MASTER'S THESIS

Unisexual Schistosomiasis in Natural Infections in Nigeria

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ABSTRACT

Urogenital Schistosomiasis is a neglected tropical disease caused by digenic trematodes of the species *S.haematobium* and the disease is widespread in rural areas lacking portable water and efficient sanitation. Typically, this parasite reside as pairs within the venous plexus around the bladder. However, recent research indicates instances where these worms can exist singly in human hosts, resulting in unisexual schistosomiasis infections. Unisexual or single sex infections are asymptomatic in nature, which makes detection of these infections difficult. This study aimed to assess the effectiveness of current molecular markers and develop new gene markers for identifying unisexual infections in the Atiba Local Government Area of Oyo State.

A cross-sectional study design was used to recruit a population of 181 participants aged (18-75 years old) from four communities in Atiba LGA in October 2023. Demographic information was gathered using structured questionnaires. After obtaining informed consent, participants provided 5 ml of blood and 20 ml of urine samples, utilized for both molecular and parasitological examinations. A polymerase chain reaction (PCR) assay was developed as a potential diagnostic tool for detecting unisexual schistosomiasis in humans. The assay included two sex-specific primers for female worms (WSh1, WSh2) and male worms (AG8_1, MS3_00009462), along with an endogenous marker (GAPDH) serving as an internal control. Additionally, centrifugation and microscopy were employed to determine the prevalence of conventional (bisexual infections) in the study area.

The prevalence of schistosomiasis in Atiba LGA was 12.7%. with the majority of infections classified as light intensity at 78.3% compared to 21.7%, and a mean intensity of 35 eggs/10ml of urine. Notably, worm fragments were identified in the urine of 11 participants. The four sex specific primers were unable to amplify the *S.haematobium* DNA, hence detection of unisexual infections was not possible. The statistical analysis revealed a significant relationship ($p < 0.05$) between schistosomiasis infection and gender, with higher infection rates observed in males. However, no significant association was found between infection and age group.

In conclusion, schistosomiasis is prevalent in the area, however there is a need to develop efficient diagnostic tools to detect unisexual infections.

CHAPTER ONE

INTRODUCTION

1.1. Background of the Study

Schistosomiasis is a parasite disease that poses a threat to global public health and affecting between 230 and 250 million individuals each year (Wei *et al.,* 2018, Colley *et al.,* 2017). Its impact is profound in impoverished and rural areas, particularly those with agricultural and fishing populations. About 90% of all schistosomiasis cases worldwide are thought to originate from sub-Saharan African nations, which the World Health Organization rates as having the highest parasite load (Hastings 2016; Ajakaye *et al.,* 2017). Nigeria is the country with the highest endemicity of schistosomiasis, with around 20 million individuals needing treatment each year. Schistosoma species that affect humans include *Schistosoma haematobium, S. mansoni, S. intercalatum, S. japonicum,* and *S. mekongi* (Adenowo *et al.,* 2015).

Urogenital schistosomiasis, attributed to the parasitic infection by *Schistosoma haematobium,* with transmission typically initiated by infected individuals excreting urine into bodies of water. Eggs released with the urine develop into miracidia, which then seek freshwater snail hosts (*Bulinus species*). Within these snails, miracidia transform into sporocysts and eventually into cercariae, the infectious stage of the parasite. Human infection arises through contact with freshwater bodies contaminated with cercariae. Following entry into the human host, cercariae undergo transformation into larvae known as schistosomula, traversing the circulatory system until they reach the venous plexus encircling the bladder, where they mature into adult worms. (Salawu and Odaibo 2016; Colley et *al.,* 2017). Male and female schistosome worms exhibit pronounced sexual dimorphism, unlike other trematodes, which are hermaphroditic. As digenetic trematodes, schistosomes propagate and initiate infections in specific hosts predominantly by the production of parasite eggs by mature female worms subsequent to mating with males. (Reinholdt *et al.,* 2023). *Schistosoma haematobium* worms are found in pairs within the venous plexus of the bladder. and this pairing facilitates sexual maturity in female worms. However, schistosome worms may not exist as pairs due to loss of a sexual partner. It was popular opinion that paired schistosomes were the only ones capable of developing and surviving for decades in a definitive

host, while unisexual female schistosomes were believed to stay immature and perish quickly, after loss of male counterpart (Moné and Boissier 2004). Laboratory models using mice have however negated this claim as female worms were seen to survive independently for 52weeks in the animal host (Lu *et al.,* 2021).

Unisexual or single-sex schistosomiasis infection refers to asymptomatic infections characterized by the absence of viable egg production due to the presence of only one sexual counterpart (either a male or female schistosome worm) in a definitive host. This situation may occur for two reasons: either one sexual partner is eliminated in the definitive host due to praziquantel treatment, or the host in which they complete their life cycle becomes infected by a unisexual cercaria. Unisexual infections are often overlooked in diagnosis, particularly in Point of Care Circulating Cathodic Antigen (POC-CCA) tests and nucleic acid tests. Following praziquantel treatment, some individuals may test negative for eggs but remain positive for POC-CCA. (Lamberton *et al.,* 2014; Krauth *et al.,* 2015) and / or nucleic acid positive (Weerakoon *et al.,* 2016). This diagnosis has been linked to breakdown products of schistosoma rather than lingering single-sex infections. The potential occurrence of unisexual infections in natural populations has various implications. For instance, the definitive host may serve as a reservoir for single-sex schistosomes, potentially inflating cure rates following praziquantel treatment. Moreover, there's a significant risk of hybridization with zoonotic schistosome species, as unisexual schistosomes can remain "mate ready" and subsequently reproduce upon contact with the opposite sex. (Lu *et al.,* 2018: Reinholdt *et al.,* 2023).

The bulk of knowledge about unisexual infections is based on artificial populations created in the laboratory using mouse models. Moreover, confirming single-sex schistosome infections in definitive hosts poses logistical challenges, as detecting unisexual infections is feasible in non-human animals only through dissection or perfusion (Lu *et al.,* 2018). Currently, no reliable diagnostic method exists for identifying single-sex schistosome infections in humans. An emerging technique that holds promise for detecting unisexual infections involves analyzing cell-free schistosome DNA derived from excrement, worms, egg metabolites, and cell debris present in patients' plasma, urine, and saliva.Cell-free schistosome DNA has been proven as notable markers of active infections (Kato-Hayashi *et al.,* 2015), therefore DNA can be extracted from any suitable body fluid (e.g. plasma) amplified using sex specific primers, thus confirmation of

male or female worm infection can be possible without dissection of tissues (which is not even possible in living human subjects).

1.2. Statement of problem

The administration of praziquantel in endemic areas seems to lower or eradicate the presence of eggs in urine after a period. However other diagnostic methods such as POC-CCA, DNA based tests usually detect an active infection even in the absence of egg excretion in urine. This has been proposed to be a result of the death of one of the reproductive (male or female) after MDA or the infection of the definitive host with a single sex cercariae. Current information on unisexual schistosomiasis only exists for laboratory models and the unisexual infections in intermediate hosts (snails). Much is still unknown about the profile of unisexual schistosomiasis in natural infections in humans. At present there is no valid diagnostic method to test this claim of unisexual schistostome infections in humans.

1.3. Justification

Unisexual schistosomiasis has been studied using animal models (from literature spanning as far back as 2001-2023), but there are only three studies (Eppert *et al.,* 2002, Zu-wu *et al.*, 2012, Shi *et al.,* 2014) on natural population of unisexual schistosomes. These studies only considered the infection in the natural population of the snail intermediate hosts which only harbor the developmental phase of the parasite. Artificial murine models have helped mimic the natural population of humans because of similarity in physiology and have provided a way of increasing our understanding of unisexual infections especially about the immunology and longevity of unisexual worms in definitive hosts. However, in the real context, there are not many in-depth studies on unisexual infection in the natural *S.haematobium* human populations. Information from this study could be a good lead to understanding the presence of unisexual infections and the rate at which this infection occurs in the natural population. Furthermore, understanding the reproductive biology of unisexual schistosomes may lead to develop of more targeted and effective treatments of *S.haematobium* infections.

1.4. Research Questions

- Are there any exclusive male urogenital schistosomiasis infections that occur in natural populations?
- Are there cases of exclusive female urogenital schistosomiasis infections in natural populations?

1.5. Aim of the Study

The aim of this project is to identify unisexual *S. haematobium* infections within the natural *S. haematobium* population utilizing molecular markers.

1.6. Specific Objectives

The objectives of the study are as follows:

- To determine the prevalence of bisexual Schistosomiasis infections in the study population.
- To test male upregulated gene markers MS3_00009462 and AG_8 in the detection of male *S.haematobium* adult worms in natural UGS infections.
- To validate WSh1 and WSh2 primers' efficiency in detecting female *Schistosoma haematobium* adult worms in natural UGS infections.
- To determine the prevalence of unisexual schistosomiasis in the study population.

CHAPTER TWO

LITERATURE REVIEW

2.1. Urogenital Schistosomiasis

The World Health Organization (WHO) approximates that between 500-600 million individuals face the risk of contracting human schistosomiasis, ranking it as the second most severe parasitic disease globally after malaria (Colley *et al.,* 2014). Parasitic flatworms, known as blood flukes, belonging to the genus *Schistosoma*, are particularly prevalent in regions such as sub-Saharan Africa, parts of the Middle East, South America, and Southeast Asia. In Africa, approximately 93% (192 million) of the estimated 207 million global cases of schistosomiasis are concentrated in sub-Saharan Africa. Nigeria leads with an estimated 29 million infected individuals, followed by Ghana (19 million), the United Republic of Tanzania (19 million), and the Democratic Republic of the Congo (15 million). (Adenowo *et al.,* 2015). Schistosomiasis persists as a continuous health challenge in sub-Saharan Africa, influenced by various factors. These include socioeconomic elements such as poverty and occupational practices, proximity to water sources, and the development of irrigation systems and dams (Kone *et al.,* 2022). The three primary species of schistosomes responsible for most human infections are *Schistosoma haematobium, Schistosoma mansoni,* and *Schistosoma japonicum*. While both *S. haematobium* and *S. mansoni* are prevalent in Africa and the Middle East, *S. mansoni* is the sole species found in the Americas, whereas *S. japonicum* is limited to Asia, notably in China and the Philippines (Colley *et al.,* 2014).

Mature *Schistosoma haematobium* worms commonly reside as paired males and females within the vesical venous plexus of the human host. Female worms lay eggs, which migrate through tissues, infiltrating the lining of the mesenteric veins, and eventually penetrating the bladder walls to be excreted via urine (Abdulkareem *et al.,* 2018). The treatment of schistosomiasis predominantly involves the use of a single anthelmintic medication, praziquantel (PZQ). Nevertheless, even with global endeavors aimed at eradicating this disease, rates of reinfection can be distressingly elevated and the emergence of drug resistance remains an ongoing concern (Abubakar *et al.,* 2022). *Schistosoma haematobium* has two necessary hosts in its life cycle; a molluscan intermediate host (*Bulinus species*) where the adult parasite multiplies asexually and a

mammalian definitive host (Paniker and Ghosh, 2017). Adult mature pairs of monogamous male and female migrate to urogenital venules where the females deposit millions of eggs each day. Within the ovum, a ciliated miracidium develops, while some eggs traverse the blood vessel wall into the bladder lumen and are eventually expelled in the urine (Nation *et al.,* 2020). Those eggs released into the water mature into miracidia, which then seek out specific freshwater snail hosts guided by chemical and optical signals. (Salawu and Odaibo, 2016). Within the snail host, the miracidium transforms into an unciliated form called the mother sporocyst. Subsequently, it undergoes asexual reproduction, producing multicellular daughter sporocysts, which ultimately develop into bifurcated cercarial larvae. Under suitable temperature and light conditions, snails often release cercariae, typically during daylight hours. These cercariae then navigate the water environment before attaching to a definitive host upon contact with the water. Following penetration through the skin, the cercariae mature into schistosomula, which then migrates through the liver, portal vein, and ultimately into the mesenteric vein. There, it develops into sexually mature adults, form pairs and produce eggs, initiating a new cycle of infection. (Mehlhorn, 2016).

2.2. Implications of Schistosomiasis Diagnostic Tests in Transmission

Various diagnostic methods have been developed to identify schistosomiasis infections. Commonly utilized techniques include the Kato-Katz (KK) method (for *S. mansoni*) and urine microscopy (for *S. haematobium*), along with point-of-care circulating cathodic antigen tests and antibody assays (Casacuberta-partal *et al.,* 2021). These tests possess limitations and exhibit differing levels of sensitivity in detecting infections. The gold standard for detecting infection is the excretion of parasite eggs, although it is constrained in its capacity to detect pre-patent infections. (Kato-Hayashi *et al.,* 2015; Barenbold *et al.,* 2017). A comparison of Kato-Katz (which detects the eggs) and POC-CCA techniques (detects worms) in *S. mansoni* revealed that it is possible for a patient to harbour worms without eggs, following effective anti-worm treatment. This is especially a concern in areas of low intensity transmission areas characterized by low egg excretion and higher detection of worms by POC-CCA (Infurnari *et al.,* 2017). This presents several challenges in high transmission regions, as determining the current status of schistosomiasis in highly endemic areas becomes challenging due to the superficially low parasite load detected after mass drug administration, despite the continued high rates of parasite

transmission. (Ajibola *et al.,* 2018). Several hypotheses have been put forward to explain this phenomenon, including the insensitivity of the KK stool exam in cases of low-intensity infections, potential destruction of worms upon treatment with praziquantel, temporary suppression of oviposition due to damage to some worms, and the occurrence of single-sex infections involving either stunted female-only infections or exclusive male infections (Colley *et al.,* 2017). However, the possibility of unisexual infection cannot be discounted, as numerous studies using unisexual murine models conducted by various researchers (Sombetzki *et al.,* 2022; Reinholdt *et al.,* 2023) have reported immune-modulatory effects associated with such infections. Similar to dioecious schistosomes, unisexual schistosomes are known to elicit immunological responses in their hosts. While various immune evasion strategies have been observed, certain unisexual organisms display distinct patterns of immune modulation. A Th1/Th2 imbalance induced by single-sex schistosome infection, which can occur in either sex, triggers immunological responses in the host (Zhong and Jin, 2023).

Mone and Boissier (2004) stated that male worms possess the ability to modulate the immune response, either through antigen production or by triggering inflammatory pathways. Furthermore, research by Sombetzki *et al.,* (2017) demonstrated that an 11-week infection with female worms resulted in a reduction of egg-induced hepatic fibrosis, mediated by Cytotoxic T-Lymphocyte-associated protein (CTLA-4). This protein acts as an immune checkpoint receptor, dampening immune responses (Buchbinder *et al.,* 2016). Conversely, male worms did not elicit anti-fibrotic effects or induce CTLA-4 production. Similarly, female worms also provoked lesser organ alterations compared to their male counterparts, possibly attributed to their CTLA-4-mediated effects. Moreover, in contrast to infections in definitive hosts harboring dual-sex schistosomes, single-sex infections can result in an excess of either males or females, which may remain unpaired throughout their lifespan or coexist alongside paired worms within a definitive host. (Wang *et al.,* 2022). In addition, paired worms have consistently exhibited greater susceptibility to praziquantel (PZQ) in mice, indicating their heightened vulnerability compared to single-sex schistosomes (Yu *et al.,* 2021).

2.3. Unisexual Schistosomiasis Infections

Unisexual schistosomiasis infection denotes asymptomatic cases characterized by the absence of viable egg production due to the presence of only one sexual counterpart (either a male or female schistosome worm) in a definitive host. It has been believed that unisexual infections do not occur in nature, as pairing of bisexual counterparts facilitates female reproductive maturity. It has been argued that unpaired female schistosomes, resulting from single-sex exposure or subsequent loss of a male mate, inevitably starve and have a limited survival duration (Boissier *et al.,* 2004). Nevertheless, recent studies show that unisexual schistosome worms can survive in mice for up to 52 weeks (Lu *et al.,* 2021; Sombetzki *et al.,* 2022). Most eradication efforts have focused on bisexual infections due to their symptomatic nature, as evidenced by observable egg excretion. Furthermore, infections may persist in populations despite intense eradication efforts, mainly due to asymptomatic unisexual infections that do not produce eggs (Zhong and Jin, 2023).

Numerous studies have successfully modeled unisexual infections experimentally in laboratory. However, recent findings have illuminated the occurrence of unisexual infections in natural settings as well. For instance, unisexual infections in definitive hosts may arise from the infection of a single-sex miracidium originating from a molluscan host. In a field investigation conducted by Shi *et al.,* (2014) in the hilly regions of Anhui, China, from a subset of 46 snails selected out of a larger pool of 67, it was observed that 21 snails were infected with female schistosomes, 23 with males, and only 2 with both sexes. Moreover, Zu-Wu *et al.,* (2012) reported unisexual infections in a cohort of 400 sentinel mice deployed in 2012 along the Yangtze River in the Hubei region of China to identify *S. japonicum* transmission sites. Among the 22 mice found to be infected with schistosomes, 14 were exclusively colonized by adult male schistosomes, and two were exclusively colonized by adult female schistosomes.

Unisexual infections may occur when either the paired male adult worm is eradicated by praziquantel therapy, leaving the female worms in the final host's body, or vice versa. Since the male and female paired schistosomes share a groove, it stands to reason that males would be exposed differently to praziquantel and consequently perish, while females remain unharmed (Webster, 2016). Unisexual schistosomiasis likely contributes to the persistence of infections post-treatment. However, there is currently no reliable diagnostic method for identifying human single-

sex schistosome infections in unisexual schistosomiasis. Single-sex schistosome infections are often identified through morphological observations upon dissection, a method feasible only in non-human hosts. Due to the shorter length of unpaired female worms compared to bisexually mated females, identifying unpaired female worms during dissection can sometimes be challenging (Lu *et al.,* 2018).

2.4. Morphology of Unisexual Schistosomes

Biology acknowledges that an organism's structure influences its function. The morphology of unisexual worms might explain why infections of schistosomes have often gone unnoticed or unreported. Morphologically, male and female bisexual mated worms differ, with females typically being longer and more slender. Schistosomes typically develop into cylindrical worms, ranging from 7 to 20 mm in length, featuring two suckers at each end, a blind digestive tract, a complex tegument, and reproductive organs (Melhorn, 2016). The male holds the female in its gynephoric canal, and both male and female worms permanently inhabit the mesenteric venous plexus, as seen in species such as *S. mansoni* and *S. japonicum,* or the peri-vesical venous plexus in the case of *S. haematobium* (Sun *et al.,* 2022).

Unisexual worms are approximately one-third the size of bisexual worms, making them more challenging to identify due to their smaller stature. According to Lu *et al.,* (2018), solitary females are notably shorter than paired females, whether they have never been paired or have lost their male counterpart. In male schistosomes, the testes of solitary worms are slightly smaller than those of bisexually infected mated male worms, with no significant physical difference apparent between them and females (Beckmann *et al.,* 2010). The development of the ovary in single-sex schistosomes differs from that in bisexual schistosomes. In *S. japonicum,* mature ovaries appear in bisexual worms at 18 days post-infection and develop vitelline glands, while in unisexual *S. japonicum* worms, both ovaries and vitelline glands remain underdeveloped (Zhong and Jin, 2023).

2.5. Reproductive Potential of Unisexual Schistosome

Experimental evidence demonstrates that single-sex schistosomes retain the ability to mate and reproduce under certain circumstances. Reinholdt *et al.,* (2023) illustrated that single worms were capable of pairing several months following unisexual infection and initiating egg-laying in mice

infected with either 100 male or female cercariae, and subsequently re-infected with the opposite sex for an equal duration after 12 weeks. Similarly, Lu *et al.,* (2018) documented a comparable outcome after exposing mice to exclusively female *S. japonicum* cercariae initially, followed by exclusive male *S. japonicum* cercariae five weeks later. Furthermore, it was observed that the reproductive potential persisted for up to 52 weeks, as evidenced by one-year-old male or female unpaired *S. japonicum* worms exposed to cercariae of the opposite sex. Six weeks after the second exposure, pairs of adult worms and their eggs were discovered in the liver tissues (Lu et al., 2021).

Conversely, Wang *et al.,* (2022) considered the reproductive potential of unisexual schistosomes from a different perspective. The authors utilized mice aged three months to evaluate the efficacy of PZQ treatment (doses: 0mg/kg, 200mg/kg and 400 mg/kg) against single-sex S. japonicum. Results from the study indicated that there was no observed treatment effectiveness on female schistosomes. However, a notably greater reduction in worm burdens was observed in male schistosomes at the PZQ dosage of 400 mg/kg. Additionally, both male and female schistosomes that survived PZQ treatment retained their normal reproductive potential.

2.6. Sex specific primers for Discrimination of male and female Schistosomes at Developmental stages.

Schistosomes, among trematodes, are unique due to their distinct sexes. While males and females can only be morphologically distinguished in adulthood, their gender is determined by genetics, with males having ZZ chromosomes and females having ZW chromosomes. However, during the larval stages (miracidia and cercariae), no discernible morphological differences are observable (Chevalier *et al.,* 2016). The W chromosome, specific to females, serves as the basis for all molecular techniques developed for sexing schistosome larvae. Three DNA repeated sequences D9, W1, and W2 have been isolated and described from the female W chromosome (Chevalier *et al.,* 2016). Despite all *Schistosoma* species sharing a similar sex chromosome system (ZZ, ZW), sex-specific primers vary among them. For instance, sex-specific markers for *S. mansoni* could not amplify the sex-specific region in *S. haematobium* and *S. bovis* (Kincaid-Smith *et al.*, 2016).

For identifying the sex of *Schistosoma mansoni* larvae, the specificity of the W1 sequence to females has been established. Consequently, primers W1, W2, and Sm23 were successfully designed to amplify the sex-specific region of the W chromosome and detect DNA in samples. In

male cercariae, W1 and W2 amplicons were absent, while they appeared as bands in agarose gels in female cercariae (Gasser, 1991; Moné and Bossier, 2001). The efficacy of these primers was validated by identifying cercariae from molluscs using W1 and W2 primers, followed by infecting mice with the cercariae. Subsequently, adult worms were identified by perfusion of the mice, revealing that cercariae exhibiting bands produced female worms, while those without bands produced male worms (Moné and Bossier, 2001). The utilization of W1, W2, and Sm23 primers for sexing cercariae has been adopted by several recent authors in studies involving *S. mansoni* (Koloskwi *et al.,* 2017; Sombetkzi *et al.,* 2022; Reinholdt *et al.,* 2023).

Xu *et al.,* (2019) developed a multiplex PCR method for sexing S. japonicum cercariae, utilizing Tsexu2 and Tsexd2 primers initially designed by Zhao *et al.,* (2010). Additionally, Xu *et al.,* (2019) designed de novo primers (Pf5F and Pf8R) based on a specific 26 bp deletion found in the female-specific sequence compared to the male sequence. The Pf5F and Pf8R primers were designed to bridge the 26-bp gap, enabling the distinction of female cercariae. Another pair of primers, Pm0F and Pm8R, targeting the Z chromosome, was also included in the multiplex PCR. While male cercariae could be successfully differentiated from females using multiplex PCR, the results for female cercariae were not sufficiently precise, making it challenging to distinguish mixed-sex cercariae. Due to the limitations of the multiplex technique in distinguishing female cercariae from mixed infections, Li *et al.,* (2020) developed an alternative technique. This new method leveraged female-specific genes located on the W chromosome of female *S. japonicum.* Through their research, twelve genes were identified, and primers were designed based on these gene sequences. However, out of the twelve primers identified, only three (SjF4, SjF6, and SjF9) were found to be effective for sexing cercariae using a duplex PCR technique. This new approach offers several advantages, including the ability to differentiate female cercariae from mixed infections, eliminating the need for DNA extraction, and being relatively fast (requiring only 2 hours).

Kincaid-Smith *et al.,* (2016) developed sex-specific primers to differentiate between *S. haematobium, Schistosoma bovis,* and hybrid *S. haematobium x S. bovis* adult worms and cercariae. Initially, the authors attempted to use existing primers designed for *S. mansoni* to distinguish the sex of *S. haematobium* cercariae and adult worms, but these proved ineffective. While it is relatively easy to morphologically differentiate between male and female adult worms,

such distinctions are not possible at earlier developmental stages such as cercariae. Hence, the development of sex-specific primers for *S. haematobium* became necessary, particularly for experiments involving populations of unisexual cercariae. These primers were designed based on the W-specific region of the female *S. haematobium* genome, as the genome quality of *S. bovis* was subpar. Leveraging sequencing data from a field-derived hybrid strain of *Schistosoma haematobium* and *Schistosoma bovis*, the authors identified five female-specific markers within the *S. haematobium* genome. Three of these markers were found to be applicable to *S. bovis* females (both worms and cercariae), which was expected due to the close evolutionary relationship between the two species. However, two markers, Wsh1 and Wsh2, failed to amplify sequences in either female or male *S. bovis* specimens. Nevertheless, all markers functioned effectively for the *S. haematobium x S. bovis hybrid,* aligning identically with the *S. haematobium* sequence.

Stroehlein *et al.,* (2022) pioneered the creation of the latest genome sequence, UoM_Shae.V3. Their investigation uncovered distinctive sex-linked transcription patterns in both male and female adult worms. Notably, they found that around 1512 transcripts showed significantly higher abundance in female adults compared to their male counterparts. Furthermore, females possessing the ZW chromosome system displayed over 1963 sexually upregulated transcripts, with 670 and 279 transcripts exhibiting upregulation in males and females, respectively.Some of the transcripts showing higher abundance in females are associated with progesterone-mediated oocyte maturation and are specific to particular developmental stages; for example, MS3_00007597.2 is specific to the cercarial stage, while MS3_00007199 is exclusive to the schistosomula stage. These sex-linked transcripts in adult worms hold promise as potential gene markers for molecular sexing of adult worms.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area: The study was conducted in four selected communities of Atiba local government area (LGA), Oyo State, Nigeria: Asamu, Alagbon, Ile-tuntun and Alagbede. Atiba LGA covers a land area of 1,757square kilometers and a population of 168,246 as at 2006 census (NPC, 2006). It lies between latitudes 7°50'30°N and longitudes 3°57'00E. The residents of these communities are Yoruba speaking; however, majority of the dwellers in the communities are immigrants from other states. The major occupations in these communities are fishing and farming, although some are residents practice other occupations (traders and artisans). The communities were chosen for this study because of their proximity to free flowing water bodies in these areas and to a dam at Asamu. The water provides fishing, irrigation and other needs to residents of these communities. Basic infrastructures such as accessible roads, potable drinking water, electricity, healthcare, modern toilet facilities and efficient telecommunication system are lacking in these communities.

3.2. Sample size determination

The sample size was determined using the Cochrane formula; $N = Z^2p\ (1\text{-}p)/\ d^2$ (Abubakar *et al.*, 2022). ; P = 5.5%, 0.055 (Awobode *et al.*, 2015)

Where N= sample size, Z= statistical level of confidence, p = expected prevalence from a previous study, d= margin of error; P = 5.5%, 0.055 ; d= 0.05; Z= 95%, 1.96). The sample size of 80 was calculated using the Cochrane formula, however the sample size was adjusted to 194 reduce bias.

3.3. Study design

A cross- sectional study was conducted consisting of male and female individuals of different age groups (18-75). Simple random sampling was employed for this study and sample collection was carried out in October 2023.

3.3.1. Inclusion Criteria

Participants were included in this study based on these criteria:

- The participant must be at least 18 years and not older than 75 years old
- The participant must reside in the area.
- Participants must voluntarily provide blood and urine samples.

3.3.2. Exclusion Criteria: Participants were excluded from this study if they did not give their consent; and if they were below 18 years of age.

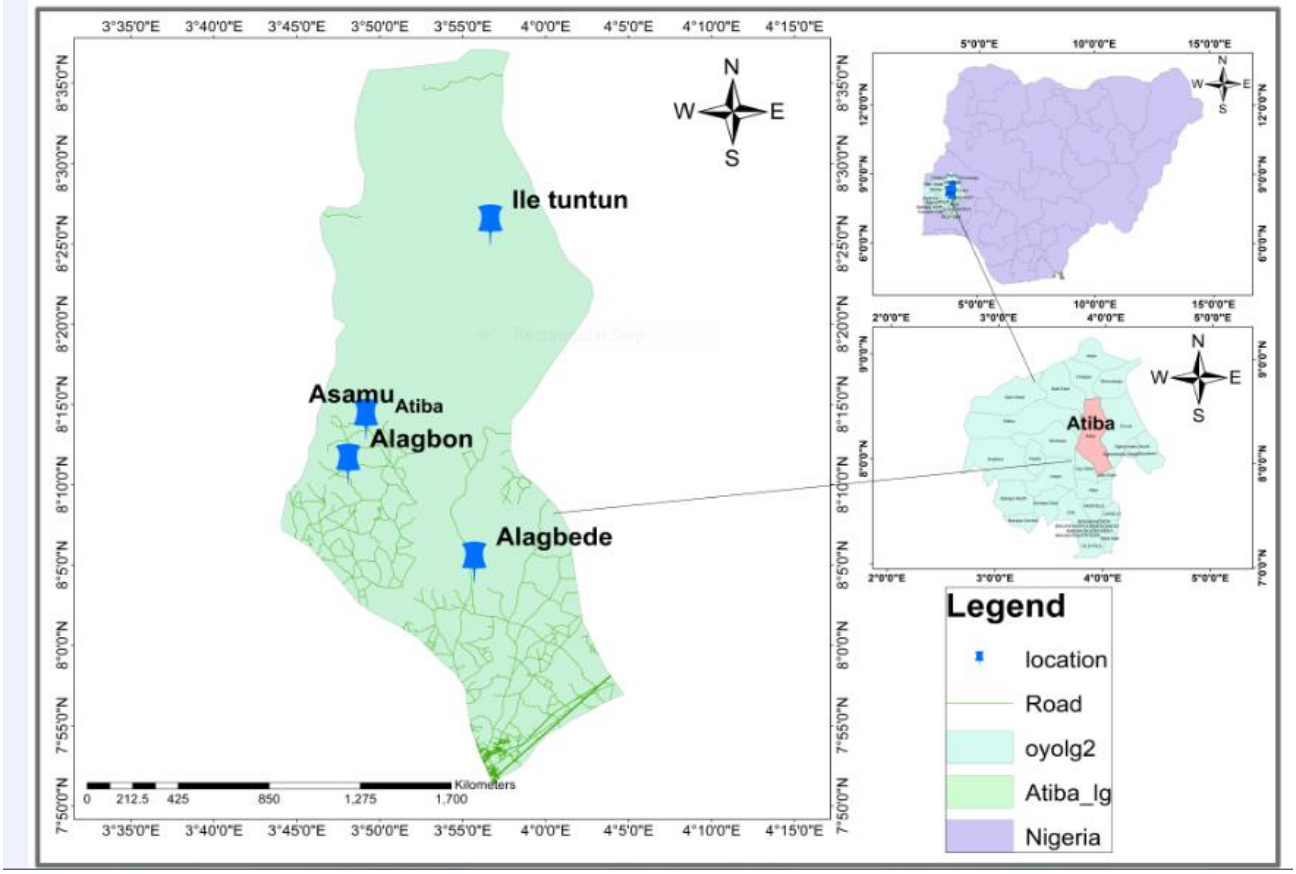


Figure 1: Map showing Study Area

3.3.3. Ethical Consideration

Ethical approval was obtained from the joint University of Ibadan/ University College Hospital ethical review committee. Participation in the study was voluntary and informed consent forms were administered to the volunteers.

3.3.4. Questionnaires

Information which included socio-demographic variables (age, sex, religion and occupation), lifestyle factors (smoker, nonsmoker, secondary smoker) occupational history, water source types (well, bore hole, river) was collected through questionnaires (see appendix 1).

3.4. Sample collection

Blood samples (5ml) were collected by venepuncture into Ethylenediaminetetraacetic acid (EDTA) bottles. After allowing the bottles to stand for some hours, plasma was obtained by aspiration of the supernatant using a pasteur pipette. Subsequently the packed cell and plasma were transported on ice to the laboratory. Urine samples were collected in universal bottles from participants between 10.00 am and 12.00 pm when maximum egg excretion occurs and stored at -20℃

3.5. Analysis of Samples

3.5.1. Parasitological Examination

The detection of *S. haematobium* eggs in urine was conducted through urine microscopy using the standard centrifugation method. The number of eggs in each 10ml of urine was tallied to determine the intensity of infection: 0 egg/10 ml of urine was deemed negative, 1-49 eggs/10 ml of urine indicated a light intensity of infection, and ≥50 eggs/10 ml of urine signified a heavy intensity of infection (Onile *et al.,* 2016). Additionally, a urinalysis reagent strip was utilized to analyze the urine samples, enabling the detection of blood and other analytes.

3.5.2 DNA Extraction

Genomic DNA from 48 plasma and 4 urine samples were extracted with the Magmax^{TM} DNA Multisample Ultra 2.0 Kit (Applied Biosystems by Thermofisher scientific), according to the manufacturer's instructions. 20µl of enhancer solution was added to 200µl of sample followed by addition of 20µl of proteinase K in an Eppendorf tube. The mixture was vortexed for 35 seconds and thereafter incubated on a heating block at 65°C for 20 minutes. After incubation 432µl of DNA binding mix was added to each sample tube and inverted five times to mix (the DNA binding mix was prepared by adding 400µl of binding solution to 32µl of DNA binding beads). After inverting the tubes, the samples were centrifuged for 3minutes in a mini centrifuge at 1000 revolutions per minute (rpm) for 3 minutes.

Subsequently, the tubes were positioned on a magnetic rack, and the supernatant was carefully drawn off from each tube, ensuring the magnetic beads remained undisturbed. Following this, the tubes were relocated to a plastic rack, and 1ml of wash I (provided in the kit) was introduced into each sample tube. The mixture was then vortexed for 30 seconds and centrifuged for 1 minute at 1000 rpm. After centrifugation, the tubes were returned to the magnetic rack, and the supernatant was meticulously removed. For the second wash of the DNA binding beads, wash II was prepared using 80% ethanol, with 500 µl added to the tubes. The tubes were again vortexed for 30 seconds, centrifuged for 1 minute at 1000 rpm, and placed on the magnetic rack, followed by careful removal of the supernatant. Next, the beads were air-dried by incubating them at 65°C for 20 minutes. Next, 100 µl of elution buffer was added to each sample tube, which was then incubated at 70°C for 5 minutes. Following the incubation, the samples were centrifuged at 1000 rpm for 5 minutes. The tubes were then transferred to a magnetic stand, and the eluates containing the purified genomic DNA were transferred to PCR tubes for storage. Finally, the DNA yield was determined using a nanodrop spectrophotometer (Thermo ScientificTM ND-2000).

3.5.3. *Schistosoma haematobium* Sex Specific Primers Used for the PCR

Female-specific primers Wsh1 and Wsh2 were utilized to detect female *S. haematobium* worms in human plasma and urine samples due to their specificity for detecting female *S. haematobium* and lower likelihood of detecting other *Schistosoma* species. These primers have previously demonstrated the capability to differentiate female *S. haematobium* cercariae, (which are morphologically indistinguishable) and isolated adult worms. The WSh1 and WSh2 primers were synthesized based on primer sequences from Kincaid-Smith *et al.,* (2016).

Male-specific primers (MS3_00009462 and AG8_1) were newly designed from two gene markers reported to be upregulated in male *Schistosoma haematobium* compared to female worms (Stroehlein *et al.,* 2022). Gene sequences were retrieved from the National Center for Biotechnology Information (NCBI) gene bank, and the NCBI primer blast tool was utilized for primer design. Among several primer pairs, those with the most favorable flanking regions (as shown in plates 1-4) and the highest proportion of guanine and cytosine nucleotides (GC content) were selected (Basu, 2015). Additionally, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, a well-known housekeeping gene, was designed as an internal control, following the method described by Kincaid-Smith *et al.,* (2016).

A supplementary primer (DRAI) was included in the study to enhance the DNA content of circulating free *Schistosoma haematobium* DNA in human samples. DRAI serves as a molecular marker for detecting urogenital schistosomiasis (UGS) from urine and plasma samples by amplifying the 121 bp tandem repeat DRAI sequence of *S. haematobium* (Keller *et al.,* 2020). The primer sequences were obtained from Hamburger *et al.,* (2001) and synthesized accordingly.

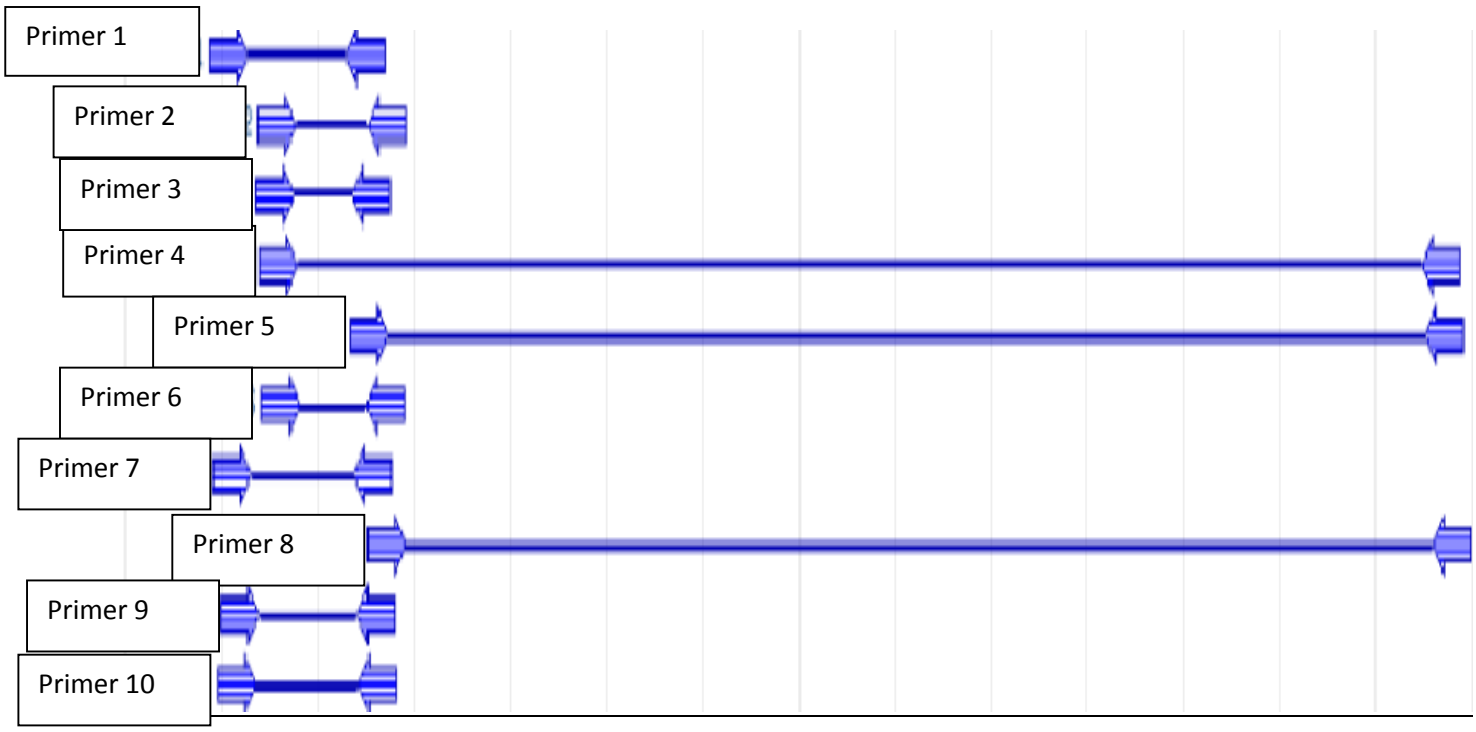


Plate 1: Pictorial view of all primer pairs for Ag_8 primer

Table1: Properties of primer pair (9) selected for design of AG8_1 primers

	Sequence (5'-3')	Template strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	CGGTGGCATTGACTCTGGTT	Plus	20	450	469	60.6	55.0	3.0	0.0
Reverse primer	AATCGTCCTCTGCCCTCTTG	Minus	20	539	520	59.46	55.0	2.0	0.0
Product Length	90								

Products on intended targets

> XM_051216088.1 *Schistosoma haematobium* Severin (AG8_1), mRNA

Product length= 90

Forward primer 1 CGGTGGCATTGACTCTGGTT 20
Template 859 ……. 878

Reverse primer 1 AATCGTCCTCTGCCCTCTTG 20
948……..929

*self comp.- self complementarity

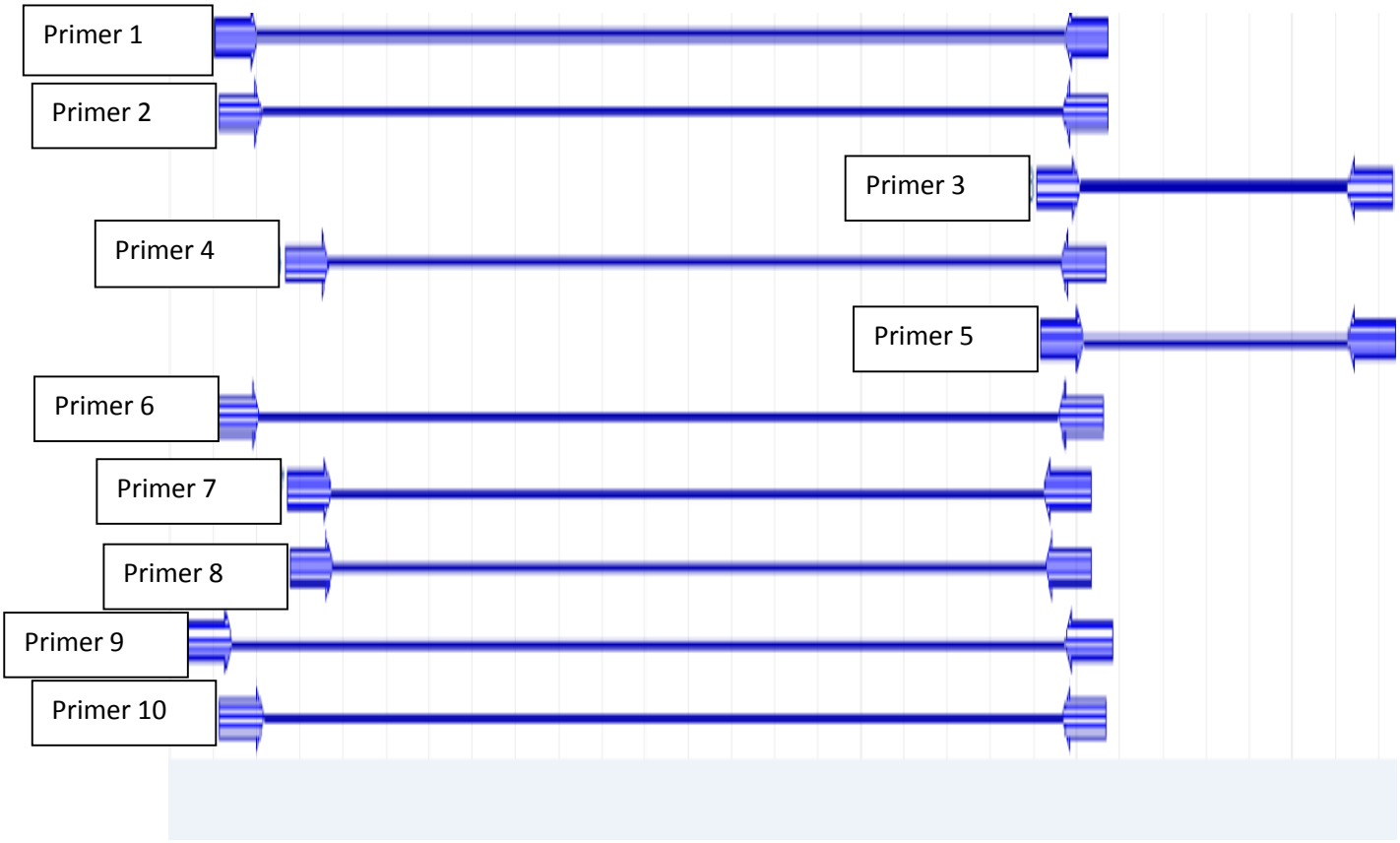


Plate 2: Pictorial view of all primer pairs for MS3_00009462 primer

Table 2: Properties of primer pair (7) selected for design of MS3_00009462 primers.

	Sequence (5'-3')	Template strand	Length	Start	Stop	Tm	GC%	Self comp.	Self 3' comp.
Forward primer	GCACCGAGGTATTTCCACCC	Plus	20	115	134	60.0	55.0	4.0	0.0
Reverse primer	CGCTTTAGTAGTGTGGCTTGG	Minus	22	486	465	59.9	55.0	2.0	0.0

Product Length= 372

Products on intended targets

> XM_012943991.2 *Schistosoma haematobium* uncharacterized protein (MS3_00009462), mRNA

Product length= 372

Forward primer 1 GCACCGAGGTATTTCCACCC 20
Template 863 ……. 882

Reverse primer 1 CGCTTTAGTAGTGTGGCTTGG 20
1234…….. 1213

*self comp.- self complementarity

Table 3: List of Primers used in this study.

Name	Forward Primer(5'-3')	Reverse Primer(5'-3')	Size(bp)
WSH1[a]	GCGTTCCGTTTAAAACATCG	GTCCATGTGAGGGAATTTCG	187
WSH2[a]	GAATCGATGACACTGGCGTA	CCACTGTCCTTCGGAATTGT	239
MS3_00009462[b]	GCACCGAGGTATTTCCACCC	CGCTTTAGTAGTGTGGCTTGG	411
AG8_1[b]	CGGTGGCATTGACTCTGGTT	AATCGTCCTCTGCCCTCTTG	90
GAPDH[c]	CGACCATTGATGCAGCTAAA	TTCCAAAATCCCCTTCATTG	558
DRAI[d]	GATCTCACCTATC-AGACGAAAC	TCACAACGATA-CGACCAAC	121

[a] represents female specific primers

[b] represents male specific primers

[c] represents internal control primer

[d] represents supplementary primer

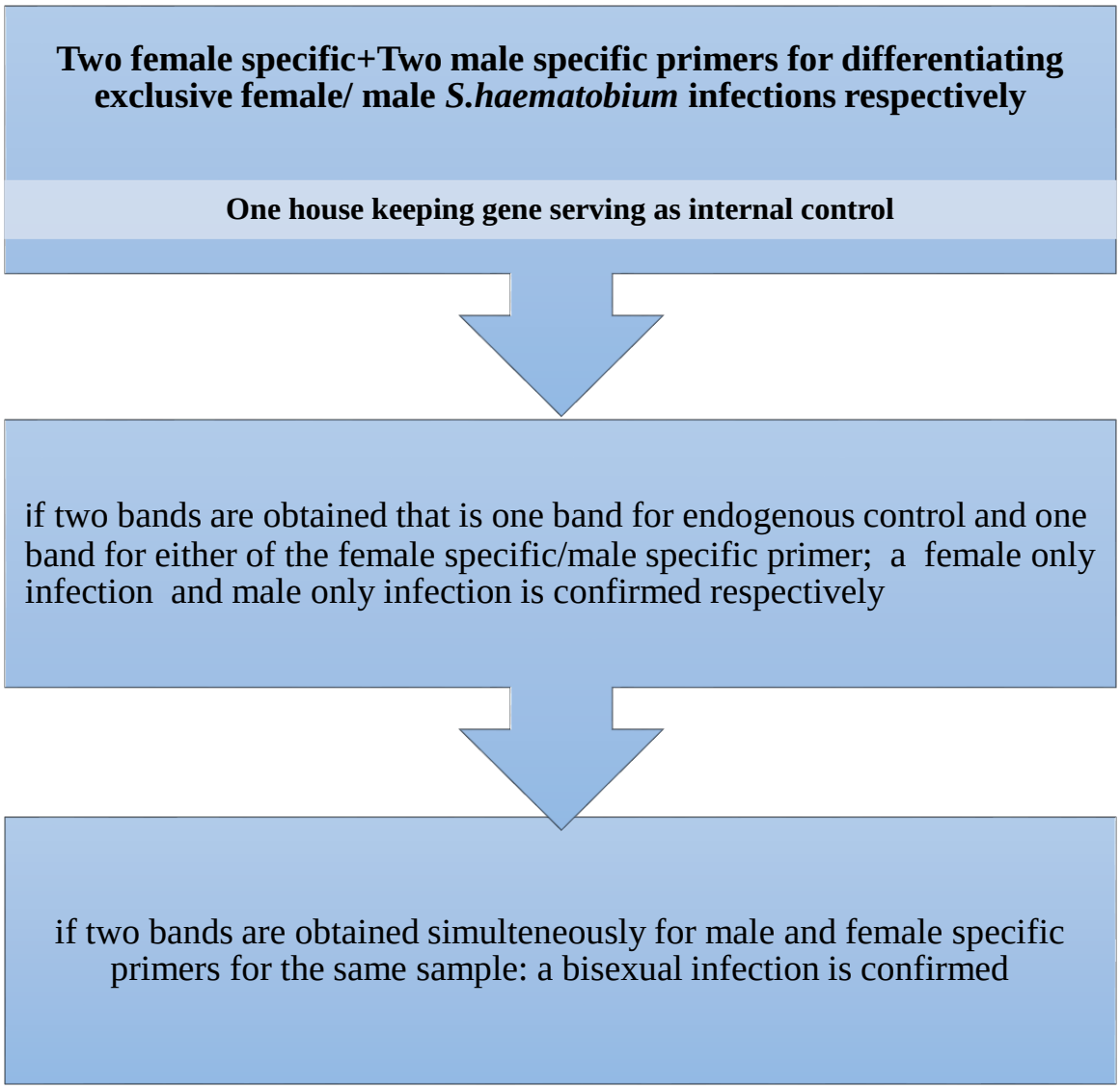


Figure 2: Hypothetical framework for the discrimination of male and female *S.haematobium* worms from plasma and urine DNA using molecular markers (Source: self developed)

3.5.4. PCR Amplification of *S.haematobium* Sex Specific Markers

3.5.4.1. *In silico* Amplification

In silico PCR was carried out to validate the primers before using the primers for wet laboratory work. The primers were amplified *In silico* on the University of California Santa Cruz (UCSC) genome browser. The reference genome sequence (UoM_Shae V3 Feb 2022) served as the template for *In silico* PCR amplification.

3.5.4.2. Amplification using Multiplex Assay

The four sex-specific primers and an internal control primer were initially employed in a multiplex PCR setup. Additionally, a touchdown PCR technique was utilized, employing an Eppendorf Mastercycler Nexus SX1, wherein individual annealing temperatures for each primer were determined using the formula 4(C+G) + 2(A+T), ranging from 52°C to 58°C. The PCR reactions were conducted in a final volume of 25 µl, comprising 0.4 µl of each primer, 12.5 µl of 1x GoTaq green master mix, 5 µl of template DNA, and 6.7 µl of molecular-grade water. The reaction protocol involved an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 10 minutes. Subsequently, PCR products were analyzed by electrophoresis on 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer containing 5 µl of SafeView™ Classic intercalating dye, with size estimation using a 100 bp DNA ladder. Gel electrophoresis was conducted at 100V for 30 minutes, and the resulting bands were visualized under ultraviolet (UV) light using an EmbiTec PrepOne™ Filter P1-1002 transilluminator.

3.5.4.3. Amplification using Single-Plex PCR assay

Conventional PCR was conducted using extracted DNA, with samples grouped into 11 pools to enhance DNA content. DNA amplification was carried out using an Applied Biosystems 2720 thermal cycler. The PCR reactions were prepared in a final volume of 25 µl, comprising 0.4 µl of each primer (forward and reverse), 12.5 µl of 1x GoTaq green master mix, 5 µl of template DNA, and 6.7 µl of molecular-grade water. The amplification protocol involved an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing

at 57°C for 30 seconds, and extension at 72°C for 10 minutes. Subsequently, PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer containing 5 µl of SafeView™ Classic intercalating dye. Size estimation was performed using a 100 bp DNA ladder, and the gel was visualized under ultraviolet (UV) light using an EmbiTec PrepOne™ Filter P1-1002 transilluminator.

3.5.4.4. Amplification using Nested PCR

The circulating cell-free *Schistosoma haematobium* DNA was found in relatively low concentrations even after sample pooling. To enhance the detection sensitivity, a *Schistosoma*-specific primer (DRAI) was initially employed to amplify *Schistosoma haematobium*-specific segments within the human DNA. This first-round amplification aimed to increase the proportion of circulating cell-free *Schistosoma haematobium* DNA. Subsequently, a nested PCR approach was utilized, where two sex-specific primers one targeting females (WSH2) and the other targeting males (MS3_00009462) were tested in the second round. Given the nested PCR strategy, only samples showing positive results from microscopy were selected for this subsequent validation step.

First round of PCR

Samples were processed in 25µl volumes, comprising 12.5µl of master mix, 1µl each of the amplification primers (DRA1 forward and reverse), 5µl of template DNA, and 5.5µl of nuclease-free water. The annealing cycles consisted of an initial denaturation at 95˚C for 15 minutes, followed by 33 cycles of denaturation at 95˚C for 30 seconds, annealing at 53˚C for 1.5 minutes, and extension at 72˚C for 1 minute. The PCR was performed using the Applied Biosystems 2720 thermal cycler, with a final extension step at 60˚C for 5 minutes. PCR products were analyzed on a 2% agarose gel in tris-acetate-EDTA (TAE) buffer, containing 5µl of Safeview intercalating dye, at 120v for 40 minutes. A 50 bp-DNA ladder was utilized for estimating the size of PCR products, and documentation was carried out using a gel documentation system (Vilber Quantum).

Second Round of PCR

The second round PCR was conducted in a total volume of 25µl, comprising 12.5µl of 1x Go Taq green master mix, 5µl of the first round PCR product, 0.4µl each of the primers (WSH2 forward and reverse, and MS3_00009462 forward and reverse), and 6.7µl of nuclease-free water. The PCR conditions included an initial denaturation phase at 95˚C for 5 minutes, followed by 35 cycles at 95˚C for 30s, 57˚C for 30s, 72˚C for 40s, and a final extension at 72˚C for 10 minutes. PCR products were visualized on 2% agarose gels at 120V for 30 minutes using a 50 bp-DNA ladder as the molecular size marker. Gel visualization was conducted using a gel documentation system (Vilber Quantum).

3.6. Statistical Analysis

Data collected from both the questionnaire and urinalysis in the field was first entered into a Microsoft Excel spreadsheet. Subsequently, the data was exported into Statistical Package for the Social Sciences (SPSS) 20.0 for analysis. Descriptive statistics, such as percentage values, were utilized to analyze the questionnaire data, which was then presented in a frequency table. Pearson's chi-square and p-values were employed to assess significant relationships between variables.

CHAPTER FOUR

RESULTS

A total number of 194 participants were recruited in this study from the four study areas (Asamu, Alagbon, Ile-tuntun and Alagbede). However, 13 participants were excluded from this study as a result of their inability to provide information and samples important to the research. Some five participants from Alagbon were included in this study even though some of their demographic data was not available because they were Hausa and it was important to have data from another ethnic group. Thus, data of 181 participants from the four study areas was included in the analysis: Asamu (73), Alagbon (51), Ile tuntun (29) and Alagbede (28). Parasitological analysis was conducted in the field, whereas molecular analysis was performed on a subset of samples, comprising 48 plasma (45 negative and 3 positive samples) and 4 positive urine samples previously identified through microscopy. The positive samples were chosen to validate the primers as the presence of eggs signifies sexual reproduction, confirming the presence of both male and female adult worms.

4.1. Prevalence of *S. haematobium* by gender and age group in the Natural Population

The prevalence of *S. haematobium* infection in Atiba LGA was 12.7%. There was a significant difference ($P< 0.05$, $P= 0.021$; $X^2=11.6$) in *S.haematobium* infection in males and females in Atiba (Table 7). Statistical analysis also indicated that there was no significant difference ($P< 0.05$, $P= 0.223$; $X^2=4.4$) between age groups in *S.haematobium* infection (Table 8).

4.2. Intensity of *S.haematobium* infection in relation to age groups and gender

The overall mean intensity of infection was 35 eggs/10ml of urine from the four study areas. The mean intensity by gender for the four communities was 37.9 eggs/10ml for males and 34.1 eggs/10ml of urine for females. The age group 18-30 had the highest mean intensity of infection with a value of 57.2 eggs/10ml of urine followed by the above 50 age group with 18 eggs/10ml of urine. The age group 31-50 was last with a mean intensity of 17.5 eggs/10ml of urine.

4.3. Urinalysis

A total of seven analytes was determined in urine by urinalysis. The highest frequency of haematuria (blood) was recorded in Asamu while the frequency of leukocytes and proteinuria was highest in Alagbon study area (Table 5).

4.4. DNA Yield

The DNA yield was recorded for 48 plasma samples and 4 urine samples. The highest DNA yield obtained for plasma samples was 22.17 ng/µl and 28.60 ng/µl for urine samples (Table 9a and b).

4.5.1. *In silico* amplification

The expected base pair size of all primers corresponded to the results of the *In silico* PCR amplification. The *in silico* amplification for one male specific primer (AG_8) and one female specific primer (WSh2) is shown Plate 3.

4.5.2. Amplification using multiplex assay

The sex specific primers and internal control primers were first carried out using a multiplex approach, however no amplification occurred. Also, the multiplex assay in conjunction with touch down method did not amplify the desired regions (Plate 4).

4.5.3. Amplification using Single-Plex assay

The primers were subsequently placed using a single PCR approach, there was still no amplification. This is shown in plate 5.

4.5.4. Amplification using Nested PCR

The nested PCR amplification was carried out by first amplifying DNA with DRA1 primers. Subsequently after amplification with DRAI primers the samples were amplified and bands appeared after amplification (plate 6). The amplicons obtained from the first round of PCR were used as templates in the second round, however no amplification was observed (plate 7 and 8).

Table 4: Demographic and Descriptive Statistics of the Study population.

Variables	Frequency (%)			
	Asamu	**Alagbon**	**Ile-tuntun**	**Alagbede**
Gender				
Male	41(56.2)	28(53.8)	9 (31.0)	15 (53.6)
Female	32(43.8)	23(44.2)	20(69.0)	13(46.4)
Age				
18- 30	27(37.0)	11(21.2)	9(31.0)	10(35.7)
31-50	36(49.3)	16(30.8)	10(31.5)	12(42.9)
Above 50	9(13.7)	19(36.5)	10(34.5)	6(21.4)
Education				
Nas	20(28.8)	21(40.4)	10(34.5)	6(21.4)
Cps	3(4.1)	1(1.9)	2(6.9)	6(21.4)
Ncps				1 (3.6)
Ncss	15(20.5)	10(19.2)	8(27.6)	2(5.1)
Csc	33(45.2)	12(23.0)	8(27.6)	13(46.4)
Others	1(1.4)	-	1(3.4)	-
Occupation				
Farming	28(38.4)	24(46.2)	17(58.6)	7(25.0)
Fishing	26(35.6)	9(17.3)	3(10.3)	8(28.6)
Rice farming	1(1.4)	-	-	-
Others	16(21.9)	9(17.3)	6(20.7)	5(17.9)
Farming and fishing	2(2.7)	2(3.8)	3(10.3)	7(25.0)
Water source				
River	63(86.3)	43(82.7)	29(100)	28(100)
Well	6(8.2)	-	-	-
Pipe borne water	2(2.7)	2(3.8)	-	-
Others	-	2(3.8)	-	-
Water source visit				
Once daily	39(53.4)	6(11.5)	1(3.4)	5(17.9)
More than once daily	30(41.1)	24(46.2)	24(82.8)	20(71.4)
Weekly	2(2.7)	-	-	1(3.6)
Once a year	-	1(1.9)	-	
Monthly	-	-	2(6.9)	2(7.1)
Never	-	11(17.3)	1(3.4)	
Smoking status				
Smoker	8(11)	9(17.3)	4(13.8)	6(21.4)
Non smoker	64(87.7)	35(67.3)	25(96.6)	22(78.6)
Second hand smoker	6(8.2)	16(30.8)	1(3.4)	3(10.7)

Nas- never attended school, Cps-completed primary school, Ncps-never completed primary school, Ncss- not completed secondary school, Csc- completed primary school.

Table 5: Urinalysis

Analytes	Frequency (%)			
	Asamu	Alagbon	Ile- tuntun	Alagbede
Blood	12(16.5)	3(5.7)	7(24.1)	1(3.6)
Leukocyte	9 (15.1)	17(32.7)	7(24.1)	4(14.3)
Nitrate	3(4.1)	0	1(3.4)	1(3.6)
Proteinuria	5(6.8)	6(11.55)	1(3.4)	4(14.3)
Ketone	22(30.1)	1(1.9)	1(3.4)	2(7.1)
Bilirubin	19(26.1)	17(32.7)	26(89.6)	6(21.4)
Glucose	0	0	0	0
Urobilinogen	68(93.1)	4(7.7)	2(6.8)	18(64.3)

Table 6: Schistosomiasis infection and intensity of infection determined by Microscopy

Microscopy	Frequency (%)				
	Asamu	Alagbon	Ile tuntun	Alagbede	Atiba LGA (Total)
Positives	14(19.2)	3(4.8)	5(17.2)	1(2.6)	23(12.7)
Female	3 (4.1)	1(2.0)	5(17.24)	0	9 (10.2)
Male	11(15.1)	2 (3.9)	0	1(2.6)	14(15.1)
Heavy infections ($N \geq 50$)	3 (13.0)	0	2(8.7)	0	5 (21.7)
Light infections ($N \leq 50$)	11(47.8)	3(13.0)	3(13.0)	1(4.3)	18 (78.3)
Worm fragments in urine	7(9.6)	1(2.0)	3(10.3)	0	11 (6.1)

Table 7: Relative risk estimate of Schistosomiasis infection associated with gender

		Schistosomiasis infection			
		Negative	Positive	Total	
Atiba LGA	Female	79 (43.6)	9 (5)	88 (48.6)	X^2=11.6
	Male	79 (43.6)	14(7.7)	93(51.4)	P=0.021
	Total	158(87.3)	23(12.7)	181	
Asamu	Female	29 (39.7)	3 (4.1)	32 (43.8)	
	Male	30 (41.1)	11(15.1)	41(56.2)	
	Total	59 (80.8)	14 (19.2)	73	
Alagbon	Female	21(41.2)	1(1.9)	22 (43.1)	
	Male	27(52.9)	2 (3.92)	29 (56.9)	
	Total	48(94.1)	3 (5.9)	51	
Ile tuntun	Female	15(51.7)	5 (17.2)	20 (69)	
	Male	9 (31.0)	0	9 (31)	
	Total	25 (86.2)	5 (17.2)	29	
Alagbede	Female	13 (46.4)	0	13 (46.4)	
	Male	14 (50)	1 (3.6)	15 (53.6)	
	Total	27 (96.4)	1 (3.6)	28	

Table 8: Relative risk estimate of schistosomiasis infection associated with age group

		Schistosomiasis infection			
		Negative	Positive	Total	
Atiba LGA	18-30	46 (25.4)	11 (6.1)	57 (31.5)	X^2 =4.4
	31-50	68 (37.5)	6 (3.3)	74 (41)	P= 0.223
	> 50	39 (21.5)	6 (3.3)	45 (24.9)	
	Nil	5 (2.8)	0 (0)	5 (2.8)	
	Total	158 (87.3)	23 (12.7)	181	
Asamu	18- 30	18 (24.7)	9 (12.3)	27 (37)	
	31-50	34 (46.6)	2 (2.7)	36 (49.3)	
	> 50	7 (9.6)	3 (4.1)	10 (13.7)	
	Total	60 (82.2)	13(17.8)	73	
Alagbon	18-30	10 (19.6)	1(1.96)	11(21.6)	
	31-50	15 (29.4)	1 (1.96)	16 (31.4)	
	>50	18 (35.3)	1 (1.96)	19 (37.3)	
	Nil	5 (12.3)	0 (0)	5 (9.8)	
	Total	48 (94.1)	3 (5.9)	51	
Ile tuntun	18-30	8 (27.6)	1(3.4)	9 (31)	
	31-50	8 (27.6)	2 (6.9)	10 (34.5)	
	> 50	8 (27.6)	2 (6.9)	10 (34.5)	
	Total	25(86.2)	5 (17.2)	29	
Alagbede	18-30	10 (35.7)	0 (0)	10 (35.7)	
	31-50	11 (39.3)	1 (3.6)	12 (42.9)	
	>50	6 (21.4)	0 (0)	6 (21.4)	
	Total	27 (96.4)	1(3.6)	28	

Table 9a: DNA yield for urine samples

Sample ID	ng/µl	A260	A280	260/280
P1	28.60	0.572	0.313	1.82
P2	16.81	0.336	0.166	2.20
P3	14.38	0.288	0.113	2.55
P4	9.63	0.193	0.114	1.69

Table 9b: DNA yield for plasma samples

Sample ID	ng/µl	A260	A280	260/280
1	4.31	0.086	0.076	1.13
2	7.11	0.142	0.091	1.56
3	13.83	0.277	0.319	0.87
4	1.86	0.037	0.0.010	3.88
5	4.66	0.093	0.132	0.71
6	7.32	0.146	0.182	0.8
7	3.16	0.063	0.084	0.75
8	6.65	0.133	0.122	1.09
9	3.90	0.078	0.079	0.99
10	17.48	0.35	0.208	1.68
11	2.56	0.051	0.059	0.87
12	3.43	0.069	0.064	1.07
13	7.17	0.143	0.142	1.01
14	4.63	0.093	0.148	0.62
15	2.33	0.047	0.045	1.03
16	11.05	0.221	0.125	1.77
17	10.36	0.207	0.251	0.83
18	7.39	0.148	0.167	0.88
19	2.24	0.045	0.042	1.06
20	7.07	0.141	0.206	0.69
21	7.56	0.151	0.167	0.90
22	4.89	0.098	0.102	0.96
23	4.05	0.081	0.079	1.03
24	3.26	0.065	0.059	1.11

25	1.72	0.034	0.015	2.23
26	4.65	0.093	0.081	1.15
27	22.17	0.448	0.305	1.45
28	1.50	0.03	0.029	1.05
29	3.02	0.060	0.060	1.00
30	5.91	0.118	0.124	0.95
31	2.46	0.049	0.054	0.91
32	5.25	0.105	0.097	1.09
33	6.20	0.124	0.108	1.15
34	4.55	0.091	0.071	1.28
35	4.00	0.080	0.77	1.04
36	2.01	0.040	0.045	0.90
37	2.34	0.047	0.049	0.96
38	2.95	0.059	0.057	1.04
39	4.03	0.081	0.057	1.42
40	2.59	0.052	0.024	2.20
41	2.67	0.053	0.027	1.97
42	4.67	0.093	0.103	0.90
43	2.01	0.040	0.035	1.14
44	4.08	0.082	0.108	0.75
45	9.37	0.187	0.183	1.02
46	2.79	0.056	0.052	1.08
47	15.05	0.301	0.184	1.64
48	10.21	0.204	0.105	1.94

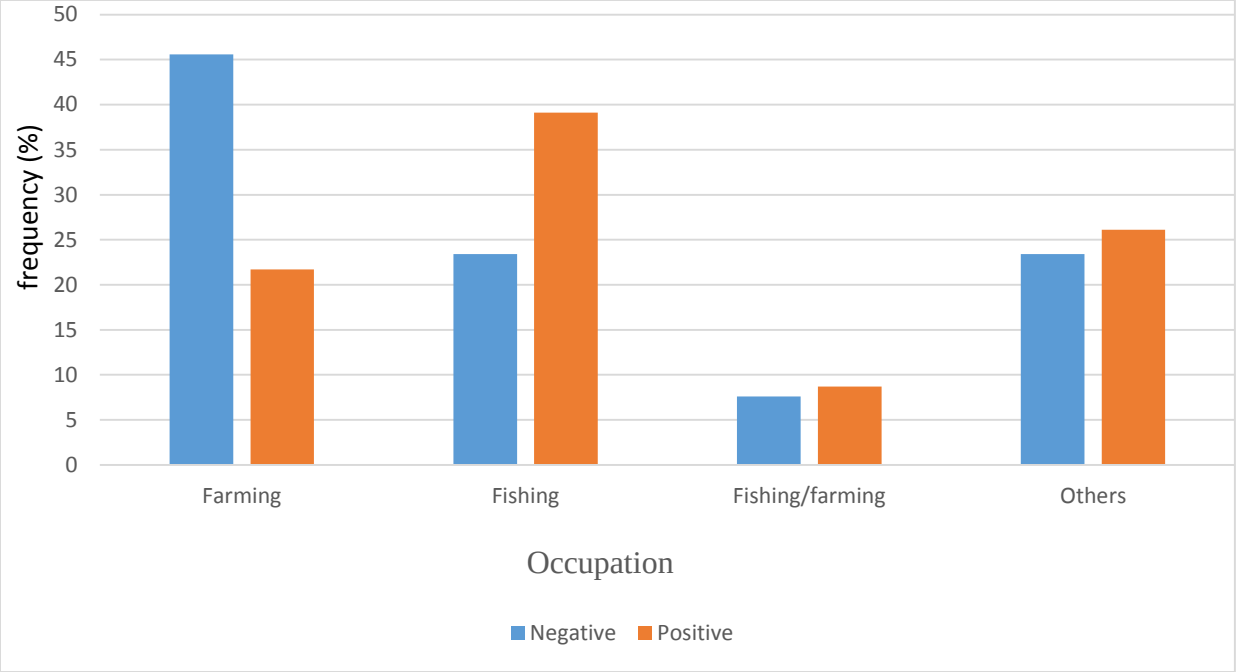
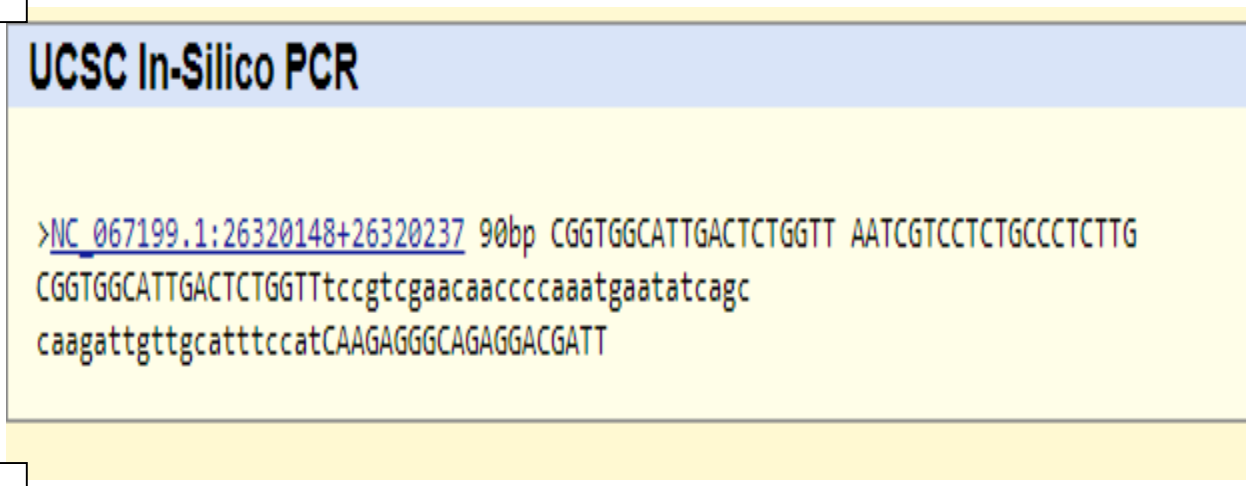


Figure 3: Prevalence of Schistosomiasis infections among different occupational groups in Atiba LGA.

A



B

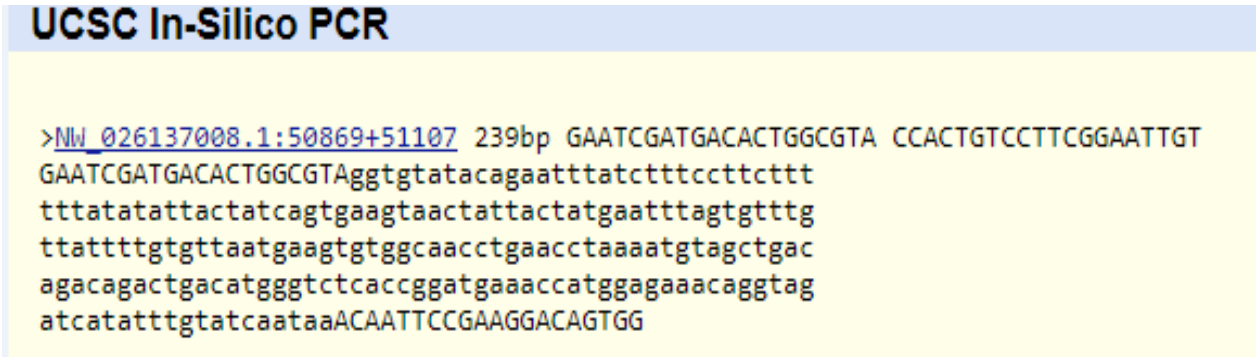


Plate 3: (A) *In silico* amplification of AG8_1primer showing the expected base pair (90bp). (B) *In silico* amplification of WSh2 primer showing the expected base pair (239bp).

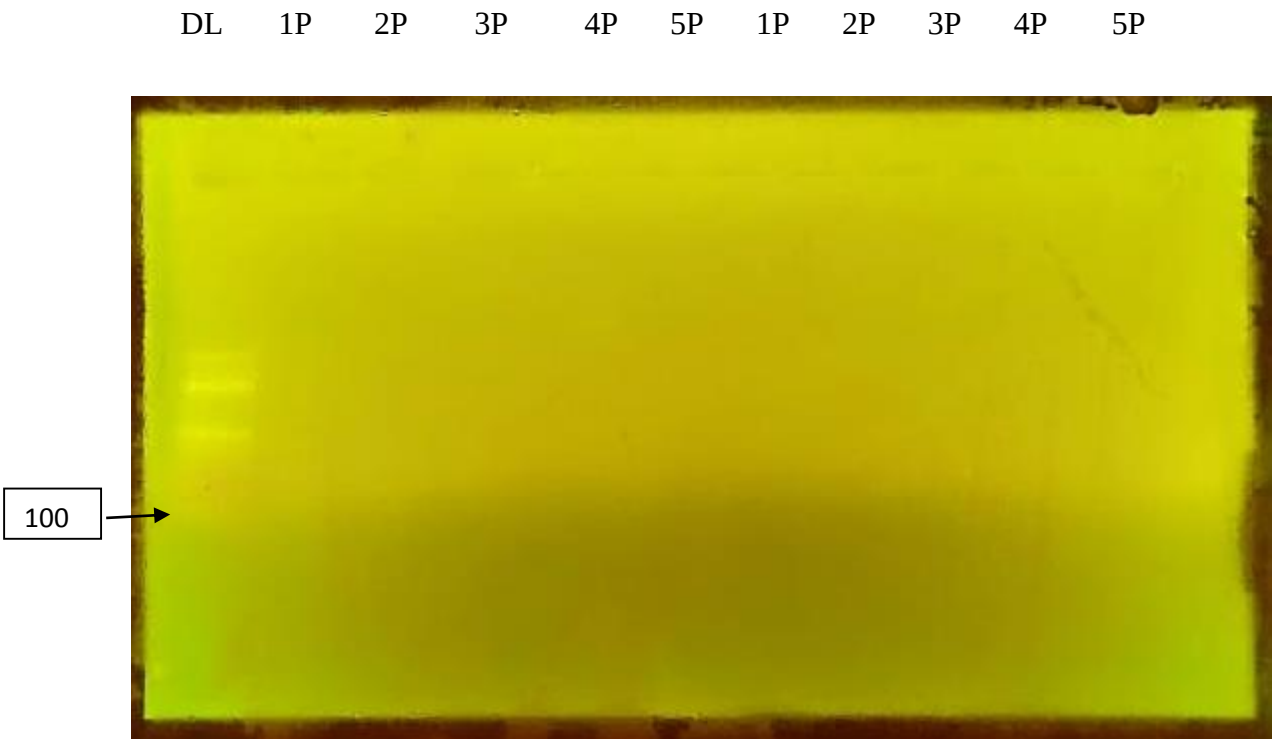


Plate 4: Agarose gel after multiplex PCR assay using all five primers**.** DL- 100bp DNA ladder, samples contained (1P- GAPDH, 2P-WSh1, 3P- WSh2, 4P- AG8_1, 5P- MS_00009462)

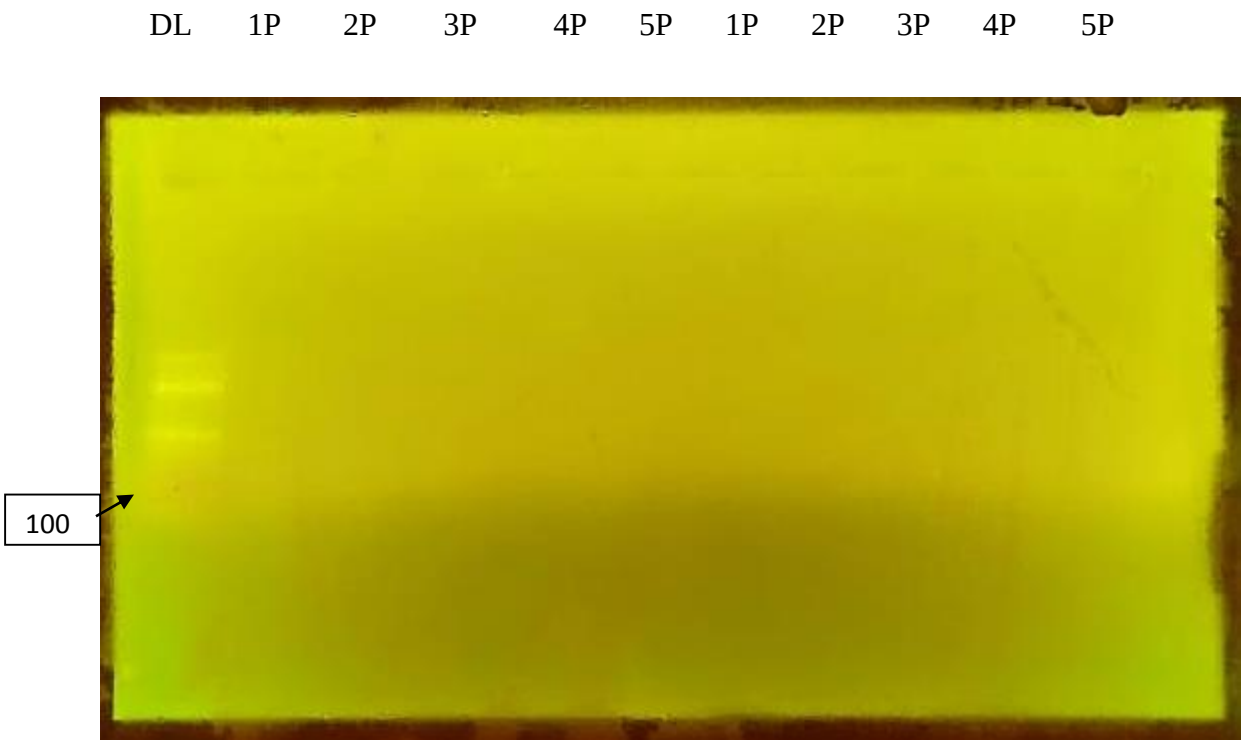


Plate 5: Agarose gel after single-plex assay using all five primers**.** DL- 100bp DNA ladder, samples contained (1P- GAPDH, 2P-WSh1, 3P- WSh2, 4P- AG8_1, 5P-MS3_00009462)

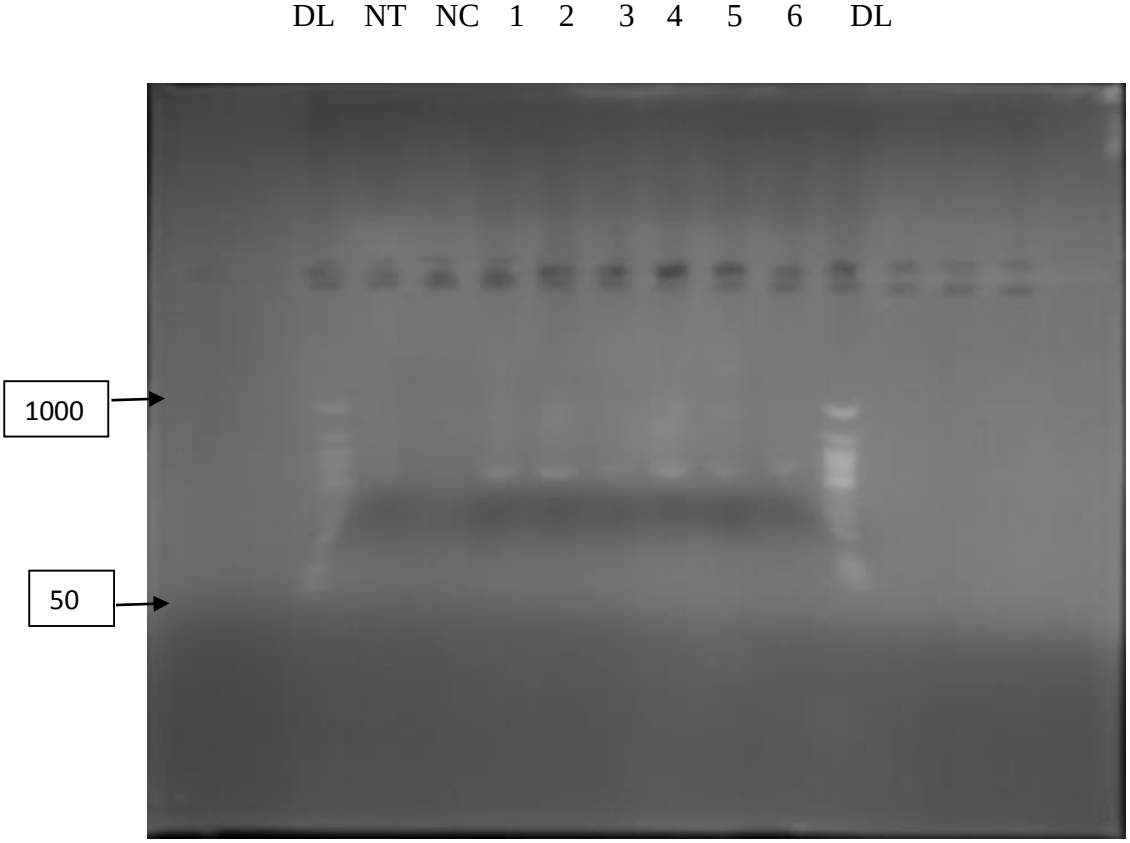


Plate 6: PCR products after amplification with DRA1 primer**.** DL- 50bp DNA ladder, NT-No template control, NC-negative control, 1-6 (DNA samples)

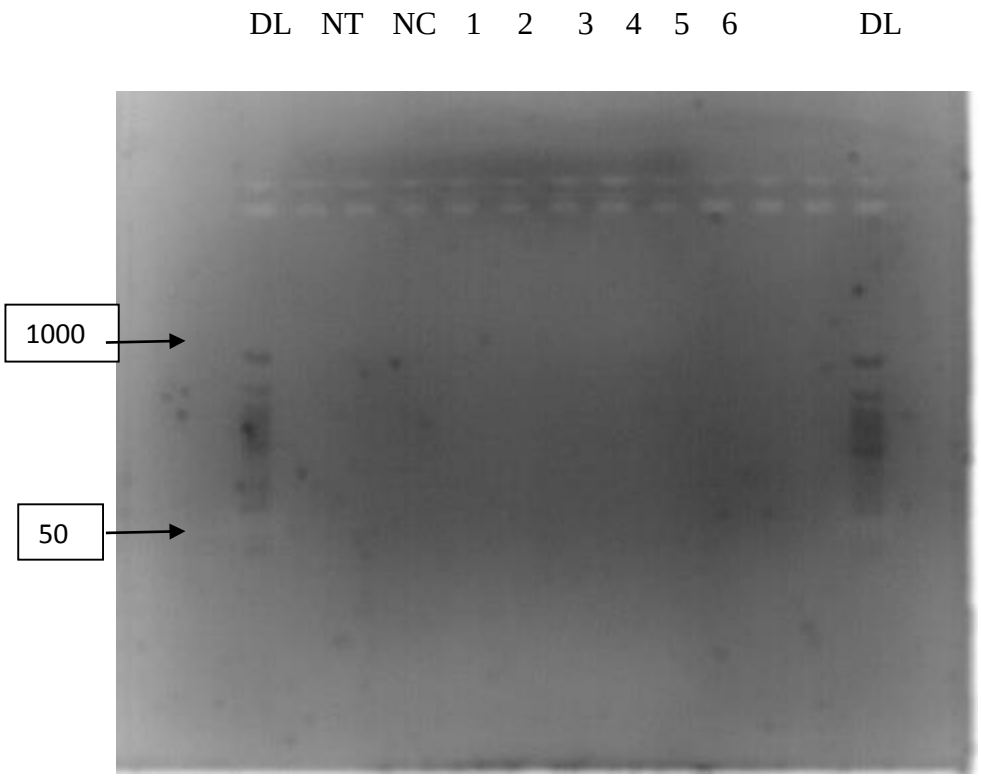


Plate 7: Agarose gel stained with safe view showing absence of amplification of male specific primer after nested PCR. DL- DNA ladder, NT-No template control, NC-negative control, 1-6 (DNA samples)

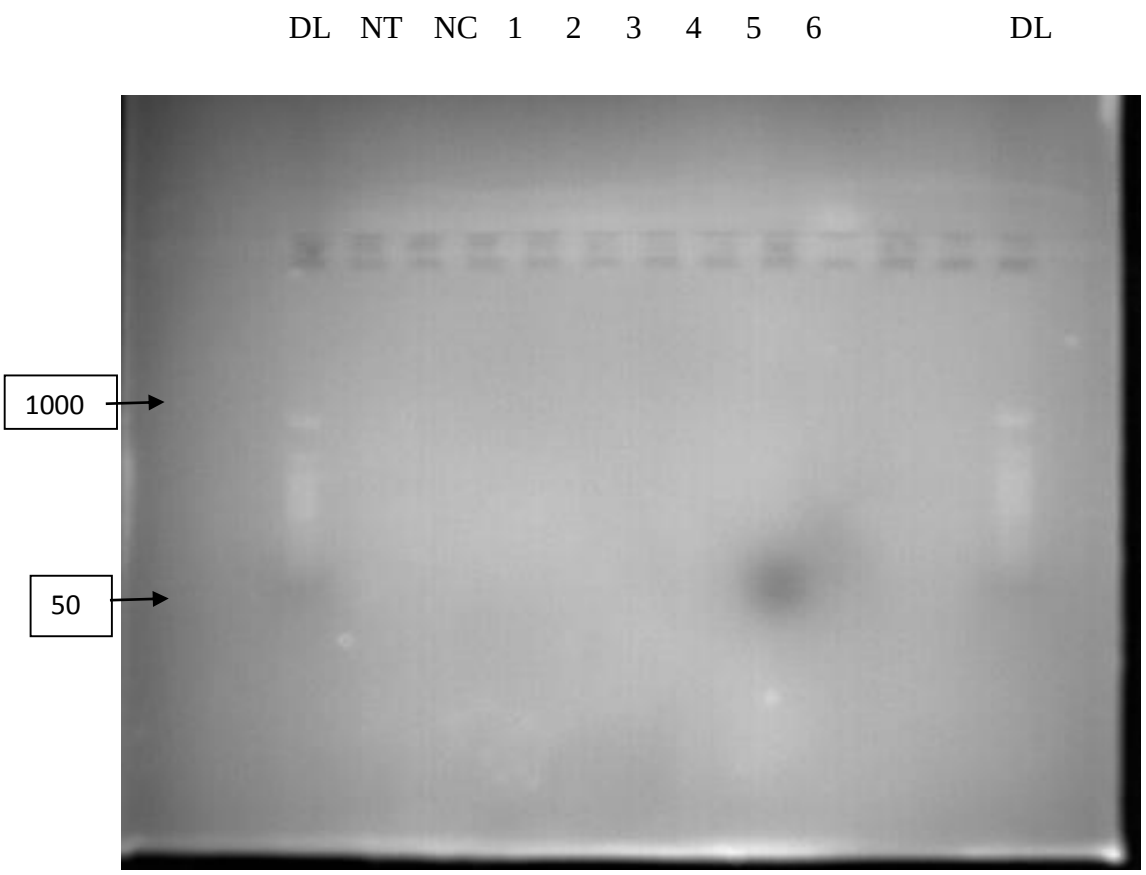


Plate 8: Agarose gel stained with safe view showing absence of amplification of female specific primer after nested PCR. DL- DNA ladder, NT-No template control, NC-negative control, 1-6 (DNA samples)

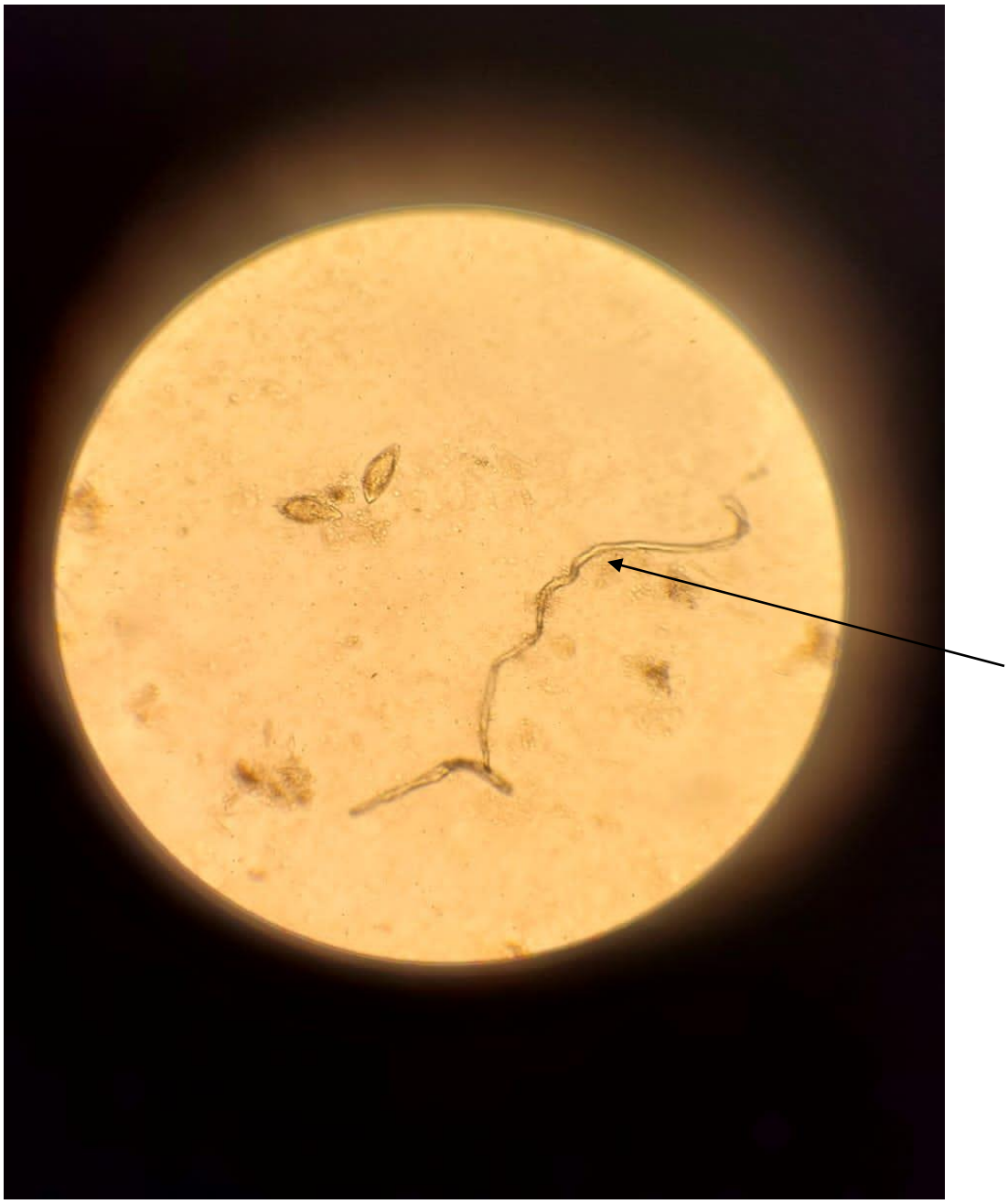


Plate 9: Microsopy image showing *S. haematobium* eggs and worm fragments suspected to be *S. haematobium.* Arrow points to worm fragments suspected to be *S.haematobium* found alongside eggs in urine samples.

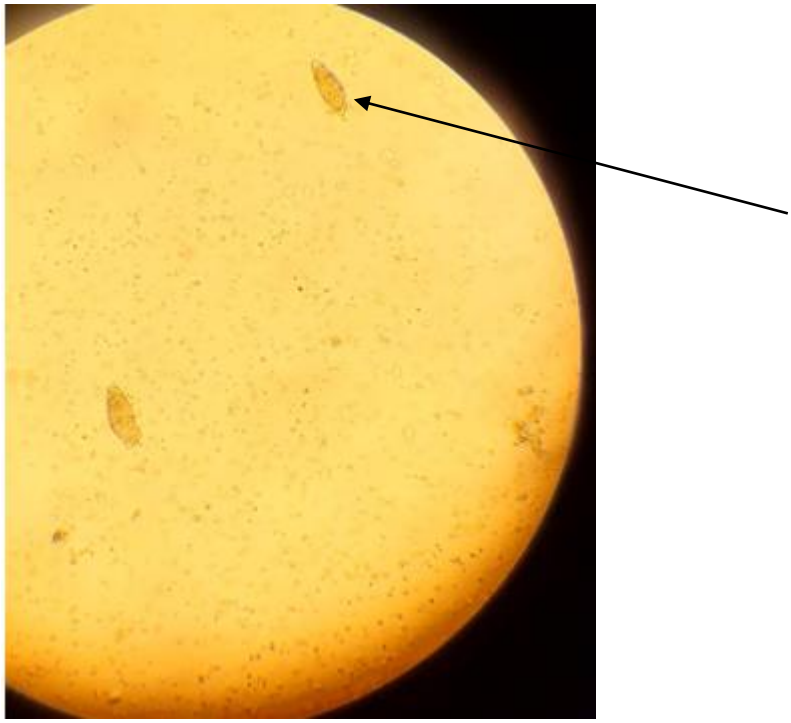


Plate 10: Microscopy image showing *S.haematobium* eggs found in a urine sample. Arrow points to *S.haematobium* eggs having terminal spine.

CHAPTER FIVE

DISCUSSION

Schistosomiasis continues to pose a substantial global health challenge, particularly in rural areas with restricted access to clean water and proper sanitation (Odeniran *et al.,* 2020). This study aimed to assess the occurrence of single-sex schistosomiasis infections and to evaluate the prevalence of urogenital schistosomiasis in four communities within the Atiba Local Government Area of Oyo State. The study areas are situated alongside rivers, which serve as the primary water source for the residents who utilize it for various purposes such as drinking and bathing. Microscopy results revealed an overall schistosomiasis infection prevalence of 12.7%, with a mean intensity of 35 eggs/10 ml of urine across the four study sites. This prevalence surpassed the previously reported rate among female residents in Oyo State (5.5%) (Awobode *et al.,* 2016), yet remained lower than rates reported among adults in Eggua, Ogun State (14.4%) (Anumudu *et al.,* 2019), and Oyan Dam, Ogun State (44.1%) (Otuneme *et al.,* 2019). This variation may be attributed to the routine administration of praziquantel by the Oyo State Ministry of Health to the inhabitants of these communities.

An intriguing observation noted across the three study areas (Asamu, Alagbede, and Ile tuntun) was the presence of worm fragments suspected to be adult *S. haematobium* worms in the urine samples. According to the gold standard of schistosomiasis diagnosis, urine samples from infected individuals typically yield only eggs (Ajibola *et al.,* 2018), suggesting a potentially high worm burden in the region. Additionally, it was noteworthy that while some individuals exhibited a high worm burden, others tested negative for eggs despite sharing the same water source. This phenomenon may be attributed to unisexual infection (Colley *et al.,* 2014).

The prevalence of schistosomiasis infection was higher among males, with 14 cases (7.7%) compared to females, with 8 cases (5.0%). This elevated prevalence among males can be attributed to their involvement in activities such as fishing, swimming, and irrigation, which increase their

exposure to water bodies. Similar findings of higher schistosomiasis prevalence among males have been reported by Umar *et al.,* (2016), Akinneye *et al.,* (2018), and Kone *et al.,* (2022). While *Schistosoma haematobium* infection is not gender-specific but results from exposure to contaminated water, it's worth noting that in regions where women are primarily engaged in farming or fishing, a higher prevalence of schistosomiasis among them has been documented (Umar *et al.,* 2016; Bakuza *et al.,* 2017).

Fishing, predominantly practiced in these communities, exposes individuals to frequent contact with water bodies, elevating the risk of schistosomiasis among fishermen. The prevalence of schistosomiasis was notably highest among individuals engaged in fishing (Figure 3), consistent with previous findings by Jafaru *et al.,* (2023) and Odeniran *et al.,* (2020) linking fishing occupation to increased schistosomiasis prevalence. Moreover, the spatial distribution of *S. haematobium* infection is heavily influenced by patterns of water contact, with heightened risk associated with frequent and intense interaction with water bodies (Ndassi *et al.,* 2019). Among the study areas, Alagbede exhibited the lowest infection frequency due to its distance from the river source, while Asamu, closest to the dam, displayed the highest infection rate and recorded the highest frequency of urine samples containing worm fragments. Moreover, the frequency of light infections, totaling 18 cases (78.3%), exceeded that of heavy infections, which accounted for 5 cases (21.7%), a trend consistent with findings from a prior study by Onile *et al.,* (2016). This disparity may be attributed to the potential presence of acquired immunity among adults in the community, stemming from prolonged exposure to schistosomiasis (Onile *et al.,* 2016).

Regarding age groups, the highest infection rates were observed in the 18-30 age group. This mirrors results obtained by Anumudu *et al.,* (2019) and Adeboye *et al.,* (2021), where the highest prevalence of infection was identified in the 20-29 and 18-30 age groups, respectively. This age bracket exhibited a high prevalence due to its significant representation in the community's agricultural workforce, actively engaged in agricultural practices (Anumudu *et al.,* 2019).

Unisexual infections often go undetected, particularly during diagnosis, and many egg-negative samples that test positive for nucleic acid have been linked to breakdown products of *Schistosoma* (Lu *et al.,* 2018). While the concept of unisexual infections has been validated in artificial mouse populations, much about their occurrence in natural populations remains unexplained. Diagnosing

single-sex infections poses several apparent challenges, notably the inaccessibility of worms in humans compared to murine models, makes it difficult to clearly discern patterns in human infections. In murine models of unisexual infection, researchers can exclusively study the immunogenic effects of male or female worms on the pathogenicity of S. haematobium (Reinholdt *et al.,* 2023). However, detecting such patterns in humans is a daunting task as the absence of eggs in urine may result from various factors besides unisexual infection. De Noya *et al.,* (2007) conducted a study in Venezuela to measure unisexual infections in *Schistosoma mansoni*-infected populations using Enzyme linked Immunosorbent Assay (ELISA) which detects worm antibodies. However the results from the study could not determine the sex of the worm that induced the antibody response detected in positive samples identified in their study. Such responses could also arise from bisexual infections.

Due to the unavailability of a diagnostic technique to test the validity of unisexual infections, we designed a PCR method based on the WSh 1 and 2 primers previously reported by Kinacaid *et al.,* (2016). Wsh1 and Wsh2 were used to detect the sex of individual *S. haematobium S. bovis,* and their hybrids parasitic stages using DNA isolated from cercariae and adult worms. Both primers were designed based on the W specific regions that are absent in male worms. One advantage of Wsh1 and 2 primers is specificity. Both primers only amplify of *S.haematobium* worms or cercariae and don't amplify other *Schistosoma* species.

In addition to the WSh1 and WSh2 primers, novel male-specific primers were developed based on two male genes, AG8_1 and MS3_00009462, which were reported to be upregulated in male *S. haematobium* by Stroehlein *et al.,* (2022). These male primers were designed to accurately detect exclusive S. haematobium unisexual infections in the population. Unlike the identification of male and female cercariae in mice, where cercariae are initially molecularly identified from mollusks and then introduced into mice, with the absence of oviposition indicating unisexual infection, the patterns in humans may differ. Unisexual infection in humans could occur due to the death of either male or female worms after praziquantel treatment and/or infection with single-sex cercariae (Zhong and Jin, 2023). There is a possibility of single-sex unpaired male and female worms in the same human, and amplification using only female-specific markers may underestimate the proportion of unisexual infection in such cases. Lastly, the GAPDH gene which is a notable housekeeping gene that has a critical function in the catalysis of the sixth step of

glycolysis (Lin and Redies, 2012) served as an internal control for the PCR procedure. The anticipated result was that the GADPH gene, acting as an endogenous marker would be present in all samples, whether they contained single-sex or bisexual infections. Also, the presence of bands from amplicons of WSh1 and WSh2 primers would denote a female single-sex infection, while bands from AG8_1 and MS3_00009462 would indicate a male single-sex infection. Furthermore, simultaneous bands from either WSh1 and WSh2 primers or AG8_1 and MS3_00009462 would suggest a bisexual infection. However, the primers showed no bands including GAPDH- the endogenous marker. This could have arisen from a number of reasons from the standpoint of the PCR procedure such as primer design and annealing temperature. The primers were validated again on NCBI using *in silico* PCR to be assured that the primers were properly designed. The annealing temperature for each primer was also calculated using the formula 4(C+G) +2(A+T) and the annealing temperature for each primer was incorporated into the annealing phase of the PCR using a touch-down PCR technique, there was still no amplification.

It was logical to hypothesize that single sex infections would imply that the circulating cell-free *S. haematobium* DNA will be present in dilute proportions because there the population of the worms will be reduced. It was logical to conclude that if there was a possibility to increase the volume of circulating cell free *S. haematobium* DNA, then there is a possibility of identifying unisexual infections using the molecular makers recruited for this study. In order to increase the volume of circulating cell free *S. haematobium* DNA, the DNA samples were first amplified with a supplementary primer (DRAI) to help increase the amplification of *S. haematobium* region in the human DNA. The amplicons obtained from using DRAI were used to test male specific and female specific primers but there were still no bands obtained on the agarose gel. The results from this attempt to identify single sex *S. haematobium* from DNA isolated from human and urine samples reveals that the markers currently available for detecting sex of *S. haematobium* are not ideal for detection of unisexual infections from human urine or plasma and more study needs to be carried out to identify the patterns of unisexual infections in humans which will ultimately aid the detection of unisexual infections.

Conclusion

Despite efforts to explore unisexual infections using molecular markers, we encountered challenges in detecting such infections from human samples. The lack of amplification observed even with supplementary primers suggests that current methodologies may not be suitable for identifying unisexual infections in humans. Hence, there is an urgent necessity for ongoing research endeavors aimed at crafting enhanced diagnostic tools and elucidate the patterns of unisexual infections within human populations.

In this study, we have highlighted the continued prevalence of schistosomiasis in rural communities facing challenges in accessing clean water and sanitation facilities, emphasizing the urgent need for effective interventions in these areas. The prevalence of schistosomiasis was notably higher among males, particularly those engaged in fishing occupations, and individuals in the age group of 18-30, likely due to recurrent exposure to contaminated water sources during agricultural activities. The presence of suspected adult *S. haematobium* worms in urine samples, particularly in areas heavily reliant on river water, underscores the pressing need for improved water sanitation and hygiene practices. Additionally, the variability in infection rates among individuals using the same water source suggests potential unisexual infections which warrant further investigation.

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APPENDIX 1

QUESTIONNAIRE

ID..... PARTICIPANT.....

……

VILLAGE: DATE: INTERVIEWED BY:

A: Demographic Characteristics of study participant

1. Sex (Tick) [ ] Male [ ] Female
2. Age : 18-30 [ ], 31-50 [ ], above 50[ ]
3. Marital Status (Tick) [ ] Single [ ] Married [ ] Separated [ ] Divorced [ ] Widow [ ] Widower, others (specify)_____

4. Religion: Christianity [ ] Islam [ ]

5. Educational background: [ ] Never attended school [ ] did not complete primary school [ ] Completed primary school [ ] did not complete secondary school [ ] Complete secondary school, others specify_____

6. Occupation: Farming [ ], Fishing [ ], Trading [ ], Rice Farming [ ], Civil servant [ ] others specify_____

7. Type of Fuel used in cooking: Fire Wood [ ], Charcoal [ ], Kerosine [ ], Gas [ ]

8. Ethnicity _____

B: Water supply and consumption habits

1. Source of water used: River [ ], Well [ ], Pond [ ], Bore hole [ ], Pipe borne Water[ ] others specify _____
2. What is the water used for? Drinking [ ], laundry [ ], Bathing [ ], Religious purpose [ ] others specify _____

3. How often do you visit the water source? Once daily [ ], More than Once Daily [ ], Weekly [ ] Monthly [ ].

4. Do you store drinking water? Water pot [ ], plastic buckets[ ], Jerry cans [ ] others specify _____

5. Do you ever process your drinking water? Filtering [ ], Boiling [ ], Freezing [ ], None [ ] others specify _____

6. Do you have a toilet facility in your house? [ ] Yes [ ] No

If 'NO' where do you defecate? [ ] Open Field [ ] Rivers [ ] Others _____

If 'YES' which kind do you have? [ ] Water cistern [ ] Latrine [ ] Others

7. Distance between toilet and water source? [ ] Far [ ] Not too far [ ] Directly opposite [ ] Beside

8. Use of water and soap after using the toilet? [ ] Regularly [ ] Occasionally [ ] Never

9. Do you walk around with barefoot? [ ] Regularly [ ] Occasionally [ ] Never

10. Do you wash your fruits and vegetables before eating? [ ] Always [ ] Not always [ ] Never

11. Do you keep any of these animals? [ ] Dogs [ ] Goat [ ] Sheep [ ] cattle [ ] others_____

12. Where do the animals stay? [ ] inside the house [ ] cages [ ] free range

13. Are the pets/ livestock treated for any worm infection? [ ] Yes [ ] No

14. If yes, how often ? [ ] Every 6 month [ ] Yearly [ ] Never

C. **LIFE STYLE FACTORS**

1. Do you smoke cigarettes? EVER [ ], NEVER[ ],

2. If yes, for how long have you been smoking.....

3. How much do you/ did you smoke? 2packs a day [ ], 5 packs a day [ ], 20 packs a day [

4. If No, does anyone smoke around you? Yes[ ], No [ ]

5. How long ago did you stop?.....

D. **MEDICAL HISTORY**

1. Any previous complaints on lower pelvic region? Yes [ ], No [ ]

2. Do you frequently experience pain during urination? Yes [ ], No [ ]

3. Have you at any time, seen blood come out with your urine? Yes [ ], No [ ]

4. If yes, how does the blood come out in the urine? Continuously with the urine[ ], with the last few drops [ ], before the urine emerges [ ]

5. Have you ever been diagnosed of schistosomiasis (atosi Aja) before? Yes [ ], No [ ]

6. If yes, when? …….

7. Have you at any time been treated? Yes [ ], No [ ]

8. With what? Herbs[ ], injection [ ], Paraziquantel [ ], unknown drugs [ ]……(tick as many as possible)

9. Have you ever taken part in our research survey before? YES[ ], NO [ ]When?.....