td>
</tr>
<tr>
<td>Tissue paper</td>
<td>386</td>
<td>68 (17.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piece of cloth</td>
<td>95</td>
<td>10 (10.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NB: Eleven (11) participants, positive for *T. vaginalis* with incomplete data were excluded from this analysis. The total sample size and positives for *T. vaginalis* used for this analysis were 481 and 78 respectively.

*History of having multiple sexual partners over the last six months at the time of this study period
Table 2.3 Association of clinical signs with *Trichomonas vaginalis* infection from GLM analysis and model averaging.

<table>
<thead>
<tr>
<th>Source</th>
<th>Z value</th>
<th>P value</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal itch</td>
<td>2.11</td>
<td>0.04*</td>
<td>0.62</td>
</tr>
<tr>
<td>Discharge colour</td>
<td>1.14</td>
<td>0.25</td>
<td>0.57</td>
</tr>
<tr>
<td>Discharge consistency</td>
<td>0.17</td>
<td>0.07</td>
<td>0.53</td>
</tr>
<tr>
<td>Discharge frequency</td>
<td>0.63</td>
<td>0.14</td>
<td>0</td>
</tr>
<tr>
<td>Strawberry cervix</td>
<td>1.56</td>
<td>0.19</td>
<td>0</td>
</tr>
<tr>
<td>Discharge colour</td>
<td>1.14</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>Vulvar redness</td>
<td>0.20</td>
<td>0.61</td>
<td>0</td>
</tr>
</tbody>
</table>

*statistically significant (P-value < 0.05).

Variables are ranked by relative importance, which is the probability if selection in the AIC best-fit model. Variables significant in the global model are shown in bold.

Table 2.4 Association of behavioural and demographic variables with *Trichomonas vaginalis* infection from GLM analysis and model averaging.

<table>
<thead>
<tr>
<th>Source</th>
<th>Z value</th>
<th>P value</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral sex</td>
<td>1.96</td>
<td>0.04*</td>
<td>0.72</td>
</tr>
<tr>
<td>Cleaning material after toilet</td>
<td>1.22</td>
<td>0.10</td>
<td>0.66</td>
</tr>
<tr>
<td>Education</td>
<td>1.46</td>
<td>0.10</td>
<td>0.53</td>
</tr>
<tr>
<td>Treatment mode</td>
<td>1.82</td>
<td>0.07</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Variables are ranked by relative importance, which is the probability if selection in the AIC best-fit model. Variables significant in the global model are shown in bold.

### 2.3 DISCUSSION

The overall prevalence of *T. vaginalis* infection in this study was 13.2% and 18.1% by wet mount microscopy and PCR respectively, with a significant difference in sensitivity between the two techniques. Regional prevalence of *T. vaginalis* infection by PCR was 21.7% and 12.8% for Volta and Greater Accra region respectively, with a significant difference in prevalence between the two regions. Vaginal itch and a history of oral sex were the only variables found among the presenting clinical signs and behavioural risk factors to have a
strong association with *T. vaginalis* infection in this study, although infection was also associated with a previous history of still birth.

### 2.3.1 Prevalence

*Trichomonas vaginalis* infection is the most prevalent non-viral sexually transmitted infection worldwide (Menezes *et al.*, 2016). In spite of this, the regional prevalence of the infection is yet to be established in most parts of the world, including Ghana; most likely due to the absence of national routine screening, management and control programmes as exists for other sexually transmitted infections. In most parts of the globe, *T. vaginalis* infection has not been considered a high health risk and has received little or no attention with regard to public health and clinical intervention (Van Der Pol, 2007). However, the high prevalence of *T. vaginalis* infection and the associated severe health sequelae observed in recent times requires a more coordinated national programme, similar to other STIs.

In this study, the prevalence of *T. vaginalis* was higher than reported in the two other studies which have been conducted in Ghana (Adu-Sarkodie, 2004; Apea-Kubi *et al.*, 2006) (see Table 2.5). These previous studies sampled from only a single hospital and used serological and wet mount techniques to detect infection. The present study, by contrast, sampled women visiting a number of hospitals and clinics, and utilised both wet mount microscopy and PCR for diagnosis. The study found PCR to be a more sensitive diagnostic method, a finding which has also been reported by other studies (Wendel *et al.*, 2002; Piperaki *et al.*, 2010; Patil *et al.*, 2012). Because our study population was drawn from women visiting STI and gynaecological clinics, the prevalence found is likely to be greater than in the general female population in
Ghana, although it is similar to prevalence reported elsewhere in sub-Saharan Africa (see Table 2.5).

Table 2.5 Prevalence of *T. vaginalis* in Ghana and other countries in Sub-Saharan Africa

<table>
<thead>
<tr>
<th>Country</th>
<th>City</th>
<th>Study group</th>
<th>Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Africa</td>
<td>Durban</td>
<td>women in high-risk</td>
<td>14.6</td>
<td>(Abbai et al., 2016)</td>
</tr>
<tr>
<td>South Africa</td>
<td>Mopani</td>
<td>non-preg women</td>
<td>20.0</td>
<td>(de Waaij et al., 2017)</td>
</tr>
<tr>
<td>Nigeria</td>
<td>Maidugri</td>
<td>Pregnant women</td>
<td>10.99</td>
<td>(Mairiga et al., 2011)</td>
</tr>
<tr>
<td>Nigeria</td>
<td></td>
<td>Pregnant women</td>
<td>18.7</td>
<td>(Oyeyemi et al., 2016)</td>
</tr>
<tr>
<td>Nigeria</td>
<td>Abeokuta</td>
<td>Pregnant women</td>
<td>10.3</td>
<td>(Etuketu et al., 2015)</td>
</tr>
<tr>
<td>Southwest Ethiopia</td>
<td></td>
<td>Pregnant women</td>
<td>4.98</td>
<td>(Eshete, 2013)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Mwanza</td>
<td>Pregnant women</td>
<td>10.41</td>
<td>(Maufi et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>High-risk</td>
<td>women</td>
<td>19.0</td>
<td>(Francis SC et al., 2014)</td>
</tr>
<tr>
<td>Kenya</td>
<td>Nairobi</td>
<td>Pregnant women</td>
<td>40.0</td>
<td>(Mullick et al., 2005)</td>
</tr>
<tr>
<td>Ghana</td>
<td>Accra</td>
<td>Pregnant women</td>
<td>2.7</td>
<td>(Apea-Kubi et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Kumasi</td>
<td>Pregnant women</td>
<td>5.4</td>
<td>(Adu-Sarkodie, 2004)</td>
</tr>
</tbody>
</table>

2.3.2 Association with clinical signs

There was very little consistency in presenting signs of infection reported by participants in this study. Only vaginal itch showed a significant association with *T. vaginalis* infection, although the colour and consistency of vaginal discharge also featured in a high proportion of the top ranked GLMs. Other studies have also found that clinical manifestation of *T. vaginalis* infection is varied, with frothy, malodourous vaginal discharge and strawberry cervix being the most useful predictive signs for clinical diagnosis of infection (Fouts & Kraus, 1980; Wølner-Hanssen et al., 1989). In this study, despite the variations in clinical signs among the study subjects, vaginal discharge and frothy malodourous discharge were presented in more than half of the cases, with less than a fifth of them presenting with strawberry cervix. This variation in clinical signs could be due to a number of reasons, including genetic polymorphism in the
parasite (Alderete et al., 1986; Rojas et al., 2004) and changes in signs with stages of the infection. For example, development of strawberry cervix is progressive with *T. vaginalis* infection, initially developing as an irritable erythema and then progressing into group of small punctate haemorrhagic spots of strawberry appearance on the vagina close to the mucosa of the cervix (Fouts & Kraus, 1980; Wølner-Hanssen et al., 1989). The clinical signs associated with *T. vaginalis* infection could also be related to the menstrual cycle (Simoes-Barbosa et al., 2005). It is also possible that clinical signs could be altered with varying incubation period of the parasite and the presence of other infectious agents (Eshete, 2013; Maufi et al., 2016). Other plausible explanations for the variability in clinical presentations among infected participants in this study could be coinfections (for example, with HIV or other infectious agents) or repeated exposure to *T. vaginalis* from infected male sex partners who are not being treated for *T. vaginalis* infection.

The absence of a positive association between most of the presenting signs and *T. vaginalis* infection is not unexpected considering the diverse aetiology of vaginal discharge but could have serious implications for a syndromic approach to managing infection, potentially resulting in under or over treatment, prolonged infection and increased spread in the population. This suggests the need for routine and structured screening and treatment approach to improve the recovery rate of *T. vaginalis* infection in asymptomatic women visiting the health and antenatal clinics in Ghana, incorporating *T. vaginalis* into the existing national health centre-based STI screening, testing and treatment programs as well as the approved national antenatal screening tests list. This will improve the recovery rate of *T. vaginalis* infection in asymptomatic and symptomatic women visiting health and antenatal clinics in Ghana.
2.3.3 Association with childbirth history

A previous history of still-birth was significantly associated with current *T. vaginalis* infection in this study. Although we had no records to indicate previous history of *T. vaginalis* infection (i.e. at the time of the stillbirths), it is possible that these women might have had the infection over a long period of time without manifesting any symptoms and that this might have contributed to the adverse pregnancy outcome. Adverse pregnancy outcomes have been associated with *T. vaginalis* infection in previous studies (Minkoff *et al.*, 1984; Draper *et al.*, 1995; Cotch *et al.*, 1997; Sangkomkamhang *et al.*, 2008). The mechanism by which *T. vaginalis* infection contributes to stillbirth is not known. An association has been found, however, between premature membrane rupture, which is sometimes associated with *T. vaginalis* infection, and intrapartum stillbirth (Yang *et al.*, 2004). Further study is required to determine the risk posed to childbirth by infection with *T. vaginalis* and to help understand the underlying mechanisms of any association.

2.3.4 Association with sociodemographic/behavioural risk factors

The only risk factor significantly associated with *T. vaginalis* infection in our study was a previous history of engaging in oral sex, although there was some evidence of association with a lower level of education, using pieces of dirty cloth as cleaning material after the toilet and douching as treatment mode for vaginal discharge. It is important to note that the term “risk factor” in this study refers only to an association with infection and does not imply a direct causal relationship.

It is not clear how engaging in oral sex may increase the risk of infection or whether there could be other underlying confounding factor(s). *Trichomonas vaginalis* is believed to be
confined to the urogenital tract and primarily transmitted through heterosexual vaginal intercourse, although other forms of transmission cannot be completely excluded– recent case of *Trichomonas vaginalis* causing oropharyngeal trichomoniasis in a male patient with a history of engaging in oro-vaginal sex with an infected female partner has been reported (Carter-Wicker *et al.*, 2016). It is possible that oral sexual activity is correlated with unmeasured, confounding factors, but this is speculative at this stage. Previous studies have found evidence for an increased risk of *T. vaginalis* infection with decreased educational level, although the association was not strong (Yoshikawa, 1981; de Waaij *et al.*, 2017). The linkage existing between level of education and sexually transmitted infection is not clearly understood. It is possible that persons with a lower level of education have limited knowledge on safe sexual hygiene and practices, although there could also be other confounding factors such as poverty and multiple sexual partners. The weak association of douching with *T. vaginalis* infection found in this study, agrees with findings from other studies (Sutton *et al.*, 2007; Luo *et al.*, 2015).

Although we found no association between infection and age, some studies have reported an increased risk of infection with aging (Yoshikawa, 1981, 1983, 1997; Casau, 2005). High prevalence among older age groups (>40 years) may be related to biological and hormonal changes resulting in thinning of the vaginal mucosa wall (which acts as barrier against infection) (Bachmann & Leiblum, 2004) increasing the risk of mucosal tear during sexual activity, thereby increasing the risk of infection. In contrast, other studies have found an increased frequency of infection among younger age groups, which could be attributed to higher sexual activity and poor adherence to safe sex practices (Ambrozie *et al.*, 2016).
CHAPTER 3
POPULATION STRUCTURE AND GENETIC DIVERSITY OF TRICHOMONAS VAGINALIS AND THE CORRELATION WITH CLINICAL PRESENTATIONS

3.0 INTRODUCTION

Trichomonas vaginalis, a protozoan parasite is the causative agent of the most prevalent non-viral sexually transmitted infection globally (Schmid et al., 2011) and infects over 275 million people each year (Rowley et al., 2012). The prevalence of the infection is, however, underestimated due to up to one half of cases being asymptomatic (Matini et al., 2017). The parasite infects both sexes with varied clinical presentations. In females, for example, the infection presentation ranges from asymptomatic to severe symptoms including vaginitis, vulva itch, vulva redness, strawberry cervix, urethritis and vaginal discharge (Wølner-Hanssen et al., 1989), coupled with adverse pregnancy outcomes such as premature membrane rupture, still birth, miscarriages and low birth weight in some cases (Draper et al., 1995; Cotch et al., 1997; Guenthner et al., 2005; Swadpanich et al., 2008; Chinyere et al., 2012). In men, T. vaginalis infection is known to cause infertility, chronic prostatitis, and non-gonococcal urethritis (Krieger, 1999; Hobbs et al., 2006).

The existence of different genotypes of T. vaginalis has become evident over the last decade (Meade et al., 2009; Conrad et al., 2012; Matini et al., 2012; Hawksworth et al., 2015; Mao & Liu, 2015; Masha et al., 2017) and may contribute to the varied clinical presentations observed in T. vaginalis infections. Although some studies have been conducted to assess the existence of genetic diversity of T. vaginalis in various countries (Meade et al., 2009; Conrad et al., 2012; Cornelius et al., 2012; Matini et al., 2012; Hawksworth et al., 2015; Masha et al., 2017),
there still remain vast regions of the globe with no existing data. This presents a major setback to appropriate and effective control, management and treatment of the infection.

*Trichomonas vaginalis* has a large genome, with an estimated size of 160 megabases and 60,000 genes over six chromosomes (Woehle *et al.*, 2014). Previous studies have reported two consistent outcomes: the existence of genetic heterogeneity at the regional and global level and the identification of two major genetic groups (types 1 and 2) (Snipes *et al.*, 2000; Meade *et al.*, 2009; Conrad *et al.*, 2012; Cornelius *et al.*, 2012). These genetic groups have been associated with different phenotypic traits: type 1 is more frequently found to be infected with *T. vaginalis* virus (TVV) and is linked with greater pathogenicity and type 2 is associated with increased metronidazole resistance (Snipes *et al.*, 2000; Conrad *et al.*, 2012). Type 1 is believed to be the ancestral lineage, with type 2 diverging from a type 1 clade (Conrad *et al.*, 2012). Their distribution has been found to be in equal proportions in regions where studies have been conducted, raising questions about the factors responsible the origin and persistence of the two types (Conrad *et al.*, 2012).

The persistence of the two genetic lineages of *T. vaginalis* and their phenotypic association suggests the need for the use of a more robust and sensitive method to assess the distinguishing features of these groups in studies on the epidemiology of *T. vaginalis* infection. Over the last two decades, several studies have utilised different techniques to study the genetic diversity of *T. vaginalis*, including restriction fragment length polymorphism (RFLP) (Oliaee *et al.*, 2017), random amplified polymorphic DNA analysis (RAPD, internal transcribed spacer analysis (ITS) (Snipes *et al.*, 2000; Ibanez-Escribano *et al.*, 2014), microsatellite analysis (Cornelius *et al.*, 2012) and multi-locus sequencing typing (MLST) (Hawksworth *et al.*, 2015; van der Veer
Multi-locus sequence typing has high discriminatory properties coupled with reproducibility and Conrad et al. (2012) used this method based on seven house-keeping genes and single nucleotide polymorphism (SNP) to identify individual sequence type (ST) combinations in *T. vaginalis* (Conrad et al., 2012).

In this study, next generation-multi-locus sequence typing (NG-MLST) was used to examine diversity in *T. vaginalis* in two countries where there is currently very limited genetic information: Australia and Ghana. The aim was to ascertain the different genotypes of *T. vaginalis* circulating in the general population in these countries. With the increasing prevalence and severe health sequelae associated with *T. vaginalis* infection, it is hoped that this genetic knowledge will contribute to the epidemiology, public health and clinical management of the parasite.

### 3.1 METHODOLOGY

#### 3.1.1 Study sites and sample collection

The sampling for Australia (Western Australia and the Northern Territory) was undertaken at Western Diagnostics, in Perth where most of the Western Australian and the Northern Territory gynaecological diagnostic cases are sent for testing. Western Australia is a state occupying the western third of Australia and bounded by the Indian Ocean to the north and west, the Great Australian Bight and Southern Ocean to the south, the Northern Territory to the north-east and South Australia to the south-east (Figure 3.1). It has a total land area of 2,529.875 sq km with Perth as its capital city. The population of Western Australia as of June 2014 stood at 2.6
million (Australia bureau of statistics, 2014) and this is made up of the indigenous Aboriginal population as well as people from different nationalities. The Northern Territory has a total land area of 1,349,129 sq km. with an estimated population of 244,000 as of June 2015. Eighty-nine archival vaginal swab samples positive for *T. vaginalis* by PCR were collected between November 2015 and June 2016. In Ghana, sampling was undertaken in the Volta and Greater Accra regions of Southern Ghana. Sampling in Ghana is described in the previous chapter (Ghapter 2.1.6).

### 3.1.2 Genomic DNA extraction

Genomic DNA from the vaginal swabs positive for *T. vaginalis* was extracted using QIAmp mini kit commercial kits (Qiagen, Australia), following the manufacturer’s instructions. Extraction was done under strictly controlled conditions (use of safety cabinet hood, laboratory protective clothing, sterile disposable gloves and sterilised pipette tips, changed in between samples) to minimize laboratory-introduced contaminations from biological contaminants or PCR amplicons.

### 3.1.3 PCR amplification and metabarcoding

*Trichomonas* multi-locus gene sequences were PCR amplified from human vaginal samples using primers specific to seven housekeeping genes (Table 3.1). The multi-locus PCR primers also contained Illumina MiSeq adapter sequences on the 5’ end, as per the MiSeq platform standard protocols (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation). Selection of the seven housekeeping genes was based on two factors: their ability to be easily amplified and sequenced on both DNA strands and their previously reported genetic diversity (Cornelius *et al.*, 2012).
Amplification of all genes was carried out as follows: 1.25 U Taq DNA polymerase, ×1 PCR Buffer (MgCl2 free), 2 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate, 2 μL of DNA template, and 1 μM of each primer with PCR-grade water to make up a final volume of 25 μL. No template controls were included in the PCR runs. The amplification protocol was adapted from Cornelius et al (2012) and was the same for all loci. Amplification was performed with one cycle of 95°C for five minutes, followed by 40 cycles 95°C for one minute, 45 s of annealing at 60°C, one and a half minutes of extension at 72°C and final extension step at 72°C for ten minutes.

Amplification of a single product was confirmed by gel electrophoresis using 2% agarose gel containing SYBR safe Gel Stain (Invitrogen, USA), visualized with a dark reader trans-illuminator (Clare Chemical Research, USA), and products corresponding to the correct length were selected for further analyses. Multi-locus amplicons from each sample were uniquely indexed with DNA barcodes and prepared for sequencing according to Illumina recommended protocols (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation).
Table 3.1 Oligonucleotide primers used for *T. vaginalis* MLST

<table>
<thead>
<tr>
<th>Locus (abbreviation)</th>
<th>Orientation</th>
<th>Sequences (5’-3’)</th>
<th>Size (bp)</th>
<th>Gene Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptopanase (P1)</td>
<td>Forward</td>
<td>CGTCAACATCGGTGGCTTCA</td>
<td>1,451</td>
<td>498</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCGACAGCGACGACATTTCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaminase (P3)</td>
<td>Forward</td>
<td>GTGCCATTACAACAGCATCG</td>
<td>886</td>
<td>451</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAAGTATAGCTCCGCTGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family T2 aparanaginase-like</td>
<td>Forward</td>
<td>GAAACAGGAGCACCAGCAGA</td>
<td>990</td>
<td>412</td>
</tr>
<tr>
<td>Threonine peptase (P6)</td>
<td>Reverse</td>
<td>TCTCTAGCAACGCAGCCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanyl tRNA synthetase (P8)</td>
<td>Forward</td>
<td>TCTGTCCAGGATGGTGTTCTTT</td>
<td>3,075</td>
<td>494</td>
</tr>
<tr>
<td>DNA mismatch repair protein (P13)b</td>
<td>Reverse</td>
<td>TCCGTGCCAGGATAAGTCTTT</td>
<td>1,758</td>
<td>491</td>
</tr>
<tr>
<td>Serine hydroxymethyltransferase (P14)</td>
<td>Forward</td>
<td>GCTGAGTGACGGTGACAGTAC</td>
<td>1,356</td>
<td>449</td>
</tr>
<tr>
<td>Manose 6-phosphate isomerase (P16)</td>
<td>Reverse</td>
<td>AGCCAGTGGCATTCTGAGTT</td>
<td>1,128</td>
<td>459</td>
</tr>
</tbody>
</table>

a Determined to have likely resulted from lateral gene transfer (Conrad et al., 2011).
b Single-copy gene used for population genetics analysis of *T. vaginalis* (Cooper & Feil, 2006; Cooper *et al.*, 2008; Cornelius *et al.*, 2012).
3.1.4 Purification and quantification of barcoded PCR products

Twenty-five microliters (25 µl) of the indexed PCR products was purified by adding 28 µl of Agencourt AMPure XP magnetic particle suspension (Agencourt Bioscience Corporation, Beckman Coulter, Beverly, Massachusetts, USA) in a reaction plate. The mixture was incubated at room temperature for five minutes. The reaction plate was then placed onto an Agencourt SPRIPlate Super Magnet Plate for two minutes to separate the beads from the solution. The supernatant was aspirated from the reaction plate on the magnetic plate and discarded. The beads were resuspended with 200 µl of 70% ethanol and incubated at room temperature for 30 seconds on the magnetic plate. The ethanol was aspirated at the end of the incubation period and the process repeated twice. The reaction plate was taken off the magnetic plate and 20 µl of elution buffer dispensed into reaction well containing magnetic beads. The mixture was incubated at room temperature for two minutes. The reaction plate was placed back on the magnetic plate for one minute to separate the beads from the solution. The final solution (purified product) was transferred into a new well plate.

The purified PCR products were quantified using Invitrogen Qubit dsDNA HS Assay kit (ThermoFisher Scientific, USA) and measured with Qubit 2.0 fluorometer (Life Technologies, USA). The quantified products were normalised and sequenced on an Illumina MiSeq using 600-cycle V3 chemistry (301 bp paired-end reads), following the manufacturer’s recommendations.
3.1.5 Quality filtering and taxonomic assignment

Raw paired-end reads were merged with USEARCH v. 9.2 (Edgar, 2017) with a minimum of 50 bp overlap and no gaps allowed in the merged alignments. Only sequences that contained perfect (no mismatches) primer sequences were retained for analysis, and primer sequences and distal bases were trimmed from the sequences in Geneious v. 11.3 (Biomatters, New Zealand). Sequences were then quality filtered to allow only reads with < 1% expected error as calculated with USEARCH v. 9.2 fastq filter algorithm, and sequences > 300 nt remained in the dataset. Sequences with ≤ 10 replicate reads were also removed from the dataset. Chimeras were removed and sequences were denoised with UNOISE2 (Edgar, 2017) into zero-radius operational taxonomic units (ZOTUs). Taxonomic assignment was performed by aligning ZOTUs to a reference database of known *T. vaginalis* MLST reference sequences downloaded from GenBank (April/2018). This step was done to classify each ZOTU to an assigned taxonomic allele sequences using BLAST algorithm (stringency set at ≥99%). Sequences from individual isolates were concatenated and aligned to identify single nucleotide polymorphisms (SNPs), and allele names at each locus assigned based on unique SNP profiles. Sequence types (STs) were assigned based on unique allelic profiles, following the 60 STs previously identified (Cornelius *et al.*, 2012) by aligning sequences with their reference sequences (GenBank accession numbers JX239643–JX239693). Allelic profiles were then assigned to isolates. The alleles were uploaded into the online sequence query software for *T. vaginalis* ([http://pubmlst.org](http://pubmlst.org)) to generate the sequence types (ST) for each isolate. New ST numbers were followed on consecutively from those previously described (Cornelius *et al.*, 2012).

3.1.6 Population genetics and phylogenetic analysis

The discriminatory power of the MLST scheme was measured with Simpson’s index of diversity (Grundmann *et al.*, 2001), using an online resource
This measured the ability of the MLST tool used in this study to differentiate and assign the unique sequences at different loci. Nucleotide diversity of sequences at individual loci were measured with Tajima’s estimator (θπ) (Tajima, 1983). The Bayesian clustering program STRUCTURE 2.3.3 (Pritchard et al., 2000) was used to infer the number of populations K that isolates belong to according to allele frequencies at each locus. This program performs model-based analysis to assign isolates to populations, making use of MLST data. The admixture model was used with correlated allele frequencies to assess ancestry, whereby each isolate draws some fraction of its genome from each of K populations for a defined set of K values. STRUCTURE was run ten times for each of 11 K values (K = 1–11) with a burn-in period of 100 iterations, followed by a further 100 iterations. The DK method was used to infer the true number of populations to which isolates belong (Evanno et al., 2005). This method uses the first and second order rates of change of the log probability of the data, LnP(D), in order to calculate DK. Graphically the DK function displays a break in slope at the true value of K, allowing its detection, and inference of the true number of populations to which isolates belong. Relationships between STs and group-founding STs were found using goeBURST 1.2.1 (Francisco et al., 2009). A phylogenetic tree assessing the evolutionationary relatedness of the T. vaginalis isolates involving and reference isolates from previous studies was created with START software using Neighbour-joining (NJ) cluster method for tree construction (Jolley et al., 2001).

3.1.7 Test of linkage disequilibrium and recombination

The standardized index of association (IAS), which tests for linkage disquilibrium or the non-random association of alleles across the genome, was calculated using LIAN v3.6 (Haubold & Hudson, 2000). The expected value of IAS for a population is linkage equilibrium is 0, and the
significance of deviations from 0 is tested by a resampling procedure which randomly shuffles alleles among haplotypes.

Linkage disequilibrium provides information on the population history, the breeding system and pattern of geographic subdivision as well as portraying the history of natural selection, gene conversion, mutation and other forces causing gene-frequency evolution (Slatkin, 2008). It is based on the assumption that, when given enough evolutionary time, random evolutionary activities will result in an equilibrium distribution of alleles at each locus. Thereby particular allelic frequency at a given locus becoming independent of alleles at other linked loci (Robinson, 1998). A major advantage of this method of analysis is that, it can detect recent positive selection, including selective sweeps (selective alleles are not fixed) (Lai, 2012). Another advantage of this method is its ability to use the effects of past recombination to achieve fine-scale gene localization (Jorde, 1995). However a limitation to this method is that, past historical events such as admixture, genetic drift, multiple mutations and natural selection can affect LD analysis output. Also, allelic heterogeneity may be present at each loci and this can limit the strength of association between a given polymorphism and an observable phenotype (Jorde, 1995).

The maximum Chi-squared test (Max X²) for recombination analysis was performed using START2 (Jolley et al., 2001). Max X² performs pairwise allelic comparisons at individual loci using polymorphic site distributions to identify recombination events (Smith, 1992). Split Tree program and Phi test were also performed for recombination analysis.
3.1.8 Association with clinical signs

For the isolates from Ghana, scores were assigned to the presenting clinical signs in our quest to group the clinical isolates in pathotypes as described in a previous study (Simoes-Barbosa et al., 2005), and modified in this study as follows; vaginal discharge (score 1), yellowish-green discharge (score 1), vaginal sores (score 1), vaginal itching (score 1), frothy malodorous discharge (score 2), erythema of the vulva (score 3) and macular colpitis (strawberry cervix) (score 4). Pathotype scores varied from 0 (non-pathogenic) to 14 (highly pathogenic).

Associations between alleles at each locus and pathotype scores, and between population membership (i.e. clonal group 1 or 2) and pathotype scores were examined with a non-parametric Wilcoxon sign-rank test, implemented in the R statistical package (R Development Core Team 2013).

3.1.9 Identification of mixed infection

Mixed infection among isolates was determined by identifying isolates with multiple genotype representation at one or more specific locus, after sequences were denoised into ZOTUs (see section 3.1.5).

3.2 RESULTS

3.2.1 Diversity detected using next generation-multi-locus sequence typing (NG-MLST)

All seven house-keeping genes were successfully sequenced in the 178 vaginal swab samples, and 36 different alleles were obtained overall (Table 3.2). A total 2,542 nucleotides were
sequenced across the seven loci for each sample using next generation sequencing (NGS). Thirty-six alleles were detected among the seven loci sequenced for all 178 isolates, the number of alleles per locus ranging from two to nine (Table 3.2). Twenty nine of the identified alleles had been previously documented in the MLST database in GenBank (http://tvaginalis.mlst.net/); thus, seven new alleles were added to these existing ones (Table 3.2). The mean nucleotide diversity over all loci was 0.0135 differences/site (Table 3.2). The discriminatory power of the MLST scheme in this study is 0.852 (95% CI= 0.832-1.00). A total of 71 SNPs were found, comprising 2.8% of 2,542 nucleotides. The number of SNPs varied by locus from two to 37, comprising 0.54% of P16 sequence length to 13% of P3 sequence length (Table 3.2). Each isolate was assigned a sequence type based on the alleles located at each of the seven gene loci in that isolate.

Forty-seven individual sequence types (STs) were distinguished among the 178 isolates, 23 of which have previously been identified (Cornelius et al., 2012). This adds 24 new STs to the already existing 78, bringing the total number of STs to 102, with STs 1-60 identified by Cornelius et al. (2012), 61-78 by Hawksworth et al. (2015) and 79-102 described in this study. Twenty STs were represented by multiple isolates. Each of the STs represented by multiple isolates was comprised of isolates from both the same and different geographic sites. The remaining 27 STs (57.5%) were represented by single isolates. The data from the study will be uploaded to http://tvaginalis.mlst.net/ on publication.
Table 3.2. Genetic diversity detected at seven loci in 178 isolates of *T. vaginalis* from Australia and Ghana

<table>
<thead>
<tr>
<th>MLST locus</th>
<th>Sequence length</th>
<th>Number of alleles</th>
<th>(\theta\pi)</th>
<th>Number of SNPs</th>
<th>Total number of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophanase (P1)</td>
<td>351</td>
<td>4</td>
<td>0.001</td>
<td>2</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Glutaminase (P3)</td>
<td>285</td>
<td>8</td>
<td>0.076</td>
<td>37</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Family T2 asparaginase-like threonine peptidase (P6)</td>
<td>318</td>
<td>4</td>
<td>0.004</td>
<td>4</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Alanyl tRNA synthetase (P8)</td>
<td>430</td>
<td>5</td>
<td>0.005</td>
<td>8</td>
<td>0 (18)</td>
</tr>
<tr>
<td>DNA mismatch repair protein (P13)</td>
<td>447</td>
<td>9</td>
<td>0.005</td>
<td>16</td>
<td>1 (12)</td>
</tr>
<tr>
<td>Serine hydroxymethyl transferase (P14)</td>
<td>339</td>
<td>4</td>
<td>0.002</td>
<td>2</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Mannose 6-phosphate isomerase (P16)</td>
<td>372</td>
<td>2</td>
<td>0.002</td>
<td>2</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

The seven MLST loci are those described by Cornelius *et al.*, (2012). Sequences were trimmed to the previously defined, locus-specific length. The number of SNPs and alleles identified among the genotypes are given. The last column shows new alleles found in this study and total number of previously identified alleles for each locus in brackets. \(\theta\pi\): Nucleotide diversity per site/locus.

### 3.2.2 Diversity among geographic regions

Polymorphism was found at most loci in amongst both Australian and Ghanaian isolates, although there was some variation between countries, with one allele at the P3, two alleles at the P6 locus and five alleles at the P8 and P13 loci found only in Ghana (Figure 3.1). One allele (allele 2) and two other alleles (alleles 8 and 10) at the P1 and P13 loci respectively were found only in Australia. Sequence type polymorphism existed amongst both Australian and Ghanaian isolates, and there was some variation between countries. Allelic diversity was most pronounced at the P13 locus, with ten different alleles, followed by eight alleles at the P8 locus (Figure 3.1).
A total of 48 sequence types were identified in both Australian and Ghanaian samples. Sequence type (ST) diversity was found amongst both Australian and Ghanaian isolates as well as between countries, with 23 STs and eight STs found only in Ghana and Australia respectively (Figure 3.2). Among the Australian isolates, nine STs were represented by single isolates, while 15 STs occurred in more than one isolate. Among the Ghanaian samples, 23 STs were represented by single isolates, while 15 STs occurred in more than one isolate (Figure 3.2). Genetic overlap was observed, with 15 STs occurring in both Australian and Ghanaian isolates (Figure 3.2).
Figure 3.1 Allelic abundance and diversity between Australian and Ghanaian isolates. TRYP: Tryptophanase (P1); GLUT: Glutaminase (P3); ALTS: FT2A: Family T2 asparaginase-like threonine peptidase (P6); Alanyl tRNA synthetase (P8); DRMP: DNA mismatch repair protein (P13); SHMT: Serine hydroxymethyl transferase (P14); M6PI: Manose 6-phosphate isomerase (P16)
Figure 3.2 Sequence type (ST) abundance and diversity between Australian and Ghanaian isolates
3.2.3 Population structure

The goeBURST analysis of all isolates revealed seven clonal complexes of two more single locus variants (Figure 3.3). Clonal complex 7 contained isolates only from Ghana. Clonal complexes 1, 2, 3, 4, 5, 6 and 8 each contained isolates from both Australia and Ghana. Phylogenetic analysis of 48 STs from Australia and Ghana and 60 reference STs from Cornelius et al., (2012) (Figure 3.4), identified cluster grouping similarity with geoBURST analysis (see figure 3.3b) and ST clustering indicated by Cornelius et al., (2012).

Population assignments using the model-based approach in STRUCTURE grouped (all isolates) into two populations (Figure 3.5a). Genotypes were assigned to a population if they had at least 70% ancestry from that population. Twenty-one genotypes were not assigned to any population based on this criterion. The two populations observed by STRUCTURE are present in almost equal frequencies in the samples, with 82 in one population (Type 1) and 94 isolates in the other population (Type 2). Assignment of the test STs and reference STs from Cornelius et al., (2012) also identified two population types (Figure 3.5b) with a similar group assignment of the reference STs as indicated earlier by Cornelius et al. (2012).

When the Ghanaian and Australian isolates analysed separately, two subgroup populations were again identified, with almost equal frequencies in the isolates representing each subgroup in the two regions (Figure 3.6a and 3.6b).
Figure 3.3 geoBURST analysis of 197 isolates representing 48 STs. geoBURST analysis showed eight clonal complexes with 10 singletons. The smaller numbers are the STs and the bold numbers are clonal groups.
Figure 3.4 Phylogenetic tree of the evolutionary relationship of 48 STs (Australia and Ghana) and 60 reference STs (in bold) previously identified by Cornelius et al., (2012). Neighbour-joining method was used for the tree reconstruction.
Isolates

Figure 3.5a Assignment of isolates into two subpopulations by STRUCTURE plot for 197 isolates (Australia and Ghana). Isolates were assigned to a subpopulation if they had 70% ancestry from that subpopulation. Eighty two isolates are assigned to subpopulation 1 (Type 1, red) and 94 to subpopulation 2 (Type 2, green). The remaining 21 isolates showed various degrees of joint ancestry and therefore could not be assigned to either subpopulation.
Figure 3.5b Assignment of isolates into two subpopulations by STRUCTURE plot for 48 STs (Australia and Ghana) and 60 reference STs. Genotype identification numbers 1-48 correspond to STs from present studies. Genotypes ids 49-108 correspond to reference STs 1-60 from Cornelius et al. (2012).
Figure 3.6a Assignment of isolates into two subpopulations by STRUCTURE (Ghana).
Isolates were assigned to a subpopulation if they had P80% ancestry from that subpopulation. STRUCTURE plot for the 93 Ghana isolates. Forty nine isolates are assigned to subpopulation 1 (type 1, red) and 36 to subpopulation 2 (type 2, green). The remaining eight isolates showed various degrees of joint ancestry and therefore could not be assigned to either subpopulation.
Figure 3.6b Assignment of isolates into two subpopulations by STRUCTURE (Australia).
Isolates were assigned to a subpopulation if they had P80% ancestry from that subpopulation. STRUCTURE plot for the 104 Australian isolates. Thirty eight isolates are assigned to subpopulation 1 (type 1, red) and 48 to subpopulation 2 (type 2, green). The remaining 18 isolates showed various degrees of joint ancestry and therefore could not be assigned to either subpopulation.
3.2.4 Linkage disequilibrium and recombination assessment

For the entire *T. vaginalis* population, the standard index of association (*I*<sub>AS</sub>) was 0.0624, which was significantly greater than zero (*P*<0.05) (Table 3.3a). Likewise, when each ST was treated as a single isolate, removing redundancy due to indistinguishable clones, the *I*<sub>AS</sub> was 0.0285, which was also statistically different from zero (*P*<0.05) (Table 3.3a). When multilocus linkage disequilibrium was analysed separately for the type 1 and type 2 isolates identified in the Structure analysis, *I*<sub>AS</sub> values were 0.1307 and 0.0659 respectively, which were both significantly greater than zero (*P*<0.05) (see Table 3.3b).

The level of multi-locus linkage disequilibrium assessed among the Ghanaian isolates using LIAN is shown in Table 3.4a. For the entire *T. vaginalis* population, the *I*<sub>AS</sub> was 0.0720, which was significantly greater than zero (*P*<0.05). Likewise, when each ST was treated as a single isolate, removing redundancy due to indistinguishable clones, the *I*<sub>AS</sub> was 0.0241, which was also statistically different from zero (*P*<0.05) (Table 3.4a). The *I*<sub>AS</sub> for population Type 1 and 2 were 0.0058 and 0.0571 respectively, which were both significantly greater than zero (*P*<0.05) (see Table 3.4b).

Among the Australian isolates (*I*<sub>AS</sub> for the entire population) was 0.0536, which was significantly greater than zero (*P*<0.05) (Table 3.5a). Likewise, when each ST was treated as a single isolate, removing redundancy due to indistinguishable clones, the *I*<sub>AS</sub> was 0.0036, which was also statistically different from zero (*P*<0.05) (Table 3.5a). The *I*<sub>AS</sub> for Type -1 and 2 were 0.0354 and 0.0918 respectively, which were significantly greater than zero (*P*<0.05) (see Table 3.5b).
There was insufficient allelic diversity to enable Max $\chi^2$ computation at any locus, except P3 where no evidence was found for recombination ($\text{Max } \chi^2 = 12.84, P=0.071$). The most significant breakpoint found was between sequence P3 ZOTU 3_allele 1 and ZOTU 5_allele3, between the 217th and 218th polymorphic site. The Phi test using concatenated sequence also found no evidence of recombination from Ghana and Australia isolates ($n=48$ STs, $P=0.069$).

### Table 3.3a Analysis of multi-locus linkage disequilibrium for Australia and Ghana isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>$I_{AS}$</th>
<th>$P$ (test that $I_{AS} = 0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td></td>
</tr>
<tr>
<td>All isolates (197)</td>
<td>2.1133</td>
<td>1.5373</td>
<td>0.0624</td>
</tr>
<tr>
<td>STs only (48)</td>
<td>1.9502</td>
<td>1.6656</td>
<td>0.0285</td>
</tr>
</tbody>
</table>

### Table 3.3b Analysis of multi-locus linkage disequilibrium between population types for Australia and Ghana isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>$I_{AS}$</th>
<th>$P$ (test that $I_{AS} = 0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td></td>
</tr>
<tr>
<td>Type 1 (94)</td>
<td>1.9375</td>
<td>1.0860</td>
<td>0.1307</td>
</tr>
<tr>
<td>Type 2 (82)</td>
<td>2.1376</td>
<td>1.5317</td>
<td>0.0659</td>
</tr>
</tbody>
</table>
Table 3.4a Analysis of multi-locus linkage disequilibrium for Ghanaian isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>(I_{AS})</th>
<th>(P) (test that (I_{AS} = 0))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Monte Carlo</td>
</tr>
<tr>
<td>All isolates (93)</td>
<td>2.3760</td>
<td>1.6592</td>
<td>0.0720</td>
</tr>
<tr>
<td>STs only (42)</td>
<td>1.9249</td>
<td>1.6819</td>
<td>0.0241</td>
</tr>
</tbody>
</table>

Table 3.4b Analysis of multi-locus linkage disequilibrium between population types for Ghanaian isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>(I_{AS})</th>
<th>(P) (test that (I_{AS} = 0))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Monte Carlo</td>
</tr>
<tr>
<td>Type 1 (44)</td>
<td>1.0059</td>
<td>1.0420</td>
<td>0.0058</td>
</tr>
<tr>
<td>Type 2 (36)</td>
<td>2.0768</td>
<td>1.5470</td>
<td>0.0571</td>
</tr>
</tbody>
</table>

Table 3.5a Analysis of multi-locus linkage disequilibrium for Australian isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>(I_{AS})</th>
<th>(P) (test that (I_{AS} = 0))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Monte Carlo</td>
</tr>
<tr>
<td>All isolates (104)</td>
<td>1.6452</td>
<td>1.2518</td>
<td>0.0536</td>
</tr>
<tr>
<td>STs only (26)</td>
<td>1.4630</td>
<td>1.4321</td>
<td>0.0036</td>
</tr>
</tbody>
</table>

Table 3.5b Analysis of multi-locus linkage disequilibrium between population types for Australian isolates

<table>
<thead>
<tr>
<th>Data set</th>
<th>Variance in allelic mismatches</th>
<th>(I_{AS})</th>
<th>(P) (test that (I_{AS} = 0))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
<td>Monte Carlo</td>
</tr>
<tr>
<td>Type 1 (38)</td>
<td>0.7476</td>
<td>0.6166</td>
<td>0.0354</td>
</tr>
<tr>
<td>Type 2 (48)</td>
<td>1.4838</td>
<td>0.9033</td>
<td>0.0918</td>
</tr>
</tbody>
</table>
3.2.5 Association with clinical signs

Seventy-eight *T. vaginalis* isolates with clinical information were scored on a 20-point scale based on the number of symptoms and signs presents. Clinical signs manifested by the 78 infected participants in this study indicated that six (6.7%) corresponded to asymptomatic infected group, 21 (23.6%) to the very low symptomatic, 39 (43.8%) to the low symptomatic and 12 (13.5%) to the high symptomatic group (Table 3.4). The most classic presented clinical symptoms in *T. vaginalis* infection, which are vaginal discharge, vaginal itch and frothy malodorous discharge, were frequently presented in this study; 61 (68.5%) infected persons presented with abnormal vaginal discharge, 45 (50.6%) with frothy malodorous discharge, 33 (37.1%) with vaginal itch and 22 (24.7) with yellowish-green discharge. However, only 15 (16.9%), 12 (13.5%) and 10 (11.2%) infected persons presented with vaginal sores, strawberry cervix and erythema of the vulva respectively (Table 3.6).
Table 3.6 The distribution of clinical isolates of *T. vaginalis* based on symptoms exhibited by the patients

<table>
<thead>
<tr>
<th>Pathotype</th>
<th>Score (no. patients)</th>
<th>Vaginal discharge</th>
<th>Vaginal itch</th>
<th>Vaginal sores</th>
<th>Yellowish-green discharge</th>
<th>Frothy discharge</th>
<th>Vulvar redness</th>
<th>Macular colpitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pathogenic</td>
<td>0 (6)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Very low pathogenic</td>
<td>1 (5)</td>
<td>21</td>
<td>07</td>
<td>02</td>
<td>03</td>
<td>02</td>
<td>01</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>2 (9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low pathogenic</td>
<td>4 (16)</td>
<td>31</td>
<td>21</td>
<td>09</td>
<td>16</td>
<td>33</td>
<td>02</td>
<td>01</td>
</tr>
<tr>
<td></td>
<td>5 (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High pathogenic</td>
<td>8 (3)</td>
<td>09</td>
<td>05</td>
<td>04</td>
<td>03</td>
<td>10</td>
<td>07</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>9 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 (0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>61</td>
<td>33</td>
<td>15</td>
<td>22</td>
<td>45</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>
3.2.6 Population clustering and pathophysiology classification

The distribution of pathotype scores among 78 isolates from Ghana is shown in Table 3.6. There were no significant associations between genotypes at any locus and pathotype score (Table 3.7), although there was a non-significant trend for genotype 2 at locus P1 to be associated with a greater score ($P = 0.11$). There was also no significant difference in pathotype score between isolates from Type 1 and Type 2 populations ($z = 1.11$, $P = 0.27$).

Table 3.7 Associations between genotypes and clinical presentation score

<table>
<thead>
<tr>
<th>Gene</th>
<th>ZOTU</th>
<th>Std Error</th>
<th>95% CI</th>
<th>F ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRYP (P1)</td>
<td>1</td>
<td>0.46</td>
<td>(3.11-4.95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.56</td>
<td>(4.08-6.34)</td>
<td>2.64</td>
<td>0.11</td>
</tr>
<tr>
<td>GLUT (P3)</td>
<td>1</td>
<td>0.45</td>
<td>(3.49-5.28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.99</td>
<td>(2.15-6.10)</td>
<td>2.64</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.81</td>
<td>(3.80-7.03)</td>
<td>1.02</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2.79</td>
<td>(4.60-6.60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTATP (P6)</td>
<td>1</td>
<td>0.39</td>
<td>(3.91-5.47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.93</td>
<td>(1.59-5.30)</td>
<td>1.52</td>
<td>0.22</td>
</tr>
<tr>
<td>ALTS (P8)</td>
<td>1</td>
<td>0.39</td>
<td>(3.77-5.31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.42</td>
<td>(0.91-6.59)</td>
<td>0.18</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.01</td>
<td>(0.99-9.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMRP (P13)</td>
<td>1</td>
<td>0.39</td>
<td>(3.83-5.38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.63</td>
<td>(0.92-5.59)</td>
<td>0.71</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.63</td>
<td>(1.08-7.59)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.82</td>
<td>(0.36-11.64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHMT (P14)</td>
<td>1</td>
<td>0.41</td>
<td>(3.60-5.23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.81</td>
<td>(3.21-6.46)</td>
<td>0.21</td>
<td>0.65</td>
</tr>
</tbody>
</table>

3.2.7 Mixed infection amongst *T. vaginalis* isolates

3.0 Multiple infections with different *T. vaginalis* genotypes were identified in 35 isolates from 35 hosts, 22 from Ghana, representing 24.7% of Ghanaian isolates, and 13 from Australia, representing 14.6% of Australian samples. Of these 35 isolates, multiple infections were detected at the P1 locus in
eight isolates (five from Ghana and three from Australia), P3 in five isolates (three from Ghana and two from Australia), P6 in 16 isolates (ten from Ghana and six from Australia), P8 in one isolate (from Australia), P13 in two isolates (both from Ghana) and P14 in three isolates (two from Ghana and one from Australia).
3.3 DISCUSSION

3.3.1 Genetic diversity

This is the first study to investigate genetic diversity in _T. vaginalis_ in Australia or Ghana. Seven single copy house-keeping genes were sequenced as previously described (Cornelius _et al._, 2012), in 178 isolates (89 from Australia and 89 from Ghana) and a high level of allelic and nucleotide diversity at all loci was found in the _T. vaginalis_ populations in both countries, consistent with the findings of previous studies (Cornelius _et al._, 2012; Hawksworth _et al._, 2015): in this study, 48 unique ST were identified among 197 genetic variants in 178 isolates, of which 24 had previously been described (Cornelius _et al._, 2012), indicating a moderately high ST percentage diversity (27%). This was, however, lower than findings in previous studies (88.2% and 83%) (Cornelius _et al._, 2012; Hawksworth _et al._, 2015). This brings the total number of _T. vaginalis_ STs for the overall MLST scheme to 104 from a total of 269 isolates genotyped so far. This moderately high ST to isolate diversity observed in this study indicates further unique STs are yet to be identified for the MLST genotyping scheme.

Among the Australian and Ghanaian isolates, 71 SNPs defining 36 different alleles, 29 of which have previously been reported by Corenelius _et al._ (2012), were identified in 178 isolates, with multiple alleles found at each of the seven loci, underlining the extent of genetic diversity in _T. vaginalis_. Cornelius _et al._ (2012), had identified 43 SNPs, defining 51 alleles in 68 isolates of _T. vaginalis_ in an earlier study. Hawksworth _et al._ (2015) likewise, had identified 23 SNPs, defining 25 alleles in 25 isolates; 21 of which had previously been identified by Cornelius _et al._ (2012).

The average nucleotide diversity in this study was 0.0135 differences/site. This is higher than that found among _T. vaginalis_ isolates in previous study (0.0035 difference/site) (Cornelius _et al._, 2012), with each
locus comprising more than one allele in this present study. The discriminatory power of the MLST scheme observed in this study was 0.852. Even though this value was slightly lower (0.996) than observed by Cornelius et al. (2012), it still indicates the strength of this MLST scheme to differentiate between isolates, underscoring the high discriminatory power of the MLST scheme, as indicated in previous studies (Cornelius et al., 2012; Hawksworth et al., 2015).

The high genetic diversity observed among isolates in this study could be due to genetic exchange or diversifying selection in a clonal population (Cornelius et al., 2010). *Trichomonas vaginalis* reproduces strictly by mitosis and no direct evidence of meiosis and sexual reproduction has been observed in the protozoan, even though meiotic recombinant genes have been found in the *T. vaginalis* genome (Ramesh et al., 2005; Malik et al., 2008). Indirect evidence for genetic recombination in *T. vaginalis* has been found in previous studies, however, (Conrad et al., 2012; Cornelius et al., 2012). Both studies found no linkage disequilibrium within type 1 and 2 isolates when the population groups were considered separately, suggesting recombination more likely occurred within types than between types.

In the present study, however, significant linkage disequilibrium was found, whether type 1 and type 2 isolates were analysed together or separately, whether Ghanaian and Australian populations were analysed together or separately, and whether individual isolates with multiple ST representation or STs considered as single isolates were used in the analysis, suggesting the likelihood of restrictive recombination within the two separate groups. *Trichomonas vaginalis* has previously been proposed to be a clonal organism which reproduces mainly by mitosis (Tibayrenc et al., 1990). This role of clonality is supported in this study, not only by the occurrence of linkage disequilibrium, but also because both Australian and Ghanaian isolates integrated into a clonal complex (CC). This inference is further
supported by over-representation of unique STs in the current study, which is a feature of clonally reproducing organisms (Tibayrenc et al., 1990; Asmundsson et al., 2006). The most abundant genotype identified in the present study was ST 58, which represented 27% and 14.6% of Australian and Ghanaian isolates, respectively.

Split Tree program was unable to identify recombination breakpoints within the MLST gene fragments in this study. The Phi test for recombination showed no evidence of recombination when concatenated nucleotide sequences were analysed. Similarly, no evidence of recombination was detected using Maximum Chisquared test.

The discrepancy observed between results from this study and previous studies could be due to a number of reasons. First, the analysis of linkage disequilibrium provides only an indirect assessment of recombination. Clonal reproduction does not produce linkage disequilibrium per se, rather it preserves allelic associations that arise through mutation and normally broken up by recombination during sexual reproduction (Tibayrenc et al., 1990; Smith et al., 1993). Linkage disequilibrium can be retained in populations, even when recombination is frequent, because of epistatic fitness interaction between loci (Smith et al., 1993; Tibayrenc & Ayala, 2015), an “epidemic” population structure (Smith et al., 1993) and restrained recombination (Tibayrenc & Ayala, 2015).

Second, possible lack of power due to small sample size could have prevented the detection of significant linkage disequilibrium in previous studies. A sample size of 178 T. vaginalis isolates has been used in this study, compared to sample sizes of 68 and 23 used in the study by Cornelius et al. (2102) and Hawksworth et al. (2015) respectively. The extent of sample size influence on population genetic
analyses, haplotype structures study and clinical research study has previously been documented (Sun et al., 2004; Suresh & Chandrashekara, 2012; Subramanian, 2016). Sun et al. (2004), observed that sample size when increased, had significant impact on the level of haplotype diversity within a chromosomal block and the total number of genetic representative markers identified. Suresh and Chandrashekara. (2012), had also observed that power proportionality increased as the sample size for the study increased, therefore, a study had a higher chance of detecting a difference between groups, if it existed. Subramanian (2016), studying the effects of sample size on population genomic analyses and the implications for the tests of neutrality had revealed that, sample size greatly influenced the measure of molecular genetic variations, using the Watterson’s estimator ($\theta$). They also observed that, the $\theta$ estimated for synonymous sites of genes using 512 human exomes was 1.9 times higher than obtained using 16 exomes, revealing the level of underestimation small sample size can cause and thus underscoring the importance of sample size in estimating a number of population genetic parameters.

3.3.2 Population genetic structure

Polymorphism among *T. vaginalis* genotypes was observed in this study (Figure 3.3a), clustering genotypes into eight major groups. This outcome is consistent with previous findings (Vanacova et al., 1997; Ryu et al., 1998; Snipes et al., 2000; Hampl et al., 2001; Rojas et al., 2004) While other studies have shown less clustering of *T. vaginalis* genotypes (Rojas et al., 2004; Mao & Liu, 2015), nine clusters were identified, suggesting an extensive genetic diversity among the genotypes. This, however, fell behind other findings in Iraq (Valadkhani et al., 2011), where 21 clusters among 40 genotypes were identified, as well as evidence of geographical clustering.
The STRUCTURE analysis of *T. vaginalis* genotypes in this study, including reference STs from Cornelius *et al.* (2012), showed two major population distributions, Types1 and 2, when the genotypes were considered to be from one region, matching with the reference STs and when analysed for the separate regions, which again agrees with previous findings (Conrad *et al.*, 2012; Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015; Mao & Liu, 2015). Besides the two population types identified, a third unassigned (divergent) group has been observed in a Bristol study (Hawksworth *et al.*, 2015). Similarly, in this study, a third unassigned group was observed, which demonstrated almost equal ancestry lineage of the Type-1 and 2 lineages amongst the Australian and Ghanaian samples. These findings suggest the existence of a group of *T. vaginalis* genotypes in circulation, which may have occurred as a result of recombination between population types in the past, followed by clonal divergence. Further study in this regard will be useful to help understand the entire process giving rise to the unassigned group.

In the current study, there were almost equal frequencies of the genotypes falling within each of the two major population types (36 in type 1 and 49 in type 2). This is similar to a number of previous studies, which also found almost equal distribution of genotypes between types 1 and 2 (Conrad *et al.*, 2012; Cornelius *et al.*, 2012), but differs from Hawksworth *et al.*, (2015), who found overrepresentation of type 2 among *T. vaginalis* isolates from Bristol, U.K.

### 3.3.3 Association with clinical symptoms

Clinical variability among infected groups was observed in this study, as with previous studies (Meade *et al.*, 2009; Abou-kamar *et al.*, 2017). It is not, however, clear whether the clinical variability observed in *T. vaginalis* infection is due to variations in phenotypic expressions of individual *T. vaginalis* genotypes or host factors. A previous study of the genetic characterisation of *T. vaginalis* isolates has
shown that the marked clinical variability manifested in *T. vaginalis* infection and disease outcomes is matched with genetic diversity in the organism, suggesting a correlation between the genetic diversity of the protozoan and presenting clinical symptoms (Meade & Carlton, 2013).

In the present study, varied clinical symptoms were observed among study groups; 6.7% of infected groups were asymptomatic, 23.6% were very low symptomatic, 43.8% were low symptomatic and 13.5% were high symptomatic. Vaginal discharge and frothy malodourous discharge were present in more than half of the cases, with more than a fifth presenting with strawberry cervix. This study found no significant associations between genotypes at any locus and clinical presentation score. The non-significant trend for genotype 2 at locus P1 could be associated with a greater clinical score (*P* = 0.11) and is worthy of further investigation. The analyses did not have a lot of power because of the relatively small sample size.

Cornelius *et al.* (2012) have suggested the two population clusters identified in *T. vaginalis* (i.e. type 1 and type 2) may be differentially associated with various infection outcomes and health sequelae. Meade *et al.* (2013), similarly, had suggested the existence of the two genetic types may be associated with different clinical signs and outcomes in *T. vaginalis* infection. In the present study, however, there was no evidence for an association of population type with clinical score. Limitations in the present study include the absence of data on the association between strain/genotype and the development of symptoms or clinical outcome.
Mixed infections

Overall, mixed infections were identified among 35 of 178 isolates (19.7%), predominantly among isolates from Ghana (14.6% and 24.7% of Australian and Ghanaian isolates) and all were limited to two different genotype assignments at the same loci, as previously described (Cornelius et al., 2012; Hawksworth et al., 2015). These were higher than the previously documented *T. vaginalis* mixed infection frequency in human hosts (11.0%) (Conrad et al., 2012). This could be due to frequent re-infection of the host or exposure to different sexual partners infected with the parasite (Cornelius et al., 2012).

Mixed infection is believed to impact on disease progression and treatment response (Perez et al., 2018). This could also be a reason for the varied presenting clinical symptoms and infection severity, similar to those found in a previous study involving *Plasmodium vivax* mixed infection and infection severity (Genton et al., 2008). Mixed infection could result in poor treatment outcome due to selective response of the different isolates to metronidazole, the treatment drug of choice for *T. vaginalis* (Theisen et al., 1995; Baldeviano-Vidalon et al., 2005). This has been demonstrated in an earlier study on stored specimens collected from persons with multidrug resistance tuberculosis, demonstrating that mixed infections with strains of different resistance phenotypes could compromise treatment outcomes using standard combination treatment regimens (Van Rie et al., 2005). In addition to compromising the effectiveness of treatment, mixed infection could also promote superinfection sometime after the primary infection, initiating disease progression (Du Plessis et al., 2001).
CHAPTER 4

GENERAL DISCUSSION

4.0 INTRODUCTION

Trichomonads which are free-living and others parasitic, inhabit different hosts and sites in a wide range of vertebrate hosts (Gould et al., 2014). Among these are parasitic trichomonads of medical importance: *Trichomonas vaginalis*, found in the urogenital, *Trichomonas tenax*, in subgingival, oral and bronchial and *Trichomonas hominis*, in the gastrointestinal tracts (Malik et al., 2011).

*Trichomonas vaginalis* is sexually transmitted and is the causative agent of the most prevalent non-viral STI with an annual global incidence of 276.4 million (Rowley et al., 2012), infecting both sexes with heterogeneous clinical presentations. Asymptomatic to severe clinical symptoms such as vaginitis, urethritis, vaginal discharge, as well as predisposing to adverse pregnancy outcome, such as preterm delivery, premature rupture of membrane, low birth weight, miscarriage and still birth have been identified in infection cases in women (Cotch et al., 1997; Petrin et al., 1998; Smith et al., 2002). Infertility, chronic prostatitis, and non-gonococcal urethritis have been indicated in male infection (Krieger, 1999; Hobbs et al., 2006). The varied clinical presentation observed in *T. vaginalis* infection could be due to the existence of genetic polymorphism of the parasite in circulation.

Genetic population studies have revealed the existence of genetic diversity among *T. vaginalis* populations (Meade et al., 2009; Conrad et al., 2012; Cornelius et al., 2012; Matini et al., 2012; Hawksworth et al., 2015; Masha et al., 2017). However, there still remain vast regions of the world with limited or no data on the genetic population of *T. vaginalis*, presenting a major challenge for the effective control, management and treatment of the infection.
The increasing prevalence and associated severe health sequelae of *T. vaginalis* infections, coupled with the poor understanding and dearth of data on the different genotypes circulating within different geographical regions, especially Ghana, highlights the importance of this study, which was aimed at genetically characterizing clinical isolates of *T. vaginalis* by next generation multi-locus sequence typing (NG-MLST); correlating the genetic diversity with presenting clinical symptoms; and assessing the association between genetic markers and clinical symptoms, and their pathogenic significance in infection. The research undertaken in the present study has both clinical and public health relevance. The investigation of associated risk factors and presenting clinical signs for *T. vaginalis* infection will provide in depth knowledge and understanding on the dynamics of *T. vaginalis* transmission and presenting clinical signs among infected persons. This will help to improve the clinical diagnosis, treatment and management *T. vaginalis* infection. Additionally, epidemiological data obtained from this study will help in policy formulation and public health education on the causation, prevention and control of *T. vaginalis* infection. Also, the outcome of this study will serve as a platform for future studies on the genetic markers of *T. vaginalis* and their role in the pathology of the infection, contributing immensely to future drug formulation and vaccine development for treatment and control of *T. vaginalis* infection.

4.1 DIAGNOSIS OF *T. VAGINALIS* INFECTION

*Trichomonas vaginalis* infection was detected in this study using wet preparation microscopy and conventional PCR. Wet mount microscopy is the primary and considered most effective and common laboratory diagnostic technique used for the detection of *T. vaginalis* because of its cost effectiveness and ease (Garber, 2005; Van Der Pol, 2016). However, results in this study revealed that the PCR
technique has significantly higher sensitivity than wet preparation microscopy, suggesting PCR can be used as a routine test for detecting *T. vaginalis* in the district and regional clinical laboratories in Ghana. District laboratories in Ghana provide services for an average population of 100,000–200,000 people in a clearly defined geographical area. These laboratories are part of the district hospitals which has a bed capacity of usually between 50 and 60 and served as the first referral point of healthcare, forming an integral part of the district health system. The district laboratories provide diagnostic and pathology services appropriate to the medical, surgical, and outpatient activities of the district hospital. Regional laboratories provide services to a larger population than the district laboratories and operate within a wider geographical coverage. They are part of the regional hospital which often has a bed capacity more than 100 and serve as a secondary point of care. The regional laboratories often perform the same diagnostic and pathology services as the primary laboratories, serve as referral points for diagnostic and pathology services which the district laboratories do not have the capacity to perform and also have an oversight responsibility over the district laboratories in that region. The regional laboratories also serve as teaching laboratories for biomedical and medical students.

Five hundred and six vaginal swab samples collected from women attending gynaecological clinics in four selected hospitals in Southern Ghana and tested for *T. vaginalis*, showed 63 were positive for *T. vaginalis* using wet preparation microscopy and 89 positive using PCR. The shortfall in wet preparation microscopy is the technique’s inability to detect immotile trophozoites of *T. vaginalis* (Garber, 2005; Stoner et al., 2013), which often results from delayed examination of vaginal swab specimens. Also, wet preparation microscopy is subjective and requires high technical experience to detect *T. vaginalis* trophozoites.
4.2 PREVALENCE, CLINICAL SIGNS AND RISK FACTORS ASSOCIATED WITH TRICHOMONAS VAGINALIS INFECTION IN SOUTHERN GHANA

The prevalence of *T. vaginalis* infection is underestimated due to up to half of the infected cases being asymptomatic (Matini *et al.*, 2012). An overall *T. vaginalis* prevalence of 13.2% and 16.8% was recorded in this study using wet preparation microscopy and PCR respectively, with a significant difference in sensitivity between the two techniques. This appeared higher than reported prevalences in some African Sub-regions (see Chapter 2, Table 2.5). Also, the regional prevalence using PCR was significantly higher in the Volta region (21.7% (95% CI 17.1-26.9%)) than the Greater Accra region (12.8% (95% CI 8.6-18.3%)). These values were found to be higher than previously reported prevalences in the Greater Accra region (2.7%) (Apea-Kubi *et al.*, 2006) and Kumasi, in the Ashanti region (5.4%) (Adu-Sarkodie, 2004). The difference in prevalence observed in this study and the previous ones could be due to the use of a more sensitive diagnostic method and the greater population coverage in this study.

*Trichomonas vaginalis* infection is associated with vaginal discharge, vulva irritation, painful urination, strawberry punctate cervix, green-yellowish frothy vaginal discharge, and pain during coitus (Wølner-Hanssen *et al.*, 1989). In this study, the most classically identified clinical presentations were abnormal vaginal discharge (68.5%), frothy malodorous discharge (50.6%), vaginal itch (37.1%) and yellowish-green discharge (24.7). Additionally, 16.9% of infected persons were presented with vaginal sores, 13.5% with macular colpitis (strawberry cervix) and 11.2% with erythema of the vulva. However, vaginal itch was the only clinical sign variable found to be significantly associated with *T. vaginalis* infection, with colour and consistency of vaginal discharge also featuring in the top ranked models using the generalised linear model analysis.
Previous studies have attributed the heterogeneity of clinical presentations observed in *T. vaginalis* infection to a number of reasons, including the genetic polymorphism in the parasite (Alderete *et al.*, 1986; Rojas *et al.*, 2004), and the infection stage in the individual (Fouts & Kraus, 1980; Wølner-Hanssen *et al.*, 1989). The activity of *T. vaginalis* is affected by menstrual activities of the host and this could be linked to the varied clinical presentations (Simoes-Barbosa *et al.*, 2005). Also, the varying incubation period of the parasite could influence the variations in clinical signs (Eshete, 2013; Maufi *et al.*, 2016).

In the current study, *T. vaginalis* infection was significantly associated with a previous history of stillbirth. These women had no documented previous history of *T. vaginalis* infection at the time of the incident. Even though these women were likely to have had the infection over a long duration which had resulted in having stillbirth, this study did not test for the presence of the anti-*T. vaginalis* IgG antibodies which has previously been detected (Sibau *et al.*, 1987). Cases of adverse pregnancy outcomes have been associated with *T. vaginalis* infection (Minkoff *et al.*, 1984; Draper *et al.*, 1995; Cotch *et al.*, 1997; Sangkomkamhang *et al.*, 2008), even though the underlying mechanism is not well established. For example, premature membrane rupture and intrapartum stillbirth which are sometimes associated with *T. vaginalis* infection has been documented (Yang *et al.*, 2004) but the underlying mechanisms leading to these are not yet fully understood. Further studies would thus help to unravel the underlying mechanisms leading to these outcomes.

Several risk factors associated with *T. vaginalis* infection in women in various parts of the world have been reported (Mairiga *et al.*, 2011; Ambrozio *et al.*, 2016; Maufi *et al.*, 2016; dos Anjos Gatti *et al.*, 2017). Among the risk factors analysed in this study (see Chapter 2, Table 2.2), a history of engaging in oral sex was the only one found to be significantly associated with *T. vaginalis* infection. This is the first
time history of engaging in oral sex has been viewed as a risk factor for *T. vaginalis* infection and that this variable could contribute to increased infection risk which is worth further investigation. Other variables that also showed some level of association with *T. vaginalis* infection are lower level of education, use of pieces of dirty cloth as cleaning material after toilet and douching as treatment mode for vaginal discharge (see Chapter 2, Table 2.4). The evidence of association between low educational level and increased risk of *T. vaginalis* infection, albeit weak, has also been reported in previous studies (Yoshikawa, 1981; de Waaij *et al.*, 2017). Even though association between level of education and STI is not clearly understood, persons with a lower level of education are likely to have limited knowledge on safe sexual hygiene and practices, which increases infection risk. Notwithstanding, other confounding factors such as poverty and multiple sexual partners may also feature. The weak association between douching and *T. vaginalis* infection was observed in this study. This finding has been reported in previous studies (Sutton *et al.*, 2007; Luo *et al.*, 2015).

### 4.3 POPULATION STRUCTURE AND GENETIC DIVERSITY OF *TRICHOMONAS VAGINALIS*

The multi-locus next-generation sequencing tool used in this study revealed the existence of high genetic diversity, using seven house-keeping genes to successfully characterise 178 *T. vaginalis* isolates obtained from vaginal swabs in Australia and Ghana. Seventy-one polymorphic nucleotide sites, 36 different alleles, 48 sequence types (ST), 24 of which were novel, were identified among 197 genetic variants in these 178 isolates, revealing a diverse *T. vaginalis* population. The ST diversity in this study was 27%, compared to previous findings of 88.2% (Cornelius *et al.*, 2012) and 83% (Hawksworth *et al.*, 2015). The reason for the discrepancy in the ST diversity between this study and the previous studies
could be due to one of two reasons: the coverage of a wider sampling area and/or sample size. In the
study by Cornelius et al. (2012), samples analysed included 16 reference strains of *T. vaginalis* obtained
from nine different states between 1939 and 1986 and 52 isolates from patients presenting to two clinics
in Jacksonville, Mississippi, from 2001 to 2010, presenting a wider sampling period. The sampled
population also included persons from diverse racial origin; African-American and caucasians.
Hawksworth et al. (2015) on the other hand, obtained their samples from a single hospital in Bristol.
Since the determination of ST diversity takes into consideration the number of STs identified and total
sample size, the smaller sample size used in the study; 68 samples (Cornelius et al., 2012) and 23
samples (Hawksworth et al., 2015), may have also contributed to the high ST diversity obtained in these
studies.

Similar to the previous studies, this study showed a large pool of genetic variants of *T. vaginalis* in the
Ghanaian and Australian populations, stressing the importance of identifying the different phenotypic
properties associated with these variants. The discovery of novel alleles STs of *T. vaginalis* amongst the
Ghanaian and Australian populations also lead to questions regarding the origin and transmission of
these genotypes, their implication in disease manifestation and treatment response, and how this may
enhance the invasion of their hosts.

Among the Australian isolates, the level of ST diversity observed was minimal. This is contrary to what
was observed among the Ghanaian isolates. The reason for the occurrence among the Australian isolates
could be because the sampled population was not truly independent and was conserved, considering that
a greater proportion of the sampled population was from the Northern Territory, a predominantly
Aboriginal community who are mostly conservative in their social interactions (Gray & Auld, 2000;
Carson et al., 2007). Ghanaian isolates were sampled from a population of a more diverse background
with a much less conservative socio-behavioural pattern (Anarfi, 2003; Agyei-Mensah & Aikins, 2010), which could promote multiple sexual practice among the population, promoting possible mixed infection, genetic material exchanges between different *T. vaginalis* strains, and natural selection. Further studies on the role of socio-behavioural patterns in determining the genetic diversity patterns of *T. vaginalis* would be helpful to confirm whether these patterns influence the genetic diversity pattern in a population and the dynamics involved in this process.

Two population groups, Type 1 and Type 2, and a third unsigned group have been identified in previous studies. (Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015). Similarly, this study identified two population types and a third unidentified group which may have resulted from the processes of recombination in the past. Previous studies have found evidence for recombination between, but not within Type 1 and Type 2 clades (Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015). This study found multi-locus disequilibrium even when analysing clades separately, as well as widespread clonal genotypes, suggesting that there is no evidence of recent recombination. However, some genotypes appeared to have elements of Type 1 and Type 2 clades (the unassigned genotypes), suggesting that recombination may have occurred in the past. The best explanation is that *T. vaginalis* is a largely clonal organism, with occasional bouts of sexual reproduction. Also previous studies have found evidence for recombination when all *T. vaginalis* variants, in which some STs had multiple representations, but not when STs were analysed as single isolates (Cornelius *et al.*, 2012; Hawksworth *et al.*, 2015). The present study found multi-locus disequilibrium even when analysing STs as single isolates, as well as *T. vaginalis* variants with multiple ST representation, also suggesting *T. vaginalis* is a highly clonal species. This observation was not different when the Australian and Ghanaian isolates were analysed separately. Population genetic and phylogenetic analyses showed that *T. vaginalis* population structure is strongly influenced by recombination.
Clonal population structure observed in the multi-locus linkage disequilibrium analysis in this study was further evidenced when STs were analysed using geoBUURST. Eight clonal complexes and 10 singleton STs were observed within the population. Additionally, over-representation of ST 58 was observed in 27% and 14.6% of *T. vaginalis* variants among the Australian and Ghanaian population. This a feature of clonally reproducing organisms (Tibayrenc *et al.*, 1990; Asmundsson *et al.*, 2006).

### 4.4 CORRELATION OF GENETIC VARIABILITY WITH CLINICAL SIGNS

Variability in clinical signs observed in *T. vaginalis* infection has been linked to the existence of genetic diversity in the parasite (Meade & Carlton, 2013). However, in the present study, there was no evidence of significant association of the two population groups or of particular alleles being associated with clinical signs of infection. These results were likely to be influenced by the small sample sizes, limiting the statistical power of the analysis. It may also imply that variation in clinical signs could be due to not only the genetic variability expressed by *T. vaginalis*, but also by a range of host factors (e.g. the immune state of the host, age, host genetic factors, etc.). Further study is required on the impact of the genetic variability of *T. vaginalis* on clinical outcomes in infection states.
4.5 IMPLICATIONS OF MIXED T. VAGINALIS INFECTION ON CLINICAL PRESENTATION AND TREATMENT

The *T. vaginalis* isolate proportion of mixed strain infection observed in this study was 14.6% and 24.7% for Australia and Ghana respectively. These values were found to be higher than the predicted mixed infection value expected to occur in humans (11%) (Conrad *et al.*, 2012). Frequent reinfection of host and exposure to multiple sexual partners infected with different strains of *T. vaginalis* are some of the identified contributors of mixed strain infection (Conrad *et al.*, 2012). The occurrence of mixed strain infection has been reported to influence disease progression and treatment response in protozoan infections (Perez *et al.*, 2018). Also, poor treatment outcome due to selective drug response of different isolates has been observed in mixed strain infection (Theisen *et al.*, 1995; Baldeviano-Vidalon *et al.*, 2005), often resulting in treatment failures and development of resistant strains (Van Rie *et al.*, 2005).

4.6 SUGGESTIONS FOR FUTURE RESEARCH

Despite the medical importance of *T. vaginalis* infection among women of childbearing age, there are no definite risk factors associated with infection. Distinct clinical signs associated with the infection still remain unclear and a further study into these variables and the association with *T. vaginalis* infection would improve public health and clinical knowledge on the transmission and pathology of *T. vaginalis* infection, as well as aid control, treatment and management of the infection. Also, further study to determine the risk *T. vaginalis* infection poses to childbirth will help understand the underlying mechanisms of any association.
The variation in clinical presentation in *T. vaginalis* infection is believed to be as a result of the genetic characteristics of the parasite. Extensive study within a larger sample size and wider population coverage (including both clinical and non-clinical samples) to assess this association should be undertaken. It would provide important knowledge on the role each of these genetic characters play in clinical manifestation and pathology of *T. vaginalis* infection. Host factors are also likely to interact with parasite genetic characteristics to influence clinical presentation and pathology of *T. vaginalis* infection. Thus, further studies are required to partition the variance in clinical signs and virulence to host and parasite factors. This may suggest new approaches in treatment and management of *T. vaginalis* infection, and vaccine production to control transmission of the infection.

### 4.7 CONCLUDING REMARKS

In this study, the polymerase chain reaction technique was significantly more sensitive than wet mount microscopy as a diagnostic tool for detecting *T. vaginalis*. Also, *T. vaginalis* prevalence is high among women attending sexual health and gynaecological clinics in selected hospitals in the Greater Accra and Volta regions of Ghana. Vaginal itching is significantly associated with *T. vaginalis* infection and there is evidence of a prior history of stillbirths in women who were currently infected. A history of engaging in oral sex is also significantly associated with *T. vaginalis* infection. This study was hospital based, thus, the findings may vary from a community-based study in the same population. Male partners of women enrolled in this study were not included in this study and therefore we were unable to assess the behavioural risks of the partners. Ano-vaginal transmission of *T. vaginalis*, which could be a potential source of the infection, could not be assessed in this study. Findings from this study will contribute to and inform a new approach in public health and clinical diagnosis of *T. vaginalis* infection in Ghana.
Multi-locus sequence typing using next-generation sequence analysis revealed the high genetic diversity of *T. vaginalis* isolates in Australia and Ghana, indicating that geographical origins could influence the genetic characteristics of *T. vaginalis*, as revealed in this study data. Although two main grouping/types of *T. vaginalis* were identified, a third unassigned group was also identified, which suggests an outcome of past recombinant activities between the two population types. Study findings also confirm geographic expansion and diversity of the *T. vaginalis* population. Linkage disequilibrium results suggest that the *T. vaginalis* populations in this study are highly clonal. Results of multi-locus linkage disequilibrium within the two separate populations indicate linkage disequilibrium, suggesting free recombination occurring between the two group type. Lastly, results show no correlation between any of the locus and clinical presentation scores, as well as between population types and clinical presentation scores. This study is the first of its kind in these regions and a more comprehensive study on larger sample sizes and wider population coverage, taking into consideration all the limitations indicated in this study to assess the extent of genetic diversity and population structure and their correlation with clinical presentations and outcomes will help improve knowledge on the epidemiology of *T. vaginalis* infections.
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APPENDIX 1
GHANA HEALTH SERVICE ETHICS REVIEW COMMITTEE

In case of reply the number and date of this letter should be quoted.

My Ref. GHS/RDD/ERC/Admin/App/16/02
Your Ref. No.

Daniel Sai Squire
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Murdoch, WA 650
Australia

ETHICS APPROVAL - ID NO: GHS-ERC: 02/11/15

The Ghana Health Service Ethics Review Committee has reviewed and given approval for the implementation of your Study Protocol titled:

“Trichomonas Spp. in Southern Ghana: Genetic Variation and Risk Factors for Infection”

This approval requires that you submit yearly review of the protocol to the Committee and a final full review to the Ethics Review Committee (ERC) on completion of the study. The ERC may observe or cause to be observed procedures and records of the study during and after implementation.

Please note that any modification without ERC approval is rendered invalid.

You are also required to report all serious adverse events related to this study to the ERC within three days verbally and seven days in writing.

You are requested to submit a final report on the study to assure the ERC that the project was implemented as per approved protocol. You are also to inform the ERC and your sponsor before any publication of the research findings.

Please note that this approval is given for a period of 12 months, beginning 5th February, 2016 to 4th February, 2017. However, you are required to request for renewal of your study if it lasts for more than 12 months.

Please always quote the protocol identification number in all future correspondence in relation to this approved protocol.

SIGNED................................

PROFESSOR MOSES AIKINS
(GHS-ERC VICE-CHAIRPERSON)

Cc: The Director, Research & Development Division, Ghana Health Service, Accra
Wednesday, 09 March 2016

A/Prof Andrew Thompson  
School of Veterinary and Life Sciences  
Murdoch University  

Dear Andrew,  

Project No.  2015/164
Project Title  Trichomonas spp. in women in Ghana: genetic variation and risk factors for infection

Thank you for addressing the conditions placed on the above application to the Murdoch University Human Research Ethics Committee. On behalf of the Committee, I am pleased to advise the application now has:

OUTRIGHT APPROVAL

Approval is granted on the understanding that research will be conducted according the standards of the National Statement on Ethical Conduct in Human Research (2007), the Australian Code for the Responsible Conduct of Research (2007) and Murdoch University policies at all times. You must also abide by the Human Research Ethics Committee's standard conditions of approval (see attached). All reporting forms are available on the Research Ethics and Integrity web-site.

I wish you every success for your research.

Please quote your ethics project number in all correspondence.

Kind Regards,

Dr. Erich von Dietze
Manager
Research Ethics and Integrity

cc: A/Prof Alan Lymbery and Daniel Squire
Human Research Ethics Committee: Standard Conditions of Approval

a) The project must be conducted in accordance with the approved application, including any conditions and amendments that have been approved. You must comply with all of the conditions imposed by the HREC, and any subsequent conditions that the HREC may require.

b) You must report immediately anything, which might affect ethical acceptance of your project, including:
   - Adverse effects on participants
   - Significant unforeseen events
   - Other matters that might affect continued ethical acceptability of the project.

c) Proposed changes or amendments to the research must be applied for, using an Amendment Application form, and approved by the HREC before these may be implemented.

d) An Annual Report for the project must be provided by the due date specified each year (usually the anniversary of approval).

e) A Closure Report must be provided at the conclusion of the project (once all contact with participants has been completed).

f) If, for any reason, the project does not proceed or is discontinued, you must advise the committee in writing, using a Closure Report form.

g) If an extension is required beyond the end date of the approved project, an Extension Application should be made allowing sufficient time for its consideration by the committee. Extensions of approval cannot be granted retrospectively.

h) You must advise the HREC immediately, in writing, if any complaint is made about the conduct of the project.

i) Other Murdoch approvals (e.g. fieldwork approval) or approval form other institutions may also be necessary before the research can commence.

j) Any equipment used must meet current safety standards. Purpose built or modified equipment must be tested and certified by independent experts for compliance with safety standards.

k) Graduate research degree candidates must normally have their Program of Study approved prior to commencing the research. Exceptions to this must be approved by the HREC.

l) You must notify Research Ethics & Integrity of any changes in contact details including address, phone number and email address.

m) Researchers should be aware that the HREC may conduct random audits and / or require additional reports concerning the research project.

Failure to comply with the National Statement on Ethical Conduct in Human Research (2007) and with the conditions of approval may result in the suspension or withdrawal of approval for the project.

The HREC seeks to support researchers in achieving strong results and positive outcomes.

The HREC promotes a research culture in which ethics is considered and discussed at all stages of the research.

If you have any issues you wish to raise, please contact the Research Ethics Office in the first instance.