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Prevalence of the related treponematoses syphilis and yaws and co-occurrence of pathogens causing similar skin lesions in Ghana

Agyekum Boaitey, Yaw

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PREVALENCE OF THE RELATED TREPONEMATOSES SYPHILIS AND YAWS AND CO-OCCURRENCE OF PATHOGENS CAUSING SIMILAR SKIN LESIONS IN GHANA

YAW AGYEKUM BOAITEY

A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

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Abstract

Yaws affects children in tropical regions while syphilis affects sexually active adults worldwide. Despite various campaigns towards eradication of yaws and elimination of syphilis, these two diseases are still present in Ghana. The agents causing these diseases, two *Treponema pallidum* subspecies, are genetically similar, but they have always been studied independently. In this context, this study aimed to assess the prevalence of these treponematoses and determine the co-occurrence of pathogens causing similar skin lesions in the Ashanti region of Ghana. A point-of-care test was used to determine their seroprevalence. Multiplex PCR and 16S rRNA gene sequencing were used to identify other microorganisms in these lesions. Additionally, 16S rRNA gene sequencing was used to compare the microbiota of yaws-like lesions with that of syphilis-like lesions. The results indicated that the seroprevalence of *T. pallidum* in individuals with yaws-like and syphilis-like lesions were 17.2% and 10.8%, respectively. Multiplex PCR results showed 9.1%, 1.8% and 0.9% of yaws-like lesions were positive for *H. ducreyi*, HSV-1 and *T. pallidum* respectively, and 28.26% of syphilis-like lesions were positive for HSV-2. 16S rRNA gene sequencing showed that 6.3% of yaws-like lesions were *Heamophilus*-positive, two yaws-like and one syphilis-like lesions were positive for *Treponema* and 3.6% yaws-like lesions were positive for *Mycobacterium spp*. Other microorganisms, including *Pseudomonas spp*, *Klebsiella spp*, *Escherichia spp, Staphylococcus spp* and *Proteous spp*, were identified as part of the microbiota of both yaws-like and syphilis-like lesions. The alpha diversity measures indicated that syphilis-like lesions were more diverse than yaws-like lesions, but the beta diversity measures suggested no significant dissimilarity between the microbiota of these lesions. Both yaws and syphilis are prevalent in the same geographical location, Ashanti region of Ghana. The presence of other medically important organisms as part of the microbiota of these lesions may prevent their complete resolution and impede the associated elimination and eradication campaigns.

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Author's declaration

I declare that the material contained in this thesis is my own work.

List of acronyms and abbreviation

CDC	Centre for Disease Control and Prevention
DNA	Deoxyribonucleic acid
DPP	Dual Path Platform
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ETs	Endemic treponematoses
FSW	Female sex workers
FTA-Abs	Fluorescent Treponemal Antibody Absorbed test
GHS	Ghana Health Service
HICs	High-Income Countries
HIV	Human immunodeficiency virus
ICT	Immunochromatographic Test.
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
IV	Intravenous
KATH	Komfo Anokye Teaching Hospital
KNUST	Kwame Nkrumah University of Science and Technology
LMICs	Low Middle- Income Countries
MHA-TP	Microhemagglutination assay for *Treponema*
MSM	Men who have sex with men

MU	Million units
PCR	Polymerase chain reaction
PO	By Mouth
QDS	Four Times Daily
RDT	Rapid diagnostic test
RIT	Rabbit infectivity test
RPR	Rapid plasma reagin
rRNA	Ribosomal ribonucleic Acid
SSH	South Suntreso Hospital
TPHA	*Treponema pallidum* hemagglutination assay
TPPA	*Treponema pallidum* particle agglutination assay
TRUST	Toluidine red unheated serum test
UK	United Kingdom
US	United States of America
VDRL	Venereal disease research laboratory

CHAPTER 1

1. Introduction

1.1 Treponemes causing syphilis and yaws and other treponematoses affecting humans

Human treponemal diseases include venereal syphilis, yaws, endemic syphilis (bejel) and pinta. The aetiological agents of these diseases are *Treponema pallidum* subsp. *pallidum* (TPA), *Treponema pallidum* subsp. *pertenue* (TPE), *Treponema pallidum* subsp. *endemicum* (TEN) and *Treponema carateum*, respectively (Giacani and Lukehart, 2014). Yaws, bejel, and pinta are known as endemic treponematoses (ETs) and are all closely related to syphilis. Deoxyribose nucleic acid (DNA) hybridization shows a high genetic similarity (99.57% - 99.98%) within the treponemes causing yaws, syphilis and bejel, which has resulted in the reclassification of these treponemes as subspecies of *Treponema pallidum* (Paster, 2010; Mikalová *et al.*, 2011). Treponemes, are bacteria that belong to the order spirochaetales, family spirochaetaceae, and genus *Treponema*. Spirochaetes are long, slender, spiral shape (Figure 1.1) motile bacteria (Giacani and Lukehart, 2014). *T. pallidum* measures 0.10 to 0.18 µm in diameter and 6-20 µm in length, making it invisible under a conventional light microscope. The genome of *T. pallidum* is a circular chromosome of about 1.14 Mb and contains about 1041 open reading frames (ORFs) (Fraser *et al.*, 1998). The organism depends on its host for multiple nutrients because of its limited biosynthetic capabilities. All species of *Treponema* are difficult to grow in traditional laboratory conditions because they have complex and restricted nutritional requirements and are microaerophilic (Fraser *et al.*, 1998).

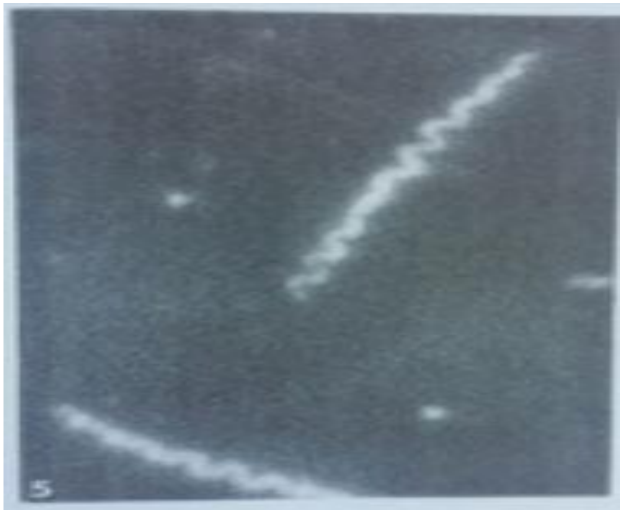


Figure 1.1 Darkfield microscope image of *T. pallidum* spiral shape (Di Virgilio *et al* 1970)

1.2 Geographical distribution of the human treponematoses

1.2.1 Yaws

Yaws is one of the neglected tropical diseases and the most common among the ETs (Giacani and Lukehart, 2014). Yaws is transmitted through skin-to-skin contact with fluid from infected people's lesions to an individual's broken skin (Perine *et al.*, 1984). Children between the ages of 2-15 years are mostly affected (peak reported incidence occurs in children aged 6-10 years); hence, they are considered as the reservoir for the infections (Perine *et al.*, 1984). Yaws occurs mainly in deprived rural populations in warm, humid, or tropical forest areas of Africa, Asia, Latin America and the Pacific, usually in countries with an average annual temperature of 27 °C, a mean annual rainfall of 1300 mm and an altitude of not more than 1800 metres (Giacani *et al.*, 2010). Additionally, yaws has a clear seasonality, and the reported number of cases rises during the rainy season compared to the dry season (Kazadi *et al.*, 2014). Transmission is enhanced by humid and warm atmospheric conditions (Kazadi *et al.*, 2014). The yaws treponemes do not survive in colder climates and places with extreme temperatures. This, alongside with the observation that yaws is commonly found in populations with a low

standard of living and poor hygiene (Mushayabasa *et al.*, 2012), seem to explain why the disease is absent in Europe and higher latitude countries.

Mass treatment campaigns and subsequent follow-up activities in the 1950's and 1960's significantly reduced the estimated cases of yaws (50 - 150 million) in 90 endemic countries (Perine *et al.*, 1984). After 1970, many endemic countries ceased yaws control and monitoring activities, resulting in the disease's re-emergence, particularly in Africa and Southeast Asia (Perine *et al.*, 1984; WHO, 2012). Over 80,000 of suspected yaws cases are reported worldwide each year (WHO, 2021a, 2021b). From 2008-2015, about 462,000 cases of yaws were reported from 12 countries to the WHO with cases concentrated mostly in Ghana, Papua New Guinea, and the Solomon Islands (WHO, 2021b). In 2021, 123,866 cases of yaws were reported worldwide with more than 80% of cases concentrated in West Pacific region (WHO, 2021b).

1.2.2 Syphilis

Syphilis occurs all over the world and is mostly spread through unsafe sexual activities by adolescents and adults. It can also be acquired congenitally (Giacani and Lukehart, 2014). Syphilis caused significant morbidity over 500 years ago until the introduction of antibiotics after World War II. Notwithstanding effective treatment, syphilis transmission levels fluctuated throughout the 20th century until the 1980s and 1910s, when the rise of HIV infections led to changes in sexual behaviour and an overall decline in syphilis (Chesson, Dee and Aral, 2003; Fenton *et al.*, 2008). Syphilis cases have been on the rise worldwide, including in the United States and Europe (Arora *et al.*, 2016; Beale *et al.*, 2019), largely due to changes in social and behavioural preferences and probably antibiotic (azithromycin) resistance, especially among identifiable groups, such as men who have sex with men (MSM) (Chen, Callahan and Kerndt, 2002; Choi *et al.*, 2007; Heffelfinger *et al.*, 2007) and female sex workers (FSW) (Cantor *et al.*, 2016).

In 2020, about 7.1 million new cases of syphilis were reported worldwide by the World Health Organization (WHO) (WHO, 2021c) with most cases concentrated in Low-and- Middle- Income countries (LMICs), where antenatal syphilis testing is minimal (WHO, 2020a, 2020b, 2022a). In 2019, more than 95% of pregnant women who attend antenatal care were tested positive in 23

of the 78 countries that reported to the WHO. Additionally, out of these 78 countries, 38 of them reported 1% or more pregnant women testing positive for syphilis in 2019 (WHO, 2020b). Cases of syphilis among pregnant women in Sub-Saharan Africa are high, causing a significant number of stillbirths and neonatal syphilis (WHO, 2020b). Syphilis treponemes can cross the placenta and infect the foetus *in utero*, which can lead to unplanned abortion, stillbirth, or congenital infection of the baby (Giacani and Lukehart, 2014). Most babies with congenital syphilis are infected in the womb, but new-borns can also be infected through contact with active lesions at birth (Herremans, Kortbeek and Notermans, 2010). Transmission through blood transfusion has occurred (Stoltey and Cohen, 2015), but is unlikely to happen now due to better donor selection and a change to transfusing with refrigerated blood instead of fresh blood (Perkins and Busch, 2010). An estimated number of 300,000 stillbirths and neonatal deaths due to syphilis infections are reported in Africa annually (Newman *et al.*, 2013).

1.2.3 Bejel

Bejel, also known as endemic syphilis, primarily affects children under the age of 15 years. It is spread by direct skin-skin or oral contact by indirect contact through contaminated drinking vessels (Csonka and Pace, 1985). Bejel is found in dry areas such as the southern part of the Sahara Desert. Although bejel is found in a drier climate, the causative organism (*Treponema pallidum* subsp. *endemicum*) is mostly found in moist parts of the body, such as the mouth (Arslanagić, Bokonjić and Macanović, 1989). Countries that have reported cases of this disease include Mali, Niger, Burkina Faso, and Senegal (Burke, 1985; Julvez, Michault and Kerdelhue, 1998). It has also been reported in the Arabian Peninsula, more specifically in Iraq, Syria, and Saudi Arabia (Csonka and Pace, 1985). In 1995, three cases of bejel were reported from southwest Turkey, even though the disease was considered eradicated (Yakinci *et al.*, 1995). Additionally, in 2012, Iran reported a case of bejel from the southwest part of the country (Abdolrasouli *et al.*, 2013). Noda *et al.*, (2018) also reported bejel cases in Cuba from samples of six individuals who had been previously diagnosed with syphilis. Although ETs are not known to be found in Europe or the United States, cases have sporadically been reported from these regions (Pillay *et al.*, 2011; Fanella *et al.*, 2012). Local spread of bejel in refugee camps has also

been reported in Canada (Fanella *et al.*, 2012). Lack of awareness and knowledge of these diseases among health care providers in developed countries may hinder the diagnosis of imported cases. This may confuse the diagnosis of syphilis if, for example, patients move from endemic countries (e.g., Ghana) to non-endemic countries (e.g., the United-Kingdom). It is therefore important for developed countries to include these diseases into their routine diagnoses (Mitjà, Šmajs and Bassat, 2013).

1.2.4 Pinta

Pinta is seen in early to late adulthood and it is transmitted through skin-to-skin contact. It affects mostly people in forested tropical regions where the weather is warm and moist (Giacani and Lukehart, 2014).. The high atmospheric humidity found in these regions allows the causative organism to thrive. Furthermore, the environmental conditions promote lesions and support their discharge, resulting in transmission of the diseases (Giacani and Lukehart, 2014). While recent data on pinta is scarce, some cases have been reported in the 1980s in rural communities of Mexico (Meheus and Antal, 1992; Giuliani *et al.*, 2005), in Indian tribes in the Amazon area of Colombia, Brazil, and Peru (WHO, 1987; Pecher and Croce, 1988) and in Panama (Fohn *et al.*, 1988).

1.2.5 The burden of yaws and syphilis in Ghana

According to the WHO records, 15 countries including Ghana, are currently known to be endemic for yaws. Ghana recorded more than 20,000 cases of yaws annually from 2007 to 2010, making it the African country with the highest number of cases for that period (WHO, 2021b). Although the number of yaws cases dropped significantly in Ghana over the past years, the current WHO data indicates Ghana still has the highest number of cases (3367) in Africa (WHO, 2022b).

The last national yaws survey in Ghana recorded a prevalence of 0.68% (WHO, 2009). However, high prevalence of more than 10% are reported in some communities in Ghana. Two separate studies from Ghana reported a 10.9% prevalence of yaws in sub-districts of the Eastern region (Abdulai *et al.*, 2018) and a 19.5% prevalence from a school in the Eastern region (Agana-Nsiire

et al., 2014). A survey conducted in the Eastern region of Ghana by Marks *et al.*, (2017) indicated that about 13% of people believe that yaws is caused by supernatural forces and would not seek medical care if they get the disease.

Yaws is unfortunately not the only treponematose observed in Ghana, as syphilis also occurs here. Generally, about 90% of people infected with syphilis are not aware of their infection because of a lack of symptoms, and this may be a major reason explaining the global spread of the disease (Steinbrook, 2007)(WHO, 2022c). More specifically, in Ghana, Ampofo *et al.*, (2002) reported a prevalence of 13.5% of syphilis treponeme (TPA) infections among blood donors. There, in Kumasi teaching hospital, Owusu-Ofori, Parry and Bates (2011) reported a 3.5% prevalence of active syphilis infection among blood donors. Additionally, Osei-Yeboah *et al.*, (2018) also reported 2.58% prevalence of syphilis in a population-based survey in the Volta region of Ghana. The national prevalence of syphilis among pregnant women in 2020 in Ghana was also high (2.8%) (WHO, 2020b). It appears that venereal syphilis and yaws impose a heavy burden in Ghana (Ampofo *et al.*, 2002; Owusu-Ofori, Parry and Bates, 2011; Abdulai *et al.*, 2018; Osei-Yeboah *et al.*, 2018; WHO, 2020b).

1.3 Genomic differences between treponemes causing yaws (TPE) and syphilis (TPA)

Culturing of treponemes *in vivo* was not possible until recently (Edmondson, Hu and Norris, 2018) because the viability of the treponemes outside the host is very limited and they are also delicate to withstand any manipulation that can be used to preserve them (Edmondson, Hu and Norris, 2018). This explains our current limited understanding of the biology of treponemes and the diseases they cause. The development of molecular biology techniques has however began to yield essential genetic and genomic information on treponemes, especially on those causing syphilis and yaws, *Treponema pallidum pallidum* (TPA) and *Treponema pallidum pertenue* (TPE), respectively. This is probably because these bacteria are the most sampled since yaws and syphilis represent most of the reported or known human treponematose cases (WHO, 2020b, 2021c).

Interestingly, because of their distinctive clinical manifestations, the bacteria causing syphilis and yaws were initially thought to belong to two different species (Miao and Fieldsteel, 1980). DNA hybridization has however evidenced how genetically closely related TPA and TPE were and they were subsequently reclassified into two subspecies of the same species (Smibert, 1984). Despite this high genetic relatedness, the observation that one bacterium seems to be sexually transmitted (TPA) and not the other one (TPE) is striking and led to two major hypotheses proposed to explain this difference. Hudson (1965) hypothesised that these organisms were identical and that environmental conditions where the disease was contracted resulted in different clinical manifestations. However, Hackett (1963) hypothesised that these organisms are genetically distinct, though very closely related. The differences in their clinical manifestations have since been supported by genetic differences rather than environmental factors (Hackett, 1963; Miao and Fieldsteel, 1980; Centurion-Lara *et al.*, 1996, 1998, 2000, 2006, 2013; Šmajs, Norris and Weinstock, 2012; Staudová *et al.*, 2014).

The genetic difference between TPA and TPE was first reported by Noordhoek *et al.*, (1989), who observed that the structural gene encoding for two similar antigenic protein (TpF-1 in TPA and TyF-1 in TPE) differed in their predicted amino-acid sequence by a single amino-acid residue at position 40 (arginine in TyF1 [TPE] and glutamine in TpF1 [TPA]). Differences in the 16S rRNA of a TPE strain and a TPA strain were also reported by Walker *et al.*, (1995) but no location was mentioned, and different strains were not investigated. Subsequent research (Centurion-Lara *et al.*, 1998, 2006; Mikalová *et al.*, 2011; Čejková *et al.*, 2012; Šmajs, Norris and Weinstock, 2012; Strouhal *et al.*, 2018) revealed several differences in the genomes of these two organisms, consistent with Hackett's hypothesis (Hackett, 1963).

It is now evident that the TPA genome differs by less than 1200 but more than 100 nucleotide positions when compared to the genome of TPE (Mikalová *et al.*, 2011; Čejková *et al.*, 2012). The TPA genome is about one- fifth of that of *Escherichia coli* (Fraser *et al.*, 1998). The genome structure of TPE ranges from 1,139,330 to 1,139,744 base pairs (bp) based on the whole genome sequence of three strains of TPE (Čejková *et al.*, 2012) and that of TPA ranges from 1,138,006 to 1,139,631 bp based on a whole- genome sequence of three strains of TPA (Fraser

et al., 1998; Matějková *et al.*, 2008; Giacani *et al.*, 2010). Indeed, distinct clustering of TPA and TPE strains can be observed on phylogenetic tree construction using five chromosomal loci (i.e TP0136, TPO367, TP0859, TP0861 and TP0865), three highly variable loci (i.e., TP0326, TP0488 and TP0 548) and one conserved locus (16S rDNA) (Figure 1.2) (Noda *et al.*, 2018). Using restriction fragment polymorphisms (RFLP), Centurion-Lara *et al.*, (1998, 2006) reported genetic differences between TPA and TPE strains in 3 genes, including two *tpr* genes (*Tpp15*, *tprC* and *tprI*). Another study investigating these *tpr* genes, revealed differences between TPE and TPA strains at six sites around these genes (Mikalová *et al.*, 2011). Further sequence variations in the sequences of the *gpd* (Cameron *et al.*, 1999) and the *arp* (Harper *et al.*, 2008) genes have been evidenced that can distinguish between TPA and TPE strains.

A pan-genomic study of *T. pallidum* by Jaiswal *et al*., (2020) reported four pathogenicity islands specific to TPA and three specific to TPE. Additionally, genes involved in lipid and amino acid biosynthesis were only found in TPA but not in TPE when genes of the pathogenicity islands were analysed (Jaiswal *et al.*, 2020). Most of these genomic differences between treponemes causing syphilis (TPA) and yaws (TPE) are predicted virulence factors for the syphilitic treponemes, which may explain the differences in the clinical manifestations of the two diseases (Šmajs, Norris and Weinstock, 2012).

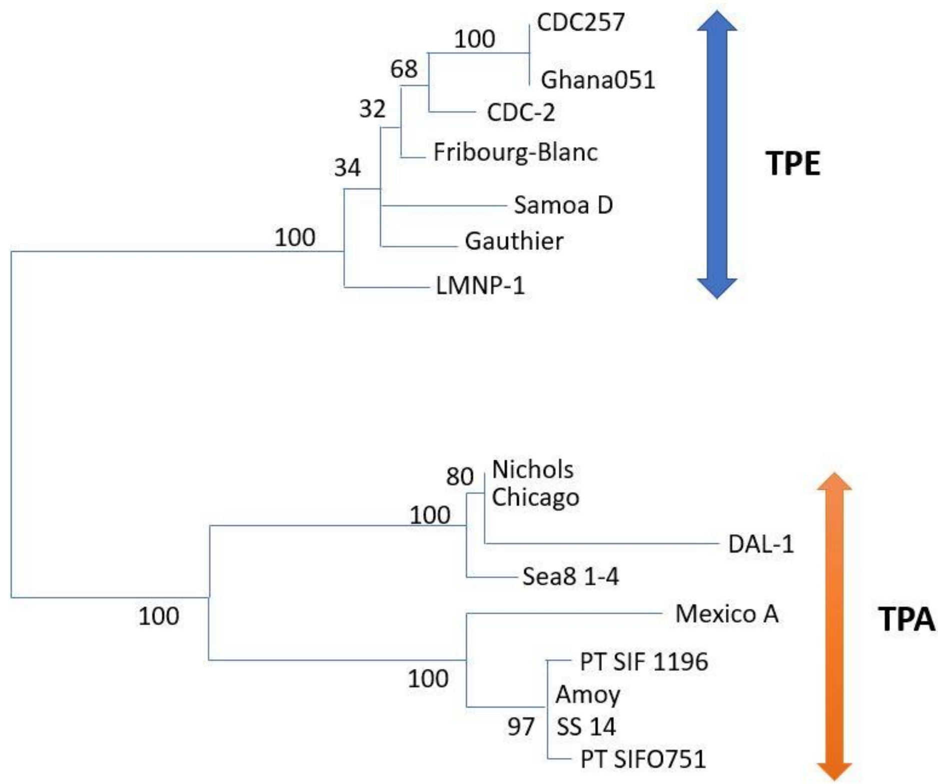


Figure 1.2 Phylogenetic tree constructed from nine gene loci of TPA and TPE strains and clinical isolates from Cuba showing distinct clustering of TPA from TPE species. The nine genes used here are TP0136, TP0326, TP0367, TP0488, TP0548, TP0859, TP0861, TP0865 and 16S rRNA (Phylogenic tree from Noda *et al*., 2018).

1.4 Clinical features of yaws and syphilis

The manifestation of treponemal infections occurs in different stages (primary, secondary, and tertiary stages) and can cause serious lesions on the skin, bone, cartilage, and affect other organs, which can lead to permanent damage resulting in death (Giacani and Lukehart, 2014). Syphilis treponemes are more invasive than yaws treponemes (Mitjà, Asiedu and Mabey, 2013)

1.4.1 Primary stage

The initial lesion of yaws (‘mother yaws’) appears as one or more non-tender papules after 9 to 90 days of exposure. The papule increases to 3-5 cm in diameter (Figure 1.3B) to become a big nodule that can ulcerate (Mitjà *et al.*, 2011). Nodules and ulcers have a granular appearance and frequently emit a yellow discharge that can dry to create a crust. About 65 to 85% of primary lesions are present on the legs, but it can be found on the buttocks, arms, hand, and face (Mitjà *et al.*, 2011). Primary yaws lesions are itchy but not painful (Koff and Rosen, 1993). There can be local swelling of lymph nodes and treponemes are highly concentrated in the early stage of the primary lesion and are extremely infectious (Sehgal, 1990). The lesion normally heals into a pitted scar with dark borders after 3-6 months even without treatment.

The primary lesion of syphilis, chancre, present as anogenital ulcer after 9-90 days of exposure (Figure 1.3A) (Singh and Romanowski, 1999) but other parts of the skin may be involved (Singh and Romanowski, 1999). The chancre may not be apparent or not recognised by the patient. The primary ulcer of syphilis (chancre) in the genital area is usually shallow, painless, basal hardening and nonpurulent (Chapel, 1978). However, extra-genital lesions present no or less basal hardening with edges projecting above the adjacent surface. Patients with lesions on the fingers, tongue, and anus experience pain. Most extra-genital ulcers are in the mouth, with one-fifth present on the lips (Chapel, 1978; DiCarlo and David H, 1997). At the primary stage, the syphilis treponemes are present in the lesion fluids and there is an enlargement of immediate lymph nodes. Lesions normally heal after 3-4 weeks of infection (Singh and Romanowski, 1999). However, healing can be delayed in individuals who are immunosuppressed, such as HIV patients (Gourevitch *et al.*, 1993).

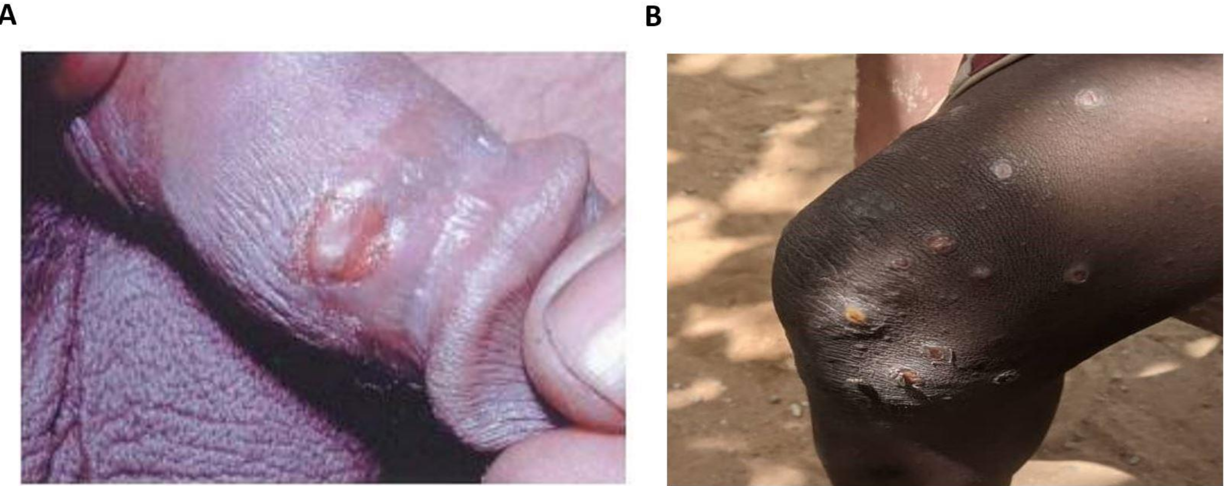


Figure 1.3 A : Genital ulcer (chancre) of primary syphilis (Goh, 2005). B: Primary lesions of yaws from this study.

1.4.2 Secondary stage

The secondary lesions of yaws appear from a few weeks to two years after the primary lesion. In secondary yaws, treponemes spread through the blood and the lymph, resulting in the advancement of the disease. The skin, bones, and lymph are mostly affected, with overall discomfort (Mitjà *et al.*, 2011). Secondary skin lesions consist of multiple smaller excrescences, often resembling the primary papule. Crusty lesions (Figure 1.4A) that may be found on the face, arms and legs are not uncommon (Perine *et al.*, 1984; Mitjà *et al.*, 2011). Hyperkeratoic plaques may form on the palms and soles and be responsible for the characteristic 'crablike' gait. Early osteoperiostiis of the proximal phalanges of the fingers (dactylitis) or long bone (i.e., forearms, tibia and fibula) may result in nocturnal bone pain and swelling (Lukehart, Fohn and Baker-Zander, 1990; Lahariya and Pradhan, 2007). Secondary yaws lesions are commonly described as "frambesiform" a raised "berry-like" lesion which may resemble the ulcer or papular rash of secondary syphilis (Perine *et al.*, 1984; Mitjà *et al.*, 2011).

About one-third of patients who advance into the secondary phase of syphilis still have some primary lesions present, which may challenge differentiating between the primary and

secondary stages (Gourevitch *et al.*, 1993). However, the second stage, which starts 4-8 weeks after the primary stage, is characterised by the extensive spread of maculopapular rash (Figure 1.4B), which matches the spread of the organism in the body. These rashes are typically known as “raw ham” or “copper coloured” (Chapel, 1978). The most affected parts are the palms and the soles. Mucous membranes, especially around the mouth, are also infected. Some patients may experience some levels of itching, but lesions are generally non-itchy (Chapel, 1978). Additional symptoms include widespread lymph node enlargement, headache, and fever. Bacteraemia is common at this stage. Syphilis treponemes are present in secondary lesions and are highly contagious. Lesions heal after some weeks, even in untreated individuals but may leave scars or hypopigmentation in the area (Chapel, 1978). Individuals infected by human treponemes may enter the latent phase of the disease before the tertiary phase if they do not receive any treatment. Patients are asymptomatic but serological tests show evidence of the disease in the latent phase (Abdulai *et al.*, 2018).

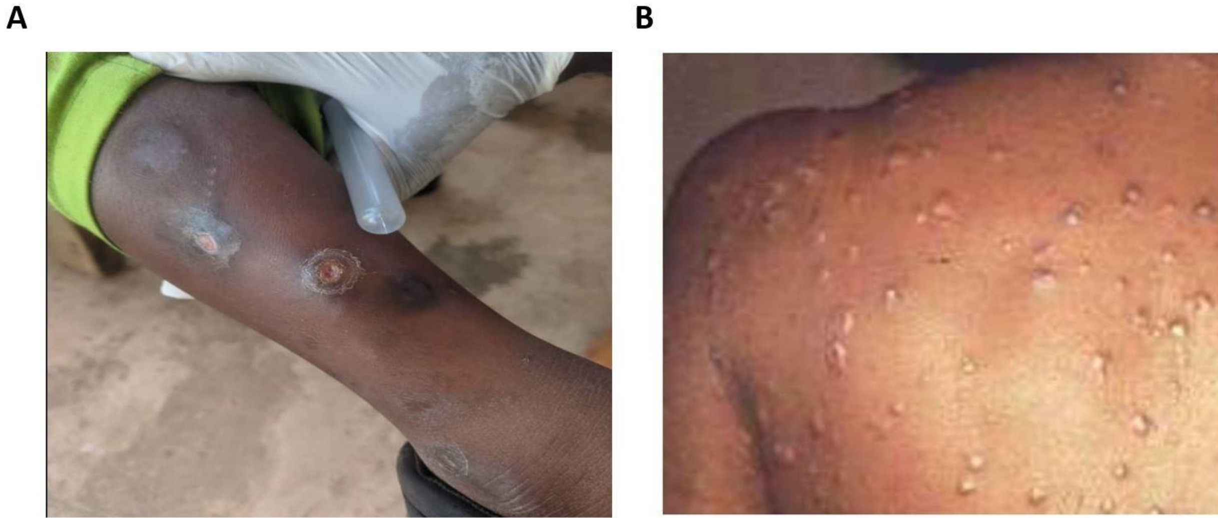


Figure 1.4. A: Crusty lesions of secondary yaws from this study. B. Papular rash of secondary syphilis (Perine *et al.*, 1984).

1.4.3 Tertiary stage

The tertiary stage of yaws has not been evidenced recently. Nevertheless, an estimate of 10% of patients with untreated yaws advance to this stage after five years or more (Koff and Rosen, 1993). As observed in earlier stages, the skin is the most affected part of the body. However, lesions of the face are common at this stage. Additionally, the joints and bones, especially in the legs, are severely affected (Perine *et al.*, 1984). Facial disfiguration, called gangosa or rhinopharyngitis mutilans, develops as the organism attacks and destroys the nose, upper jaw, palate, and pharynx. The faces of patients at this stage can develop an appearance known as goundou (Perine *et al.*, 1984; Mitjà *et al.*, 2011). Treponemes from yaws do not cause a congenital infection like that of syphilis, because yaws is found in children rather than women of childbearing age. Confirmed cases of cardiovascular or neurological diseases have not been seen in tertiary yaws (Perine *et al.*, 1984).

At least 40% of patients with untreated latent syphilis progress to late syphilis (Hunte, Al-Ghraoui and Cohen, 1993), a gradual progressive inflammatory stage in which granulomatous lesions (gummas) develop in skin, bones, liver, stomach and other organs. Deteriorating changes take place in the central nervous system causing meningovascular syphilis, and general paralysis with cerebral atrophy, psychosis, and dementia (Singh and Romanowski, 1999; Swartz, Healy and Musher, 1999; LaFond and Lukehart, 2006). Cardiovascular syphilis may lead to aortic aneurysm, and aortic valve insufficiency (Singh and Romanowski, 1999; Swartz, Healy and Musher, 1999; LaFond and Lukehart, 2006). Treponemes are not present in late-stage syphilitic lesion (Hunte, Al-Ghraoui and Cohen, 1993)

1.4.4 Congenital syphilis

Untreated syphilis can profoundly affect pregnancy outcome, resulting in spontaneous abortion, stillbirth, premature delivery, or perinatal death because the organism can cross the placenta and affect the foetus (Wendel *et al.*, 1989; Lago, Vaccari and Fiori, 2013). Infants born with congenital syphilis, depending on the severity of infection, have a rash, skin and mucous membrane lesions and often fail to gain weight, becoming marasmus. There is usually a high mortality rate (Frank and Duke, 2000; Lago, Vaccari and Fiori, 2013). In 2016, 6,100 neonatal

deaths, 41,000 preterm or low-birthweight and 143,000 early foetal and stillbirths due to congenital syphilis were reported worldwide (Korenromp *et al.*, 2017, 2019).

1.5 Other microorganisms causing similar skin lesions to yaws and syphilis

The epidemiology of yaws has been complicated by the presence of other organisms that cause similar lesions, such as *Haemophilus ducreyi*, the causative agent of the ulcerative sexually transmitted disease chancroid. *H. ducreyi* is now a common cause of non-genital skin lesions in several countries where yaws is endemic (Figure 1.5A) (Marks, Chi, *et al.*, 2014; Mitjà *et al.*, 2014; Ghinai *et al.*, 2015; Abdulai *et al.*, 2018). A wide range of other organisms cause ulcerative skin lesions like yaws does, including *Mycobacterium ulcerans*, responsible for buruli ulcer, and *Leishmania* species, causing cutaneous leishmaniasis, and tend to be endemic in regions where yaws is found. These micro-organisms and the diseases they cause also occur in Ghana (Williamson *et al.*, 2008).

Ngono *et al.*, (2021) sampled skin lesions during three outbreaks of yaws between 2017 and 2019 and performed a PCR test targeting the highly conserved genes DNA polymerase 1 gene (*polA*) for characterising TPE and haemolysin gene A (*hhdA*) for *H. ducreyi*. They reported that *H. ducreyi* occurred in most skin lesions. In this report, TPE was only detected during the first outbreak (12 (16.2%) TPE and 27 (36.5%) *H. ducreyi* out of 74 samples during the 2017 outbreak). However, *H. ducreyi* was detected in all three outbreaks, recording 70.8% and 84.6% in the second and third outbreaks respectively. Using the same gene targets (*polA* and *hhdA*), Abdulai *et al.*, (2018) reported 22.6% *H. ducreyi*, 5.7% TPE and 0% *M. ulcerans* in Abamkrom sub- district in Ghana from yaws-like lesions before total community treatment (TCT) with azithromycin. Additionally, Abdulai *et al.*, (2018) reported that 31 of the participants who were positive for both treponemal and non-treponemal tests were *T. pallidum* PCR negative. It appears now that surveillance data of yaws based on clinical and serological diagnosis alone, without molecular diagnostics (PCR and sequencing) can lead to wrong diagnosis.

Genital ulcer disease (GUD) is frequently caused by *Herpes simplex virus* (*HSV*) types 1 and 2, syphilis, and *H. ducreyi* (Table 1.1). Accurate clinical diagnosis of genital ulcer disease is

challenging in Africa since ulcers caused by these organisms resemble each other (Figure 1.6) and occurrence of mixed infections can happen (Dangor *et al.*, 1990). This is exacerbated by a lack of appropriate laboratory facilities (Dangor *et al.*, 1990). A study in Malawi reported 67% of *HSV-2*, 15% *H. ducreyi*, 6% syphilis, 6% *Lymphogranuloma venereum*, 14% mixed infection and 20% no aetiology among patients presenting with GUD (Phiri *et al.*, 2013). Another study also reported the presence of similar microorganisms except for those causing syphilis in South Africa among men presenting with genital ulcers (Chen *et al.*, 2000; Lewis *et al.*, 2012). This confirms the presence of multiple organisms in genital ulcers. In places where appropriate laboratory facilities are not available, this can lead to misdiagnosis of GUD.

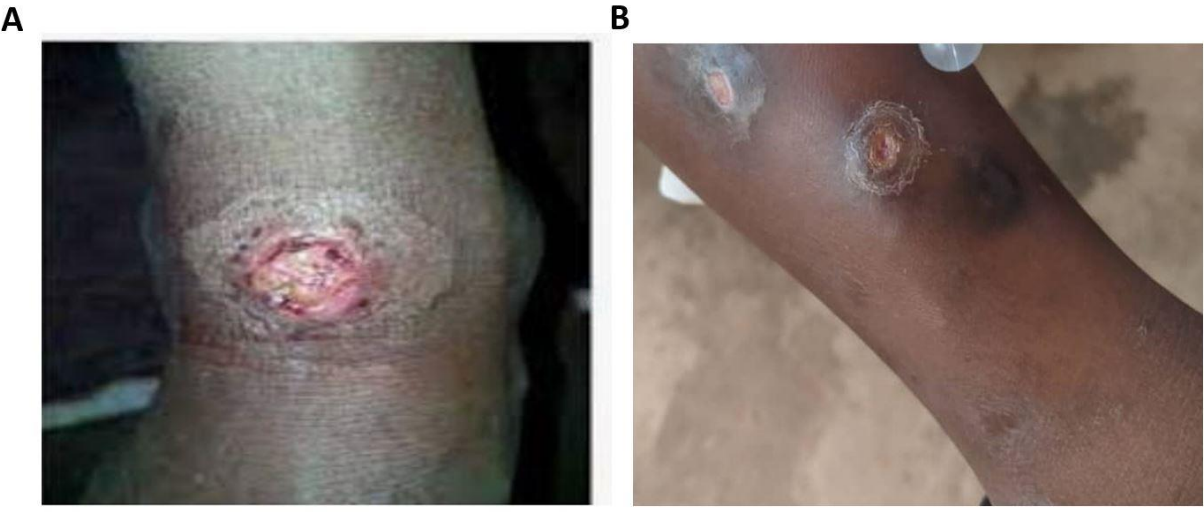


Figure 1.5. Lesion of *H. ducreyi* (A) look like that of yaws (B). Illustration of A is from (Marks *et al*., 2014) and B is from fieldwork of this study.

Figure 1.6. Genital ulcers from *H. ducreyi* (A), *HSV-2* (B) and syphilis infections(C) look similar. Illustrations A and B is from (Quizlet, 2022) and C from (Perine *et al*., 1984).

Table 1.1 Mode of Transmission, clinical features, laboratory diagnosis and treatment of common organisms that cause lesions like those of yaws and syphilis

Organism	Mode of Transmission	Clinical features	Laboratory diagnosis	Treatment
H. ducreyi	**1**. Sexually transmitted (Trees and Morse, 1995; Lagergård, Bölin and Lindholm, 2011) **2**. Skin to skin contact of children (Abdulai *et al.*, 2018)	Tender erythematous papules within 4-7 days. These papules progress to a pustular stage and subsequently ulcerate (Lewis, 2003). Ulcers (chancroid) are multiple, painful, purulent, and deep with ragged undermined edges and evidence of bleeding points in the base (Lewis, 2003). In men chancroid is normally found on the prepuce and penile frenulum. In women, chancroid typically appears as vulva ulceration (Morse, 1989; Lewis, 2003)	**1**. Culturing on selective media (e.g., Mueller-Hinton agar with 5% chocolatized horse blood) (Alfa, 2005; Lewis, 2014) **2**. PCR and sequencing (Alfa, 2005; Lewis, 2014) **3**. Serological test e.g., detection of *H. ducreyi* lipo-oligosaccharide using monoclonal antibodies (Alfa, 2005; Lewis, 2014)	Adult: **1**. Azithromycin 1 gm orally in a single dose (CDC, 2021) **2**. Ceftriaxone 250 mg IM in a single dose (CDC, 2021) **3**. Ciprofloxacin 500mg orally 2 times/day for 3 days (CDC, 2021) **4**. Erythromycin base 500mg 3 times/day for 7 days (CDC, 2021) **Children**: Azithromycin 30 mg/kg body weight (CDC, 2021)

Organism	**Mode of Transmission**	**Clinical features**	**Laboratory diagnosis**	**Treatment**
HSV-1	Oral to oral contact, skin to skin, oral-genital contact, mother to child during delivery (rare case) (Whitley and Roizman, 2001)	Asymptomatic in most cases but symptoms include painful blisters or open sores in or around the mouth (cold sores). These symptoms can reoccur periodically. Genital herpes is also normally asymptomatic but symptoms that can occur include genital or anal blisters or ulcers. *HVS-1* genital herpes does not commonly reoccur (Whitley and Roizman, 2001)	**1**. Cell culture (Lakeman, Whitley and Group, 1995) **2**. PCR (Lakeman, Whitley and Group, 1995) **3.** Serological (for only past infection) (Lakeman, Whitley and Group, 1995)	**1**. Acyclovir: 400 mg orally 3 times/day for 7-10 days (Whitley and Roizman, 2001; CDC, 2022) 2. Famiciclovir: 250 mg orally 3 times/day for 7-10 (Whitley and Roizman, 2001; CDC, 2022) **3**. Valacyclovir: 1 mg orally 2 times/day for 7-10 days (Whitley and Roizman, 2001; CDC, 2022)

Organism	**Mode of Transmission**	**Clinical features**	**Laboratory diagnosis**	**Treatment**
HSV-2	Sexually	Symptoms of *HSV-2* genital herpes include macules and papules followed by vesicles, pustules, and ulcers (Corey *et al.*, 1983). Complications are uncommon in men, but aseptic meningitis and urinary retention are common in women (Whitley and Roizman, 2001). Other complication in both men and women include paraesthesias and dysaesthesias of the legs and perineum, dysuria, localised inguinal adenopathy, and malaise (Whitley and Roizman, 2001). Recurrence of *HSV-2* genital infection is common. A third of patients are estimated to experience six recurrences annually (Whitley and Roizman, 2001).	Cell culture, PCR, serological (for only past infection) (Lakeman, Whitley and Group, 1995)	**1**. Acyclovir: 400 mg orally 3 times/day for 7-10 days (Whitley and Roizman, 2001) **2**. Famiciclovir: 250 mg orally 3 times/day for 7-10 days (Whitley and Roizman, 2001) **3**. Valacyclovir: 1 mg orally 2 times/day for 7-10 days (Whitley and Roizman, 2001)

Organism	Mode of Transmission	Clinical features	Laboratory diagnosis	Treatment
Myco-bacterium ulcerans	Unknown but insects such as mosquitoes (Johnson *et al.*, 2007; Quek *et al.*, 2007) and aquatic biting arthropods (Marsollier *et al.*, 2002) have been proposed as vector for transmission	Symptoms include painless nodules or ulcers on the limbs, to more severe forms, including edematous lesions. Bone is occasionally affected (O'Brien *et al.*, 2014)	Microscopic detection of acid fast bacilli form tissue smear, culturing, PCR and histopathology(Dreyer *et al.*, 2015)	1. Surgical debridement of affected skin and surrounding tissue, with or without subsequent skin grafting (Darie, Djakeaux and Cautoclaud, 1994; van der Werf *et al.*, 2003) 2. Rifampicin at 10 mg/kg body weight by mouth daily for 8 weeks and 15 mg/kg body weight by IM injection daily for 8 weeks (Omansen, van der Werf and Phillips, 2019)

Organism	**Mode of Transmission**	**Clinical features**	**Laboratory diagnosis**	**Treatment**
Leishmania	Bite of infected female sandflies (Kevric, Cappel and Keeling, 2015)	Symptoms start with skin ulcers which can affect the entire body (cutaneous leishmaniasis). Other symptoms include weight loss, fever, hepatomegally, splenomegally, pancytopenia, anaemia and lymphodeopathy (visceral leishmaniasis). (Schönian, Mauricio and Cupolillo, 2010).	Microscopy, culture, PCR and serology (Elmahallawy *et al.*, 2014).	1. Pentavalent antimonials (Bekhit *et al.*, 2018) 2. Amphotericin B (Bekhit *et al.*, 2018)

1.6 Diagnostic methods for *T. pallidum* infection

In places where laboratory facilities are not available, human treponematoses have been diagnosed based on their clinical signs and epidemiological features, including geographical location (Giacani and Lukehart, 2014). Laboratory diagnosis of treponematoses can be divided into two main groups. These are direct detection and serological tests (Figure 1.7).

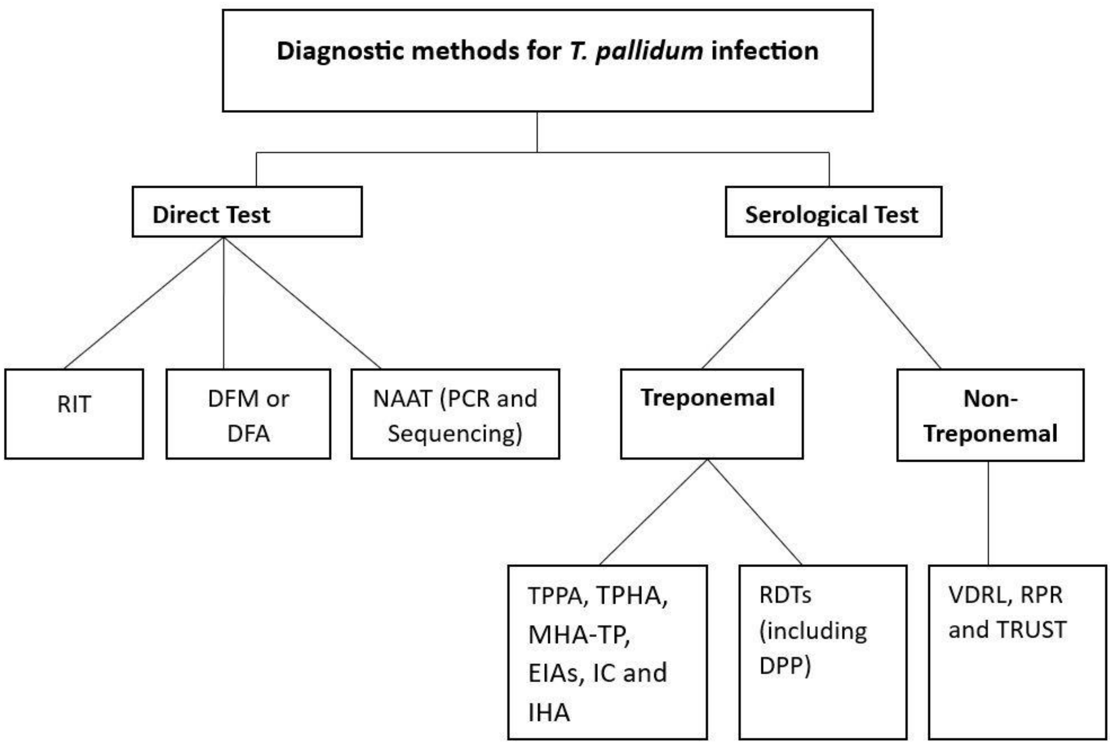


Figure 1.7 Summary of the various diagnostic methods for *T. pallidum* infection. Abbreviations used in this figure are defined as follow: VDRL= Venereal Disease Research Laboratory test, TPHA = *Treponema pallidum* Haemagglutination test, RPR= Rapid Plasma Reagin test, DPP= Duah Path Platform syphilis screen and confirmation test, RDT=Rapid Diagnostic Test, MHA-TP= Microhemagglutination assay for *Treponema,* EIA= Enzyme immunoassay, TRUST for Toluidine Red Unheated

1.6.1 Direct detection methods

In cases of bacterial infections, the affected body part can usually be sampled and then placed on specific media to identify the species responsible for those infections. Such routine laboratory approaches cannot be applied to infections due to treponemes since they remain challenging to grow on artificial media (Edmondson, Hu and Norris, 2018). Direct detection can be achieved by darkfield microscopy (DFM) or direct fluorescence antibody (DFA) testing, rabbit

infectivity test (RIT) (Theel, Katz and Pillay, 2020) and currently nucleic acid amplification test (NAAT) (Matějková *et al.*, 2009; Pětrošová *et al.*, 2012; Lu *et al.*, 2017; Marks *et al.*, 2018).

The rabbit infectivity test (RIT), which involves inoculating patient serum into rabbit testis, is the gold standard for the direct detection of *T. pallidum*. RIT is highly sensitive with a limit of detection of 1-3 treponemes (Tong *et al.*, 2017) even though some studies suggest the RIT sensitivity is below 50% in individuals with primary syphilis or neuroinvasive conditions (Hay *et al.*, 1990; Lukehart, Fohn and Baker-Zander, 1990).

Due to instability of treponemes in samples, darkfield microscopy (DFM) must be done immediately after taking a sample from skin lesions. The process involves the direct transfer of serous exudates (free of red blood cells) from primary and secondary lesions onto a sterile microscopic slide. Samples are examined within 20 minutes after collection for the presence of treponemes, where both morphological and motility characteristics of spirochetes can be assessed. The accuracy of the results of DFM depends on the time of examination (within 20 minutes) and the competence of the technician examining the sample (Theel, Katz and Pillay, 2020). The sensitivity of DFM is between 75% to 100% while the specificity is between 94% to 100% among patients with primary syphilis when compared to clinical and laboratory findings (serology and/or NAAT) as the standard (Hook *et al.*, 1985; Romanowski *et al.*, 1987; Grange *et al.*, 2012). Furthermore, in secondary syphilitic lesions, sensitivity of DFM varies from 58% to 71% while the specificity is 100% when compared to clinical and laboratory findings (serology and/or NAAT) as the standard (Lee *et al.*, 1991; Buffet *et al.*, 2007; Grange *et al.*, 2012). *Treponema denticola* commonly occurs within the oral cavity flora in most healthy individuals, and because this treponeme is morphologically indistinguishable from *T. pallidum*, DFM is not recommended for oral lesions (Larsen, Steiner and Rudolph, 1995).

As an alternative to DFM, direct fluorescence antibody (DFA) testing can be performed on serous fluid taken from syphilitic lesions, as well as other physiological fluids (e.g., cerebrospinal fluid and amniotic fluid). This process involves fixing the specimen with ethanol onto a microscopic slide, then staining with fluorescently conjugated monoclonal or polyclonal antibodies to *T. pallidum* and examining the slide for the presence of fluorescent spirochetes

(Theel, Katz and Pillay, 2020). The option to submit an air-dried or acetone-fixed specimen to the laboratory for examination without requiring observation immediately is a significant advantage of DFA over DFM. Additionally, while technical expertise is still required, DFA interpretation is much easier (i.e., the presence or absence of adequate fluorescence) (Theel, Katz and Pillay, 2020).

Different types of NAAT (molecular assays) have been developed and evaluated for the purpose of detecting the presence of *T. pallidum* in different body tissues and during different stages of the disease. These molecular assays differ in methods, design, and gene targets (Theel, Katz and Pillay, 2020). For diagnosis methods, the classic polymerase chain reaction (PCR), nested PCR, quantitative PCR, and reverse transcriptase PCR have been largely used (Cummings *et al.*, 1996; Centurion-Lara *et al.*, 2006; Chi *et al.*, 2015; Gayet-Ageron *et al.*, 2015; Theel, Katz and Pillay, 2020). Methods for molecular epidemiology of *T. pallidum* have been achieved by restriction length fragment polymorphisms (RLFP), multilocus sequence typing (MLST), a single gene or whole genome sequencing (WGS). The singleplex and multiplex PCR are the two designs that have been used across most studies (Marfin *et al.*, 2001; Arora *et al.*, 2016; Lu *et al.*, 2017; Marks *et al.*, 2018; Beale *et al.*, 2019; Theel, Katz and Pillay, 2020).

Several gene targets including *Treponema* membrane protein A (*tmpA*), subsurface lipoprotein 4D (4D), basic membrane protein (*bmp*), *T. pallidum* 47kDa protein (*tpp47*) and DNA polymerase 1 gene (*polA*), have been used for diagnostic purposes (Miao and Fieldsteel, 1980; Hook *et al.*, 1985; Rodes *et al.*, 2000; Grange *et al.*, 2012; Gayet-Ageron *et al.*, 2015). Among these genes, *polA* and *tpp47* have been evaluated and used frequently across most studies for the molecular diagnosis of treponemes (Rodes *et al.*, 2000; Gayet-Ageron *et al.*, 2015; Theel, Katz and Pillay, 2020). Both genes (*polA* and *tpp47*) demonstrated a specificity between 97-100% (Rodes *et al.*, 2000; Gayet-Ageron *et al.*, 2015; Theel, Katz and Pillay, 2020) and a sensitivity between 82-100% for early infection (Zhou *et al.*, 2019). Sensitivity can also depend on the type of specimen used for the analysis rather than just the target genes. High sensitivity (75% - 95%) has been achieved using lesion exudates (Palmer *et al.*, 2003; Heymans *et al.*, 2010; Gayet-Ageron *et al.*, 2015) and amniotic fluid (75%-100%) (Grimprel *et al.*, 1991; Nathan *et al.*, 1997; Hollier *et al.*, 2001) compared to cerebrospinal fluid (50% - 77%) (Gayet-Ageron *et al.*,

2015; Castro *et al.*, 2016) and whole blood/serum/plasma (12% - 55%) (Marfin *et al.*, 2001; Gayet-Ageron *et al.*, 2009, 2015; Martin *et al.*, 2009; Cruz *et al.*, 2010) at the primary stage of the disease. Noteworthy, in the latent stage of the disease, treponemes can be found in capillary beds (Theel, Katz and Pillay, 2020) and a higher sensitivity (54%-66%) to detect *T. pallidum* DNA using *tpp47* and/or *polA* genes was achieved when capillary blood from ear lobe was used (Castro *et al.*, 2007; Lu *et al.*, 2017).

Recent advances in molecular biology techniques have contributed to lower the costs of whole genome sequencing and different approaches are now available to distinguish between *T. pallidum* strains. This has so far mainly been achieved with next generation sequencing, including Illumina sequencing (Pětrošová *et al.*, 2012; Staudová *et al.*, 2014; Tong *et al.*, 2017; Marks *et al.*, 2018), 454 pyrosequencing (Čejková *et al.*, 2012) and sequence by oligonucleotide ligation and detection (Čejková *et al.*, 2012). Typing of strains from clinical specimen is helpful in differentiating relapse from reinfection and to link strains to their clinical manifestation and geographical location. This further contributes to disease surveillance. Various typing methods targeting specific sets of genes have been established to differentiate strains of *T. pallidum* from clinical samples routinely. These include i) the Centre for Disease Control and Prevention (CDC) typing, which uses RFLP of *tpr* genes (*tprE, tprG, tprJ*) and determination of the number of repeat in the *arp* gene (Pillay *et al.*, 1998)), ii) the sequencing based molecular typing (SBMT), which uses Sanger sequencing of *TP0136*, *TPO548* and *23S rRNA* genes (Flasarová *et al.*, 2006), iii) the enhanced CDC typing (ECDCT), which employs RFLP of *tpr* genes (*tprE, tprG, tprJ*), determination of repeats in *arp* genes and Sanger sequencing of *TP0548* (Marra *et al.*, 2010), iv) the CDC typing with additional analysis of *rpsA* gene (CDC-*rpsA*), which uses RFLP of *tpr* genes (*tprE, tprG, tprJ*) and determination of repeats in *arp* and *rpsA* genes (Katz *et al.*, 2010), v) the multi-locus sequence typing, which also employs Sanger sequencing of *TP0136*, *TP0548, TP0705, 23S rRNA* genes (Grillová *et al.*, 2018).

1.6.2 Serological methods

The serological tests used to diagnose treponemal infection are grouped into treponemal and non-treponemal tests (Figure 1.7). Since the genomes of TPE (yaws treponemes) and TPA

(syphilis treponemes) are genetically similar the same serological tests can be used to diagnose both infections (Mikalová *et al.*, 2011; Čejková *et al.*, 2012). An individual is declared as positive for *T. pallidum* infection when both treponemal and nontreponemal tests are positive.
The principle of treponemal tests is that they detect antibodies (IgG and IgM) to specific antigenic components of *T. pallidum* (Marks *et al.*, 2014; 2016). Non-treponemal tests determine the host response to non-treponemal antigens, such as cardiolipin and lecithin, which are released by damaged host cells, as well as lipoprotein–like materials released by treponemes (Matthews, Yang and Jenkin, 1979; Reisner *et al.*, 1997).
Treponemal tests consist of assays such as the *T. pallidum* particle agglutination assay (TPPA), *T. pallidum* hemagglutination assay (TPHA), microheamagglutination assay for *T. pallidum* (MHA-TP), enzyme immunoassays (EIAs), fluorescent treponemal antibody absorption assay (FTA-ABS), latex agglutination syphilis fast test, immunochromatographic (IC) rapid strip test, and immunochemoluminescence assay. Non-treponemal tests include the venereal disease research laboratory (VDRL) or the rapid plasma reagin (RPR) assay and the toluidine red unheated serum test (TRUST) (Tuddenham, Katz and Ghanem, 2020).
The MHA-TP and TPHA use sensitised red blood cells coated with *T. pallidum* (Nichols strain) antigen, which agglutinate with anti-treponemal antibodies (IgG and IgM). The TPPA is based on the agglutination of anti-treponemal with the same treponemal antigen as MHA-TP but uses coloured gelatin particles as antigen instead of red blood cells. The FTA-ABS uses fixed *T. pallidum* antigen on a slide to bind to IgM and IgG antibodies, which is detected with fluorescently labelled antihuman immunoglobulin antibodies, identified by florescent microscopy. In the latex agglutination syphilis fast test, latex particles coated with *T. pallidum* specific antigens are used to detect *T*. *pallidum* antibodies by agglutination of the particles. The IC uses one or more recombinant antigens applied to nitrocellulose strips as capture reagents. The immunochemoluminescence assay consists of paramagnetic microparticles which are coated with recombinant *T*. *pallidum* antigen and labelled with antihuman globulin which detects antibodies against *T. pallidum* (Knight, Crum and Hardy, 2007). All the three nontreponemal tests (VDRL, RPR and TRUST) use antigens containing cardiolipins, lecithin and

cholesterol which flocculate on reaction with Treponemal antibodies (Tuddenham, Katz and Ghanem, 2020).

Recently, the rapid point of care test or rapid diagnostic test (RDT) has become popular due to its many advantages over the other serological tests: the results of RDTs are communicated within 20 minutes, they are equipment-free and user-friendly (Pham *et al.*, 2005; Zhang *et al.*, 2022). Most RDTs are treponemal based tests and they are based on the principles of immunochromatography (IC) (Zhang *et al.*, 2022). However, the dual path platform (DPP) test is an RDT which has both treponemal and nontreponemal tests on a single platform (Pham *et al.*, 2005; Zhang *et al.*, 2022). The DPP is consequently also based on the principles of immunochromatography. However, it contains two nitrocellulose membrane strips perpendicular to each other compared to the only one nitrocellulose strip found in the other (treponemal test only) IC based rapid test (Marks, *et al.*, 2016). On the DPP test package one strip receives the sample and running buffer and sample migrates to the second strip on which there are two test lines and a control line. The first test line is the recombinant *T. pallidum* antigen (treponemal test line) and the second line is synthetic nontreponemal antigen (nontreponemal test line). The third line serves as procedural control (Marks, *et al.*, 2016).

DPP test has proved effective in the diagnosis of syphilis (Yin *et al*., 2013), and several evaluations of its performance in diagnosing yaws have been undertaken (Marks *et al.*, 2014 REF). Compared to the conventional reference test (immunoassays), the sensitivity and specificity of the DPP treponemal test line were 93% and 98%, respectively (Causer *et al*., 2015). Compared to conventional reference test (RPR), the sensitivity and specificity of the DPP nontreponemal test line were also 90% and 97%, respectively (Zhang *et al.*, 2022). A multi-sited evaluation study of the DPP test by Yin *et al.*, (2013) showed that the sensitivity and the specificity of the DPP treponemal test line compared to the TPPA using whole blood were 96.7% and 99.3%, respectively. Additionally, the sensitivity and the specificity of the DPP nontreponemal test line compared to the TRUST using whole blood were 100% and 94.4%, respectively.

The use of RDT for treponemal infections has enabled broader access to diagnostic tests, especially in deprived and remote places, where there are no clinical laboratories. Since the

introduction of RDT, there has been a reduction in morbidity and death associated with mother-to-child transmission of syphilis in deprived settings due to improving diagnosis of this infection (Jafari *et al.*, 2013). RDT can also play a major role in improving the diagnosis of yaws in places where clinical case detection alone was traditionally used to recognise the disease (Marks, Mitjà, *et al.*, 2015).

1.7 Challenges in laboratory and rapid diagnostic methods

Thus far, diagnoses of human treponematoses have been possible either by direct detection of the organism (darkfield microscopy (DFM), rabbit infectivity test (RIT) and nucleic acid amplification test (NAAT)) or by serological tests since the organism cannot grow easily on artificial media until recently (Edmondson, Hu and Norris, 2018). However, in deprived or low resource areas where laboratories are not available, applying these direct detection methods is very challenging.

The major disadvantages of using DFM, RIT and NAAT are as follows: i) in darkfield microscopy organisms are only detected in samples of lesions in the early stages of the disease. Also, the expertise and logistics for DFM are not available in most places, especially in remote areas where yaws is common (Marks *et al.*, 2015), ii) the RIT method of detection of treponemes is also expensive, time-consuming, and delays results (Giacani and Lukehart, 2014), iii) NAAT methods are expensive and cannot be used routinely in low- and middle-income countries, where treponemal infections, especially yaws, are common. This contributes to explaining why, worldwide, but specifically in yaws endemic areas, diagnosis of treponemal infections have mostly been achieved by a combination of clinical symptoms, knowledge of the epidemiology of the disease (Marks *et al.*, 2015) and serological tests (i.e., the treponemal and the non-treponemal test).

While serological assays do not require sophisticated equipment, and they can easily be used in low resource areas as the RDTs - including the DPP - have exemplified, they do have their own limitations. Treponemal tests are highly specific and sensitive at all stages of the disease, except the very early primary stage (Marks, *et al.*, 2016). However, treponemal tests frequently remain positive for life following infection, irrespective of treatment or natural clearance (Marks, *et al.*,

2016). The lack of discriminatory ability of these treponemal tests implies that anyone, including pregnant women who tested positive for treponemal antibodies for the first time is likely to test positive again even after successful treatment, leading to unnecessary antibiotic treatment.

The non-treponemal tests are less specific but reflect active disease more accurately as they become negative following treatment or after progression into the latent stage, though some latent stages can still remain positive (Marks, *et al.*, 2016). However, non–treponemal tests are known to be less specific, with a higher rate of biological false-positives (e.g., in pregnancy, hepatitis B infection, acute malaria, measles, systemic lupus erythematosus and rheumatoid arthritis). Additionally non-treponemal tests, like treponemal tests cannot detect the very early stage of the treponemal infection since the body has not yet produced enough detectable antibodies in the serum (Marks, *et al.*, 2016). Early detection of *T. pallidum* infection matters to avoid the development of this multistage disease into latency or tertiary stage. If *T. pallidum* is not detected in the primary stage by serological test and the disease progresses into the tertiary stage, irreversible outcomes, such as general paralysis (in syphilis) (Singh and Romanowski, 1999; Swartz, Healy and Musher, 1999; LaFond and Lukehart, 2006) and facial disfiguration (in yaws) can occur (Perine *et al.*, 1984).

Another key challenge common to any serological testing is the inability of differentiating between treponeme subspecies. Differentiation between subspecies is crucial especially in places where both yaws and syphilis co-occur because an individual who has been infected with yaws at childhood may test positive for syphilis at adulthood since the treponemal test remain positive for life, and it cannot differentiate between the yaws and syphilis treponemes. This is likely to happen in places where both yaws and syphilis are prevalent, which may lead to wrong diagnosis and epidemiological information. NAAT (PCR and DNA sequencing) are the only methods currently available that target genetic signatures supposed to be specific to each subspecies (Centurion-Lara *et al.*, 1998, 2013; Šmajs *et al.*, 2011; Čejková *et al.*, 2012; Pillay *et al.*, 2016) as well as detecting early *T. pallidum* infection. The lack of laboratories equipped to perform molecular diagnosis in areas where yaws and syphilis co-occur limits management,

control and consequently eradication and elimination of these diseases (Seña, White and Sparling, 2010).

1.8 Treatments of *T. pallidum* infection

Prior to the real breakthrough in the treatment of treponemal infection with penicillin by Mahoney, Arnold and Harris (1943) various treatments such as mercury, arsphenamine, and other preparations with heavy metals (bismuth compounds) were used. Arsphenamine, which was developed by Paul Ehrlich in the early years of the 20th century, was the first drug manufactured to treat syphilis (Perine *et al.*, 1984). However, the long period of injectable administration of arsphenamine coupled with its toxicity made this drug unfavourable (Perine *et al.*, 1984). According to the CDC's 2010 guidelines for the treatment of sexually transmitted diseases (Workowski and Bolan, 2015) and the UK's 2015 guidelines for management of syphilis (Kingston *et al.*, 2016), penicillin G (Figure 1.8) is the drug of choice for the treatment of all stages of syphilis (Table 1.2). Penicillin G is now widely used to treat syphilis (WHO, 2016), and it was also the first drug of choice for mass treatment of yaws before 2012 (WHO, 2012). The penicillin formulation (i.e., benzathine, aqueous procaine, or aqueous crystalline), dose, mode of dispensing, and duration of treatment are all determined by the stage and clinical manifestation of the *T. pallidum* infection as well as other management factors (e.g., pregnancy, HIV status, and age) (Table 1.2). Because *T. pallidum* can reside in places where treponemicidal levels of penicillin are difficult to achieve (e.g., the central nervous system, aqueous humour, and synovial fluid), using the right penicillin formulation is crucial (Workowski and Bolan, 2015). In situations (such as allergy to penicillin) where penicillin cannot be used, the alternate antibiotics are tetracyclines (doxycycline and tetracycline), macrolides (azithromycin and erythromycin) (Figure 1.9), and cephalosporin (ceftriaxone). Despite several years of penicillin's excellent efficacy (no known resistance) against treponemal infection, the use of injectables has led to the second-line oral antibiotics, including tetracyclines (doxycycline and tetracycline), and macrolides (azithromycin and erythromycin) as first-line drugs for the treatment of *T. pallidum* infections (syphilis and yaws) (Stoner, 2007; WHO, 2012).

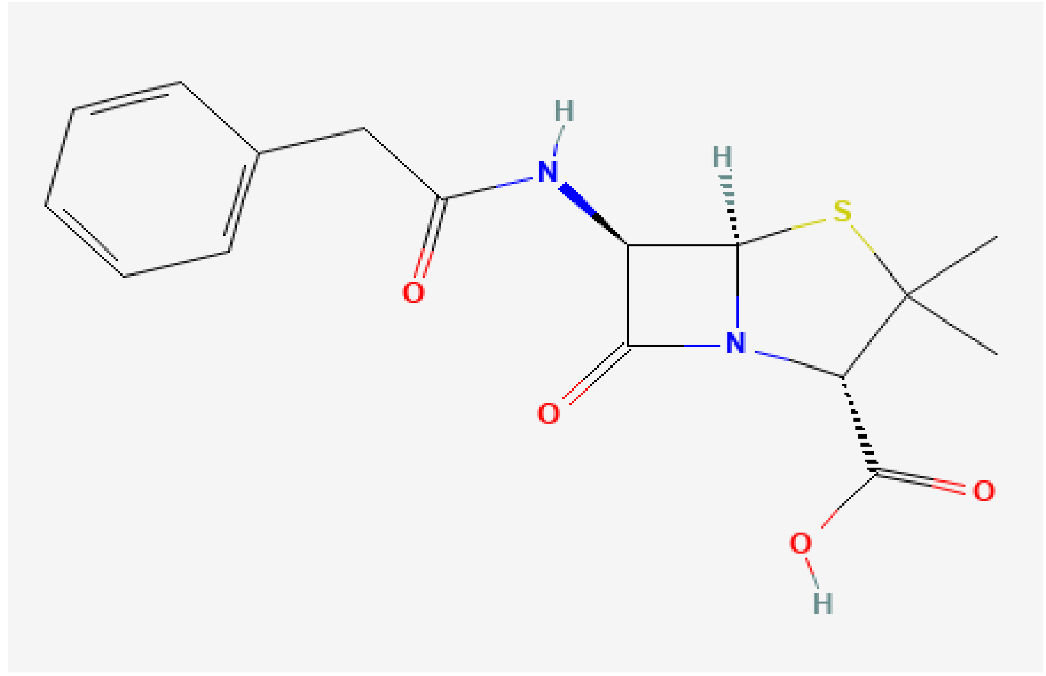


Figure 1.8. Chemical structure of penicillin G (Cha, 2004)**.** Penicillin G is the drug of choice for treating all stages of treponematoses. Penicillin administration involves the use of injectables.

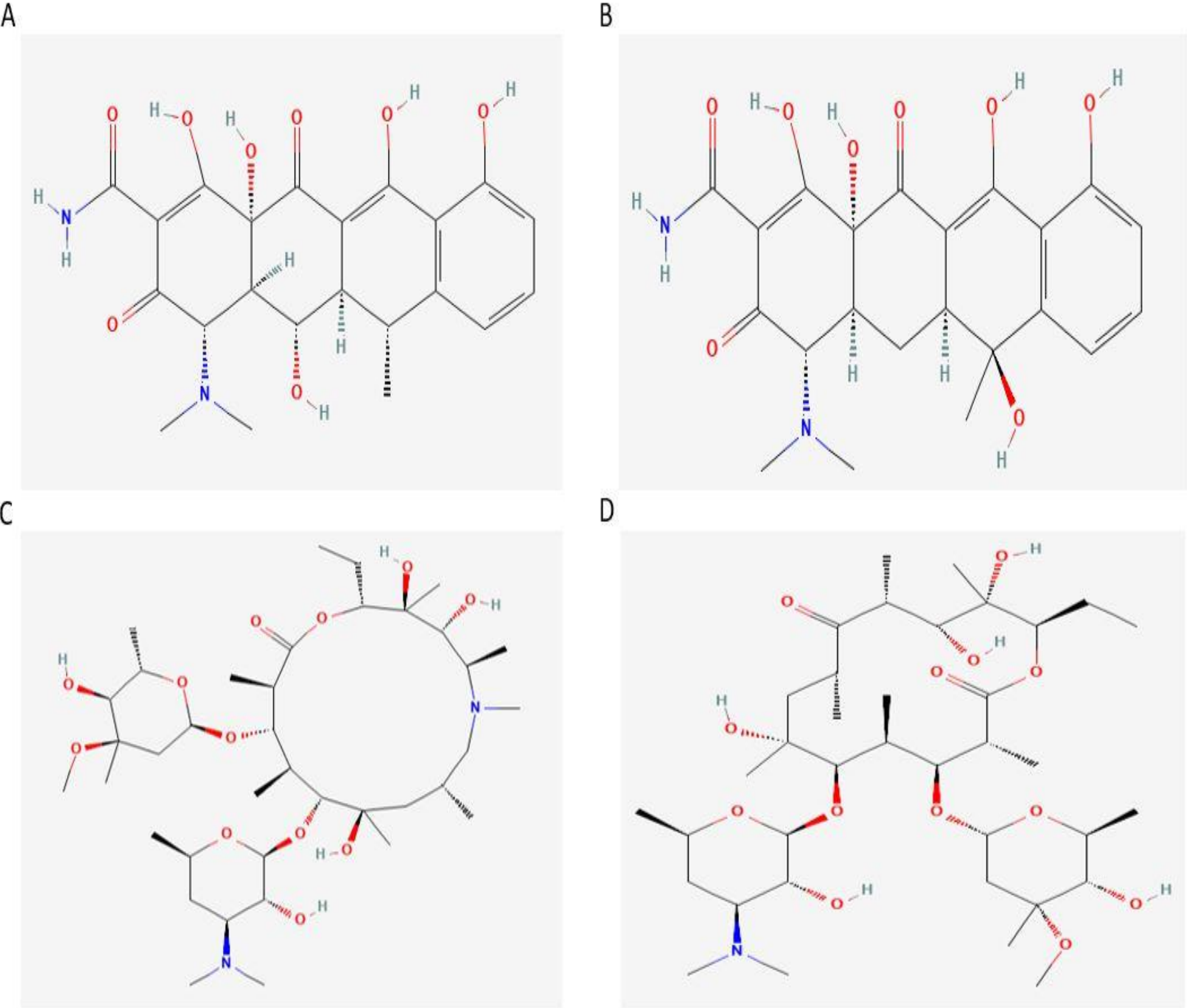


Figure 1.9. Chemical structures of (A) doxycycline (B) tetracycline (C) azithromycin (D) erythromycin (Alvarez-Elcoro *et al*, 1999; Thangadurai, 2005). Tetracycline and doxycycline belong to tetracycline group of antibiotics whiles azithromycin and erythromycin are macrolides. They are oral drugs which have been used as second-line treatment of treponematoses. Azithromycin has become the first line of choice for treating yaws after demonstrating that it is as effective as penicillin in a study in Papua New Guinea (Marks *et al.*, 2018) and Ghana (Abdulai *et al*., 2018), and it does not involve the use of injectables compared to penicillin.

Table 1.2 Recommended treatment of various stages of Treponematoses (yaws and syphilis)

Clinical stage of disease	Recommended treatment	Alternative treatment
Syphilis (primary and secondary stage)	• Adults and adolescents: Benzathine penicillin G 2.4 MU once intramuscularly (Workowski and Bolan, 2015; Kingston *et al.*, 2016; WHO, 2016)	• Procaine penicillin G 1.2 MU 10 - 14 days intramuscularly (Kingston *et al.*, 2016; WHO, 2016) • Doxycycline 100 mg twice daily orally for 14 days (Kingston *et al.*, 2016; WHO, 2016) • Ceftriaxone 1 g IM once daily for 10 - 14 days (WHO, 2016) • Azithromycin 2 g once orally (WHO, 2016) • Erythromycin 500 mg PO QDS for 14 (Kingston *et al.*, 2016)
	• Pregnancy: Benzathine penicillin G 2.4 MU once IM (Workowski and Bolan, 2015; Kingston *et al.*, 2016; WHO, 2016)	• Procaine penicillin G 1.2 MU for 10 days IM (WHO, 2016) • Erythromycin 500 mg PO QDS for 14 days (Kingston *et al.*, 2016; WHO, 2016) • Azithromycin 2 g once orally (WHO, 2016) • Ceftriaxone 1 g IM once daily for 10 - 14 days (WHO, 2016)

Clinical stage of disease	Recommended treatment	Alternative treatment
Syphilis (Tertiary stage)	• Adult and adolescent: Benzathine penicillin 2.4 MU once weekly for three weeks (three doses) (Kingston *et al*., 2016; WHO, 2016) • Pregnancy: management as in non-pregnant patient (doxycycline is contraindicated) (Kingston *et al*., 2016; WHO, 2016)	• Procaine penicillin 1.2 MU once daily for 20 days (WHO, 2016) • Doxycycline 100 mg twice daily for 30 days (WHO, 2016)
Neurosyphilis	• Procaine penicillin 1.8 MU - 2.4 MU IM once daily plus probenecid 500 mg orally QDS for 14 days) (Kingston *et al*., 2016; WHO, 2016)	• Ceftriaxone 2 g IM or IV once daily for 10 - 14 days (Kingston *et al*., 2016; WHO, 2016) • Doxycycline 200 mg orally BD for 28 days) (Kingston *et al*., 2016; WHO, 2016)
Congenital syphilis	• Aqueous benzyl penicillin 100,000 - 150,000 U/Kg/day IV for 10 - 15 days (WHO, 2016)	• Procaine penicillin 50,000 U/Kg/day IM for 10 - 15 days) (Kingston *et al*., 2016; WHO, 2016)
Syphilis in HIV positive patients	• Treatment for as appropriate for all stage of infection as in HIV negative) (Kingston *et al*., 2016; WHO, 2016).	

Clinical stage of disease	Recommended treatment	Alternative treatment
Yaws (primary, secondary, and tertiary)	Azithromycin orally at 30 mg/kg per body weight (maximum 2 g) (WHO, 2012)	Benzathine penicillin (suspected treatment failure after azithromycin): 0.6 MU single dose IM for patients under 10 years and 1.2 MU single dose IM for patients over 10 years (WHO, 2012)

MU = million units, IM = intramuscular, IV = intravenous, PO = by mouth, QDS = four times daily

Penicillin is a bactericidal beta-lactam antibiotic (Figure 1.8). Beta-lactam antibiotics interfere with the function of the transpeptidase enzyme (i.e., the penicillin binding protein [PBP]). This enzyme carries out cross-linking of the cell wall of actively growing bacteria (Zapun, Contreras-Martel and Vernet, 2008). The beta-lactam antibiotics binds to the PBP receptor, because they are similar in chemical structure, which halts the cross-linking of the cell wall by the PBP, thereby disrupting cell wall synthesis of bacteria leading to cell death (Zapun, Contreras-Martel and Vernet, 2008). Tetracyclines (Figure 1.9B) specifically inhibit the 30S ribosomal subunit of bacteria, hindering the binding of aminoacyl-transfer-RNA (aminoacyl-tRNA) to the acceptor site on the messenger RNA (mRNA) ribosome complex. When this happens protein synthesis is halted, and the bacteria cell can no longer maintain proper functioning and will be unable to grow or further replicate. This type of impairment by the tetracyclines makes them "bacteriostatic" (Roberts, 2005; Mollie C Shutter, 2022). Macrolides (azithromycin and erythromycin) (Figure 1.9 C and D) are antibiotics that stop the growth of bacteria by binding to the 23S rRNA of the 50S ribosomal subunit. Once it binds, the drug prevents the translation of mRNA, specifically the growing peptide chain, by preventing the enzyme peptidyltransferase from adding the subsequent amino acid attached to the tRNA, thereby stopping the production of new proteins in the bacteria (Vester and Douthwaite, 2001).

The dosage of benzathine benzylpenicillin is age-dependent, and the period of administration for syphilis depends on the stage of the disease. A lower dosage is used for children (Table 1.2).

A lower dosage of benzathine benzylpenicillin is used to treat yaws (Table 1.2) because penicillin can easily get into tissues invaded by yaws at a lower dosage (Perine *et al.*, 1984).

1.9 Antibiotic resistance in Treponemes causing yaws and syphilis.

As both yaws and syphilis can be treated with antibiotics, i.e. benzathine benzylpenicillin, or doxycycline, azithromycin, tetracycline, or ceftriaxone for individuals allergic to penicillin, this raises the common global challenge of facilitating antibiotic resistance acquired by pathogenic bacteria. Strategies commonly adapted by bacteria to resist the effect of penicillin include the production of lactamases that inactivate penicillin, acquisition of novel Penicillin Binding Proteins (PBPs) with low affinity for penicillin, alterations of PBPs through homologous recombination (Hanage *et al.*, 2009; Emmett and Rabadan, 2014), changes in the structure and number of porins resulting in decreased permeability to penicillin, efflux pumps that decrease the intracellular concentration of penicillin, or various combinations of these strategies. A study of *T. pallidum* genomes suggests the presence of three putative PBPs, but no distinctive lactamases (Fraser *et al.*, 1998).

Earlier reports suggested the presence of penicillin-resistant *T. pallidum* (Backhouse *et al.*, 1998), but this was not confirmed by later studies conducted by Myint *et al.*, (2004) and Douglas (2009). Myint *et al.*, (2004) suggested that, reported resistance was due to clinical relapse. Relapse of syphilis is due to treponemes that survive in the central nervous system due to the decline of therapeutic levels in body fluid and tissues. If a high dose of aqueous penicillin is given intravenously, relapse resolves completely (Myint *et al.*, 2004). This happens because *T. pallidum* can invade the central nervous system early in infection (Marra *et al.*, 2006). Thus far there is no known *T. pallidum* resistance to penicillin, and it is the only antibiotic currently recommended by the CDC and WHO for the treatment of all stages of syphilis.

Penicillin treatment, however, requires an intramuscular injection, which can be unsafe, especially in regions that are difficult to access, where treponematoses are the most prevalent (Mitjà *et al.*, 2012) and where blood transmission practices can increase the risk of blood-borne pathogens (Owusu-Ofori, Parry and Bates, 2011). This explains why the oral macrolide azithromycin has quickly been adopted to treat yaws (WHO, 2012). The efficacy of azithromycin

against *T. pallidum* was first published in 1990 after RIT (Lukehart, Fohn and Baker-Zander, 1990; Stamm and Parrish, 1990). This was supported by nonrandomised studies (Verdon, Handsfield and Johnson, 1994; Bowden *et al.*, 1995; Mashkilleyson *et al.*, 1996; Campos-Outcalt, Hurwitz and Mickey, 2002; Kiddugavu *et al.*, 2005) and three randomised controlled trials, one in Tanzania (Riedner *et al.*, 2005) and two in the United States (Hook III, Stephens and Ennis, 1999; Hook III *et al.*, 2002), where azithromycin was compared to penicillin. Since then, the single oral administration of azithromycin coupled with an efficacy equivalent to penicillin, its cost effectiveness (Blandford and Gift, 2003) and favourable side effects, made it attractive in Africa and some parts of the United States (Chen, Callahan and Kerndt, 2002; Kiddugavu *et al.*, 2005) for the treatment and prevention of syphilis.

Unfortunately, reported emergence of azithromycin resistant TPA (syphilis treponeme) (Lukehart *et al.*, 2004; Mitchell *et al.*, 2006; Martin *et al.*, 2009; Beale *et al.*, 2019) and TPE (yaws treponeme) (Mitjà *et al.*, 2018; Beale *et al.*, 2020) threatens the use of azithromycin as the treatment of choice for the treponematoses they cause. Stamm and Parrish (1990) reported that one strain (Street strain 14) of *T. pallidum* from a patient in the United States was resistant to high doses of azithromycin. *In vivo* studies of the same strain using rabbit module by Lukehart *et al.*, (2004) also confirmed azithromycin resistance. Treatment failure of syphilis infection with azithromycin has also been evident in eight patients from San Francisco between 2002 – 2003 (Klausner *et al.*, 2004). There is now widespread macrolide resistance in TPA in Europe, Asia, the United States, and Canada (Lukehart *et al.*, 2004; Mitchell *et al.*, 2006; Martin *et al.*, 2009; Beale *et al.*, 2019). Adenine to guanine mutations at either position 2058 (Stamm and Bergen, 2000) or 2059 (Matějková *et al.*, 2009) in *T. pallidum's* 23S rRNA gene result in azithromycin resistance. These mutations have also been shown to occur in rabbits' model (Molini *et al.*, 2016). These mutations confer resistance by preventing macrolide binding to the bacterial 50S ribosomal subunit, a structural component which also includes the 23S rRNA (Vester and Douthwaite, 2001; Matějková *et al.*, 2009). These two mutations are thus far the only ones known to confer azithromycin resistance in treponemes (Stamm and Bergen, 2000; Vester and Douthwaite, 2001; Matějková *et al.*, 2009; Molini *et al.*, 2016).

1.10 Eradication of yaws and elimination of syphilis

Eradication is achieved when there is a deliberate effort to reduce the incidence of a disease worldwide to zero permanently without requiring further interventions, while elimination is the reduction of a disease to no observed cases in a specific geographical location with deliberate effort, but still involves continuous intervention (CDC, 2007).

After the confirmation that a single dose of the oral antibiotic azithromycin was as efficient as the intramuscular benzylpenicillin injections for yaws treatment in Papua New Guinea in a randomized trial (Mitjà *et al.*, 2012), the World Health Organization (WHO) adopted azithromycin in its Morges strategy to have yaws eradicated by 2020 (WHO, 2012). Under this eradication program, endemic countries were tasked with stopping the transmission of yaws by 2017 and allowing three years of clinical surveillance to confirm no further transmission (WHO, 2012). However, 2020 has passed and yaws is still endemic in 15 countries (WHO, 2021a). This led to the launch of a new eradication programme in 2021 with the goal of eradicating yaws by 2030 (WHO, 2021a). Various campaigns have also been implemented to eliminate syphilis, including the CDC's national plan to eliminate syphilis through community-based efforts (CDC, 2007) and WHO's initiative for the global elimination of congenital syphilis through antenatal screening and treatment (WHO, 2008). However, syphilis still occurs worldwide (Arora *et al.*, 2016; Beale *et al.*, 2019).

The extent to which these elimination and eradication campaigns have affected the global occurrence of yaws and syphilis is investigated next, with a specific focus on the change of the number of cases through time and geographic regions. To this end, the prevalence of yaws and syphilis in published records and data downloaded from the WHO repository (i.e., number of yaws cases reported from 2008 to 2020, and latest national prevalence of syphilis in pregnant women) were reviewed, while outlining possible limitations of the reporting systems in place.

Chapter 2

2 Assessing the impact of eradication and elimination campaigns of yaws and syphilis on the number of reported cases worldwide

Assuming that the various campaigns against yaws and syphilis have had some impact on the prevalence of these diseases, it can then be hypothesised that the available data should show a significant decrease in the number of cases of these diseases with time. Additionnally, since MSM and FSW are groups specifically reported in the literature to be affected by syphilis, it was investigated if the data available shows these groups are more affected by syphilis in comparison to other groups, including inmates. To address these questions, the following data were collated:

(i) The prevalence of yaws and syphilis in the published record (studies conducted from 1988 to 2020, and published from 1991 to 2020, for yaws (n=28) (Table A1 in appendix 1.1), and studies conducted from 1994 to 2017, and published from 2000 to 2020, for syphilis (n=61) (Table A2 in appendix 1.1),

(ii) The number of yaws cases reported to WHO between 2008 and 2020 (Table A3 in appendix 1.2),

(iii) The latest national prevalence of syphilis in pregnant women recorded in the WHO repository (Table A4 in appendix 1.2).

2.1 Methods

2.1.1 Data collection

To investigate patterns in the distribution of yaws and syphilis, information from peer-reviewed articles reporting the prevalence of yaws and syphilis were first collected. An extensive search was performed using the following search engines: University of Westminster Library Search, Science Direct, PubMed, MEDLINE, Google Scholar, and Web of Science for (“yaws” OR “treponematosis” AND “prevalence OR “incidence” OR “diagnosis”) OR (“yaws” AND [each

current yaws-endemic country]) and (“syphilis” OR “treponematosis” AND “prevalence” OR “incidence” OR “diagnoses”).

Studies on the prevalence of yaws and syphilis can vary greatly in the way they have been conducted. Therefore, articles that provided information on (i) the country where the study was conducted, (ii) the number of positive cases and the studied sample size were specifically selected. These were especially important to infer the prevalence of the corresponding treponematose per country. For example, even though the study of Boock *et al.*, (2017) showed the number of positive cases for yaws, because the sample size of people screened was not explicitly given, it was not included in the set of peer-reviewed articles selected for downstream analyses. The diagnostic method(s) used (either clinical or at least one serological test), dates when studies were conducted, started and/or ended (if different), and specifically for syphilis, whether specific identifiable groups (i.e., blood donors (BD), men who have sex with men (MSM), female sex workers (FSW), drug users (DU) and inmates (prisoners)) were further recorded. Prevalence here refers to the number of participants who were positive with at least one serological test divided by the total number of participants in the study.

Articles that fitted these criteria were included, regardless of whether they had been conducted in yaws endemic countries or not. This enabled sampling information on the prevalence of both treponematoses that was not geographically restricted (syphilis) and allowed a fairer comparison between the two (yaws and syphilis). Following these criteria, a total of 89 peer-reviewed research articles were included, i.e. 28 articles related to the prevalence of yaws (Table A1 in appendix 1.1) and 61 articles related to the prevalence of syphilis (Table A2 in appendix 1.1), covering a total of 39 countries.

This published research record was reviewed in parallel to the number of cases of yaws between 2008 and 2020 from the WHO data repository (last accessed on 29.04.2022) (Table A3 appendix 1.2). To obtain information on the burden inflicted by syphilis to yaws endemic countries independent of the 89 peer-reviewed articles, the national prevalence of syphilis among pregnant women from the WHO data repository in the 39 countries (Table A4 in appendix 1.2) covered by the 89 peer-reviewed articles previously selected were recorded (last

accessed on 29.04.2022). The prevalence of syphilis in yaws endemic countries was then compared to that of countries where yaws is not endemic using data from the 61 studies that reported the prevalence of syphilis.

2.1.2 Statistical analyses

Statistical analyses and graphs were generated on R version 4.1.2. When comparing proportions, either a Chi-Square test (when lowest sample size was 5 or above) or a Fisher exact test (when lowest sample size was below 5) were applied. The R functions were either chisq.test() or fisher.test(), respectively. When comparing distributions (such as the prevalence of yaws and/or syphilis between groups), a Kruskal-Wallis rank sum test was performed using the R function kruskal.test(). When this test suggested significant differences between the groups being compared, identification of the pairs that showed significantly distinct distributions was achieved with the Dunn procedure dunn.test() from the R package dunn.test. When only two groups were compared, a Wilcoxon rank sum test with continuity correction was performed using the R function wilcox.test(). Non-parametric Spearman's rank correlation test was performed with the function cor.test() to address the association between two independent continuous variables (such as prevalence and years). The R package ggplot2 (version 3.4.0) was used for visual representation of the data.

2.2 Results and analysis

2.2.1 No significant change of the prevalence of yaws and syphilis with time

According to the published records there was no significant change of the prevalence of these treponematoses with the year when the study ended (Figure 2.1) or the year when the study started, whether syphilis and yaws are considered together (Spearman's rank correlation rho=0.045, p-value=0.6418) or separately (Spearman's rank correlation rho=0.113, p-value=0.5672 for yaws, and rho=0.016, p-value=0.889 for syphilis). Additionally, there was no significant difference between the overall prevalence of syphilis and yaws (p-value = 0.766) (Figure 2.2) even though there were significantly more studies reporting syphilis cases than

there were studies reporting yaws cases (Chi-Squared statistic = 11.378, df = 1, p-value = 0.0007433).

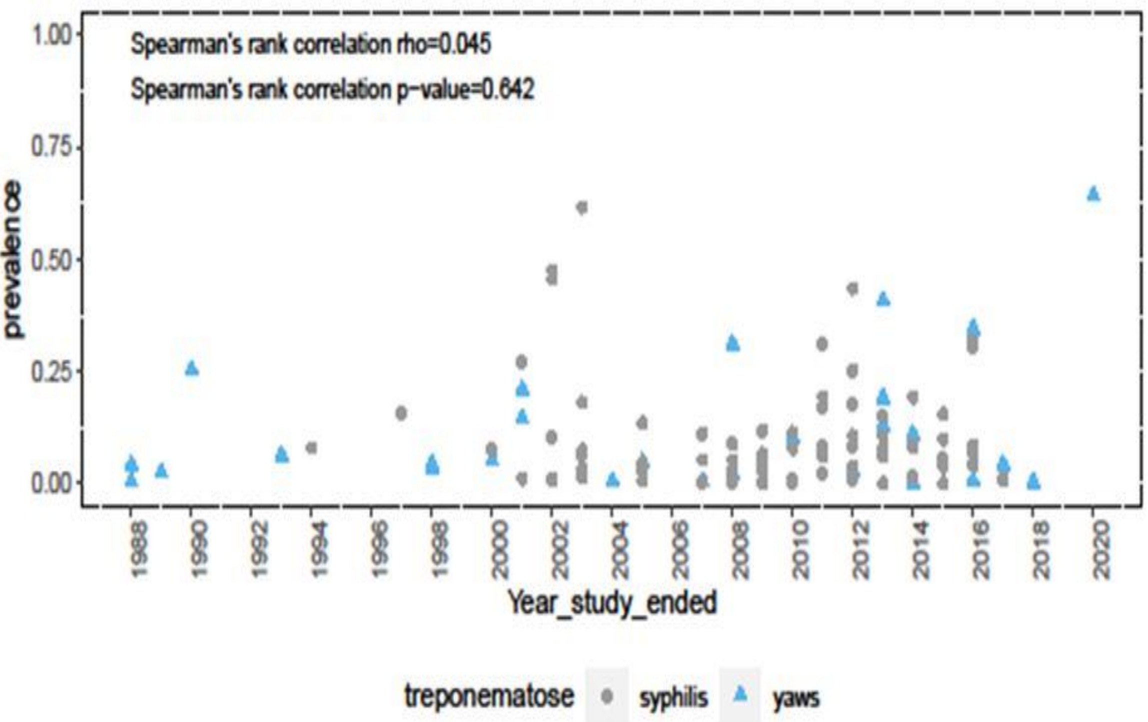


Figure 2.1 Prevalence of syphilis and yaws over time in the published record (representing 89 articles retained). Syphilis prevalence is represented in grey circles, yaws prevalence in blue triangles. The Spearman's rank correlation rho and p-value shown on the Figure refer to both diseases combined. For the treponematoses separately: Spearman's rank correlation rho=0.113, p-value=0.5672 for yaws, and rho=0.016, p-value=0.889 for syphilis. Statistical analyses and figure were generated on R version 4.1.2. Figure was generated with ggplot2 package and non-parametric Spearman's rank correlation test was performed with the R function cor.test(). Data for figure and analysis can be seen in Tables A1 and A2 in appendix 1.1.

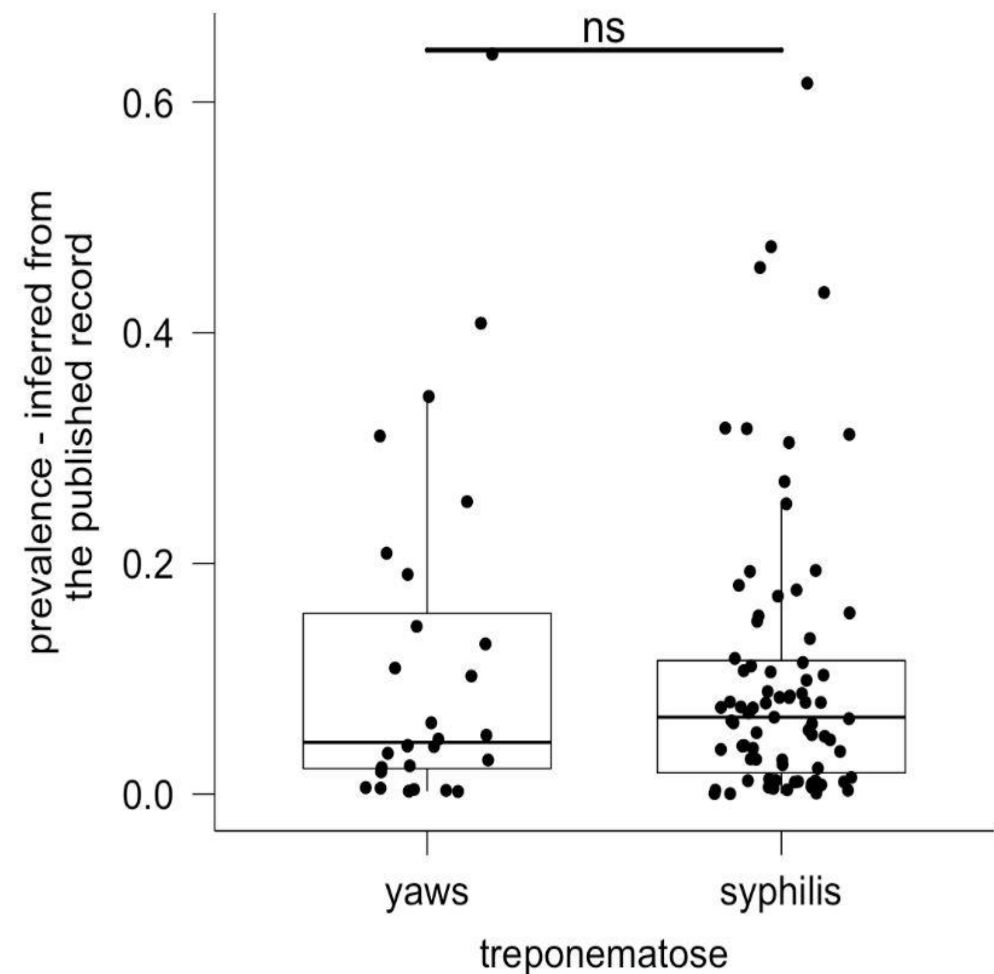


Figure 2.2. Prevalence of yaws and syphilis in the 89 peer-reviewed articles selected. The median of the prevalence of syphilis in the published record was 0.067 (inter quartile range - IQR = 0.097), and the median of the prevalence of yaws was 0.045 (IQR = 0.135). The Wilcoxon test showed that the difference was not significant (p = 0.77, effect size r = 0.03). Statistical analyses and Figure were generated on R version 4.1.2. Figure was generated with ggplot2 package and Wilcoxon test was performed with the R function wilcox.test(). Data for figure and analysis were from Tables A1 and A2 in appendix 1.1. The total number (n) = 107 here, because one article may report more than one prevalence from different locations or different specific groups.

Assuming the Morges strategy had a significant impact on the number of yaws cases reported worldwide yaws it was hypothesised that a significant difference in the number of yaws before and after 2012 (the year when the Morges strategy was implemented) should be observed. The number of cases reported to WHO rather than the prevalence was used here because according to WHO Morges strategy, an endemic country is where yaws is constantly present or at least one case of clinical and serological confirmed yaws had been reported. This is irrespective of population size. Nonetheless population sizes of the endemic countries for this period did not change significantly (population size remained stable) (Spearman's rank correlation, p-value> 0.05 for all endemic countries). To this end, the number of yaws cases reported to WHO from 2008-2020 (Table A3 in appendix 1.2) was used. Since not every country where yaws is endemic has been reporting cases consistently through the years (e.g. Central Africa Republic, the Philippines, Timor-Leste), this data was pooled per WHO region (Figure 2.3). This data does not show a significant difference in the number of yaws cases reported to WHO before and after 2012 in Africa (Wilcoxon test p-value = 0.098) or in the West Pacific (Wilcoxon test p-value = 0.466). A significant difference is however suggested in Southeast Asia (Wilcoxon test p-value = 0.003), which suggests the Morges strategy may have been efficiently applied here. However, this observation should not be over-interpreted, since Southeast Asia is represented by only two countries, and one of them (Timor-Leste) shows only two data points over the 12-year period investigated here (Figure 2.4).

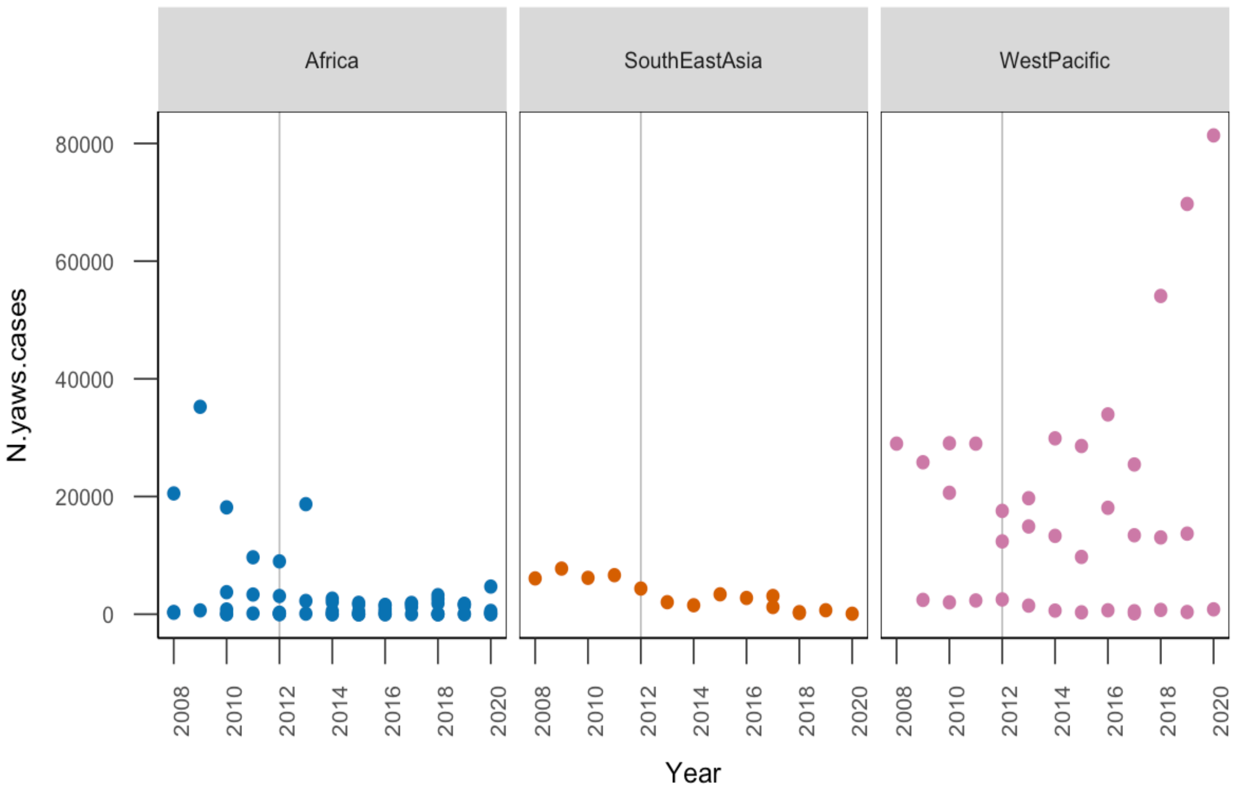


Figure 2.3 Evolution of the number of cases of yaws reported to WHO between 2008 and 2020 in yaws endemic countries, according to their classification in WHO regions. The vertical line (grey) shows 2012, the year when the Morges strategy was implemented. In Africa (blue), the median of the number of cases before 2012 was 646 (inter-quartile range - IQR = 6195) and it was 547 (IQR = 1757.5) after 2012. The Wilcoxon test does not show this difference is significant (Wilcoxon test p-value = 0.098). In the West Pacific (pink), the median of the number of cases before 2012 was 17560 (IQR = 24935.5) and it was 13363.5 (IQR = 25517.25) after 2012. The Wilcoxon test does not show this difference is significant (Wilcoxon test p-value = 0.466). In Southeast Asia (orange), the median of the number of cases before 2012 was 6178 (inter-quartile range - IQR = 548) and it was 1369.5 (IQR = 2149.25) after 2012. The Wilcoxon test shows this difference is significant (Wilcoxon test p-value = 0.003). This remains significant even after applying a Bonferroni correction for multiple testing (alpha level lowered from 0.05 to 0.05/3=0.017). N.yaws.cases= number of yaws cases. Statistical analyses and figure were generated on R version 4.1.2. Figure was generated with ggplot2 package and wilcoxon test was performed with the R function wilcox.test(). Data for the figure can be seen in Table A3 in appendix 1.2.

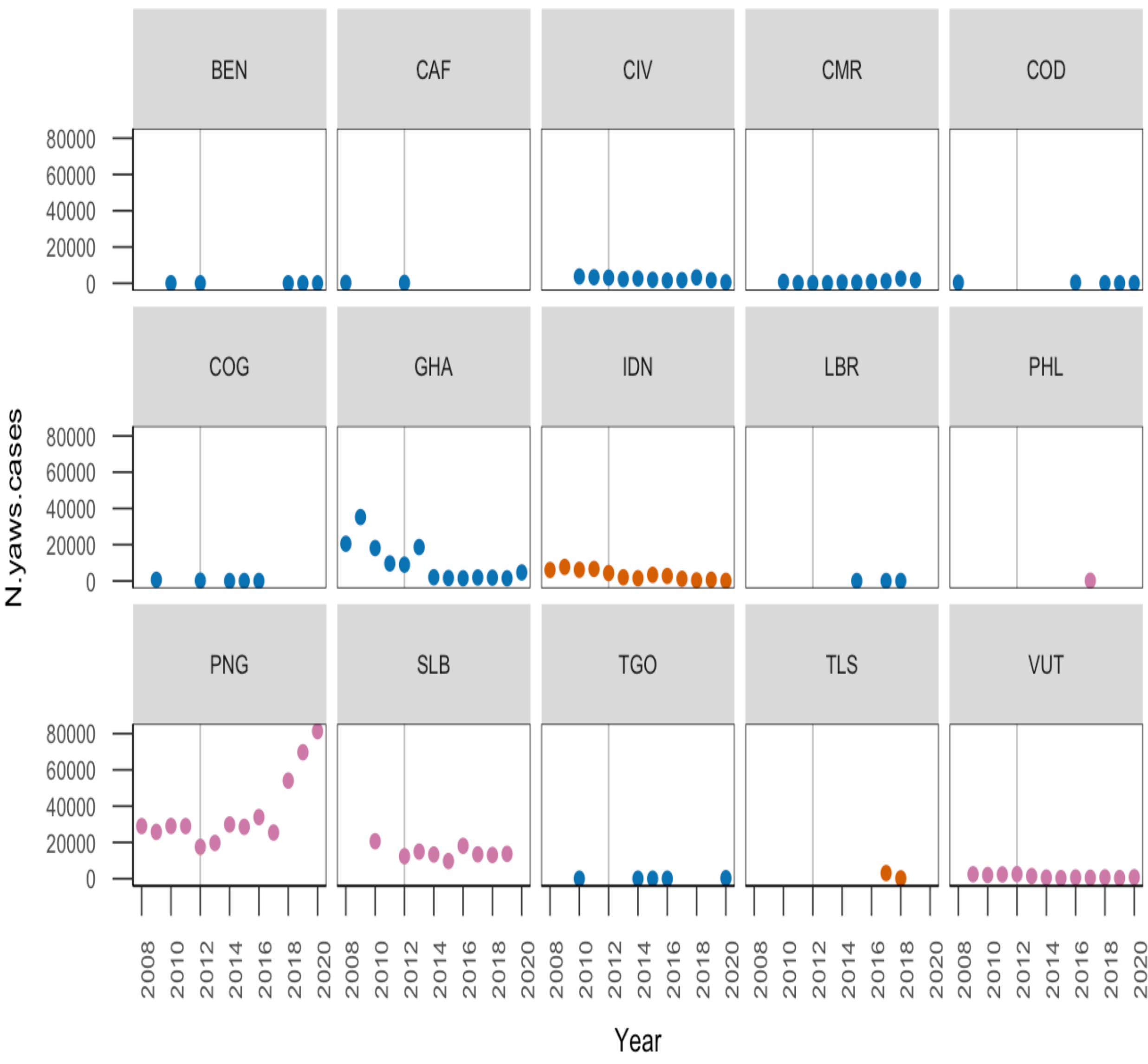


Figure 2.4. Number of yaws cases between 2008 and 2020 in the 15 countries known to be endemic for yaws. Country names are abbreviated as follows: BEN=Benin, CAF=Central African Republic, CIV= Cote d'ivoire, CMR= Cameroon, COD= Democratic Republic of Congo, COG=Congo, GHA=Ghana, IDN= Indonesia, LBR= Liberia, PHL= Philippines, PNG= Papua New Guinea, SLB= Solomon-islands, TGO= Togo, TLS= Timor-leste and VUT= Vanuatu. The colour coding shows the WHO region each country belongs to, either Africa (blue), Southeast Asia (orange) and West Pacific (pink). The vertical line (grey) shows 2012, the year when the Morges strategy was implemented. N.yaws.cases= number of yaws cases. The figure was generated on R version 4.1.2 with ggplot2 package. Data for figure was from Table A3 in appendix 1.2.

2.2.2 A lack of consistent reports to the World Health Organisation between 2008 and 2020

The number of cases of yaws for South East Asia relies only on two countries, where one of them (Timor-leste) reported data for two years over the 12 year period. This highlights an important aspect of this data: a lack of effort in monitoring or reporting yaws. More broadly, yaws has not been reported consistently throughout the 12 years (from 2008 to 2020) in eight of the 15 countries where yaws is acknowledged to be endemic (Figure 2.4): Benin (data reported for 5 years within this period), Central African Republic (data reported for 2 years), the Congo (data reported for 5 years), the Democratic Republic of the Congo (data reported for 5 years), Liberia (data reported for 3 years), the Philippines (data reported for 1 year), Timor-Leste (data reported for 2 years) and Togo (data reported for 5 years). In other words, seven out of 15 countries where yaws is currently acknowledged to be endemic have been consistently reporting the number of yaws cases to WHO: Cameroon, Cote d'Ivoire, Ghana, Indonesia, Papua New Guinea, the Solomon Islands and Vanuatu (Figure 2.4).

Inconsistencies in the reporting of yaws further appear when comparing both the published record and the WHO data. For example, Vanuatu did not report any cases to WHO in 2008, while a study conducted that year reported 95 cases from Tanna Island in Vanuatu (Fegan *et al.*, 2010). In addition, Cameroon reported 156 cases to WHO from 2012 to 2013. However, Boock and colleagues reported 188 cases in Bankim district alone in Cameroon during the same period (Boock *et al.*, 2017). Similarly, 22 cases were reported in three locations in Benin in 2012, while the national record for the same year was 11 cases (Mitjà *et al.*, 2015).

2.2.3 Syphilis and yaws are equally prevalent in yaws-endemic areas

It appears that the prevalence of syphilis has been investigated more often in countries where yaws is not endemic (Fisher exact test p-value<0.001) (Figure 2.5A). Therefore, the prevalence of syphilis in countries where yaws is endemic may be under-estimated due to limited data.

The WHO national prevalence of syphilis among pregnant women was used as an independent proxy for the prevalence of syphilis in the 39 countries covered by the 89 peer-reviewed articles. The national prevalence of syphilis among pregnant women was significantly lower in

countries where yaws is not endemic compared to yaws endemic countries (Wilcoxon test p-value = 0.0013 when the Solomon Islands - a country where yaws is endemic with 100% of antenatal syphilis - is excluded, Figure 2.5B). Assuming that the national antenatal prevalence of syphilis is a good proxy for the national prevalence of syphilis, it may be inferred that peer-reviewed studies on the prevalence of syphilis are limited in yaws endemic countries.

This is further illustrated on the map showing the distribution of the median prevalence of yaws and syphilis inferred from the 89 peer-reviewed articles (Figure 2.6) and compared to the map showing the antenatal prevalence of syphilis for those same countries (Figure 2.7). Interestingly, only four of the 16 countries where the prevalence of yaws has been reported have also reported the prevalence of syphilis (Figure 2.6). These countries are Ecuador (Hernandez *et al.*, 2017), Ghana (Adjei *et al.*, 2003, 2006; Sarkodie *et al.*, 2016), Indonesia (GERDA T Noordhoek *et al.*, 1991; Majid *et al.*, 2010; Sitanggang, 2017) and Nigeria (Olokoba *et al.*, 2009; Buseri, Seiyaboh and Jeremiah, 2010; Damulak *et al.*, 2013). Two of these countries, i.e., Ecuador and Nigeria, do not currently belong to the list of countries where yaws is endemic. Using the national antenatal prevalence of syphilis as a proxy for the national prevalence of syphilis, it may be inferred that syphilis is as prevalent as yaws in yaws endemic countries.

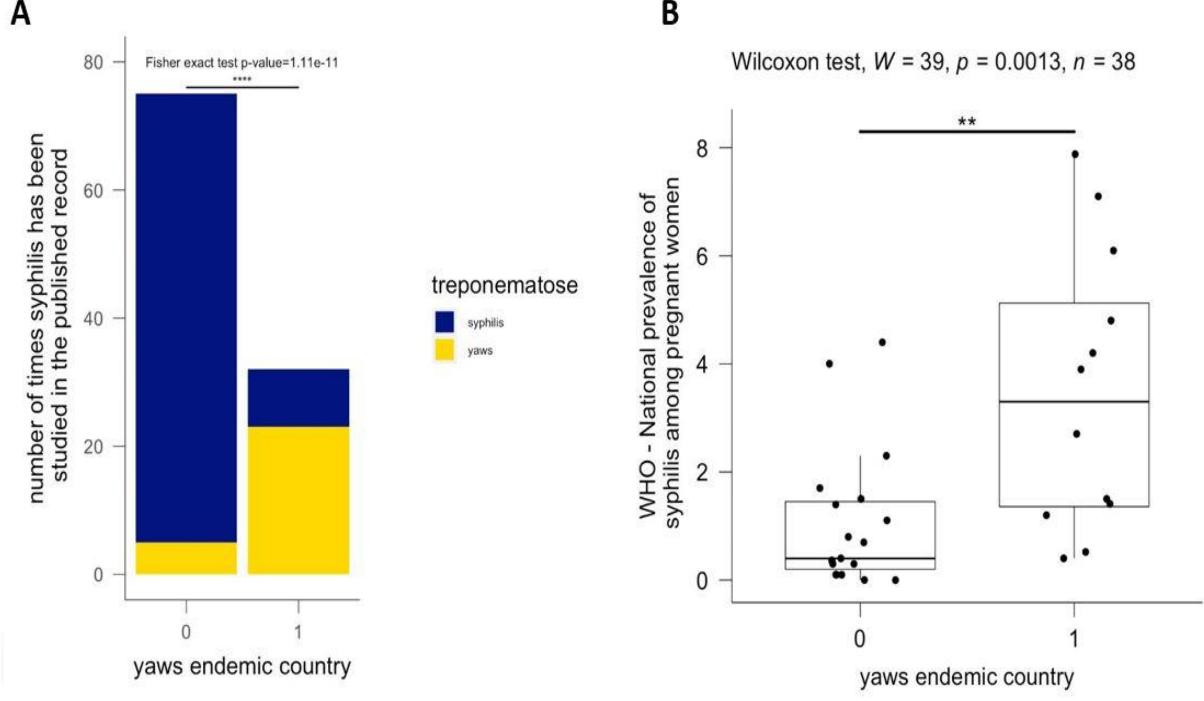


Figure 2.5 Number of studies reporting on prevalence of syphilis and the national prevalence of syphilis among pregnant women in yaws endemic (1) and non-yaws endemic (0) countries. (A) Stacked bar chart showing the number of times syphilis (navy blue) and yaws (gold) were investigated according to whether the country studied is endemic for yaws (1) or not (0). **(B)** WHO national prevalence of syphilis among pregnant women in 2020 in the same 39 countries covered by the 89 peer-reviewed articles selected, according to whether the country is endemic for yaws (1) or not (0). Excluding the outlier (Solomon Islands with 100% of antenatal syphilis), the median of the national prevalence of syphilis among pregnant women in countries where yaws is not endemic was 0.4 (IQR = 1.25), and 3.3 (IQR = 3.77) in countries where yaws is endemic. The Wilcoxon test showed that the difference was significant (p = 0.001, effect size r = 0.547). When the percentage of national prevalence of syphilis among pregnant women from the Solomon Islands is included, the median of this percentage in countries where yaws is not endemic was 0.4 (IQR = 1.25), and 3.9 (IQR = 4.69) in countries where yaws is endemic. The Wilcoxon test showed that the difference was significant (p < 0.001, effect size r = 0.574). Statistical analyses and figures were generated on R version 4.1.2. Figures were generated with ggplot2 package and wilcoxon test was performed with the R function wilcox.test (). Data for analysis and figures were from Tables A1, A2 in appendix 1.1 and A4 in appendix 1.2.

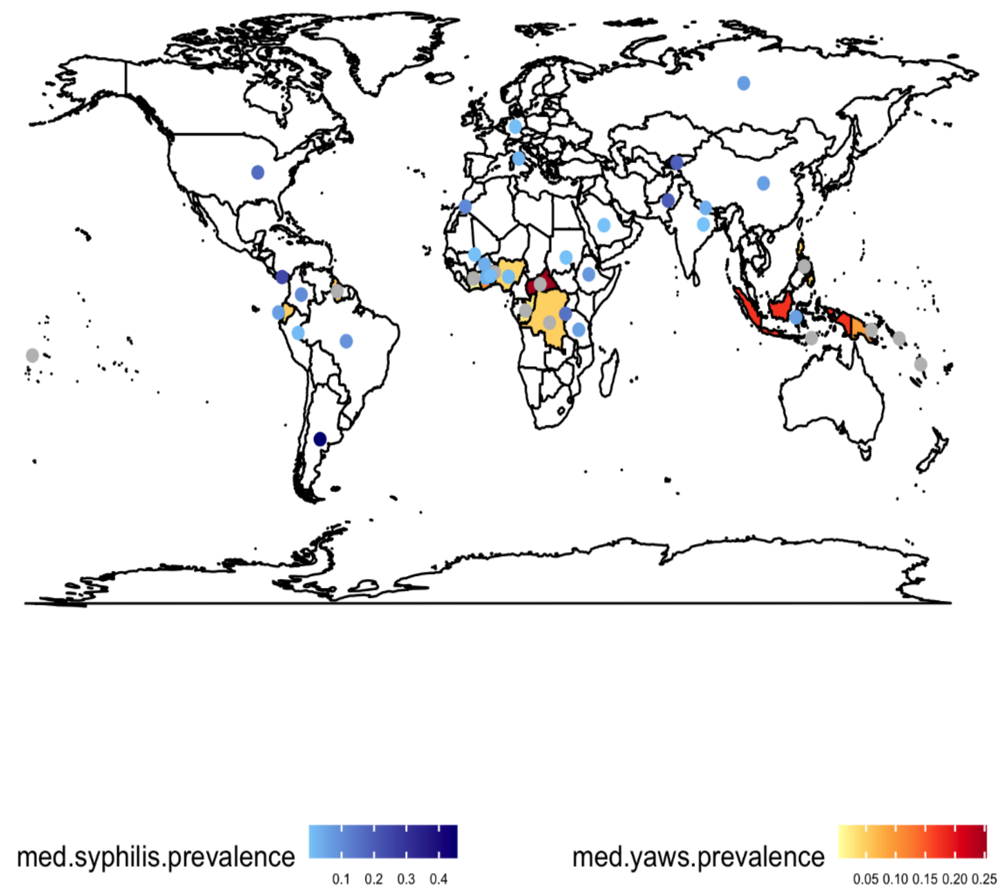
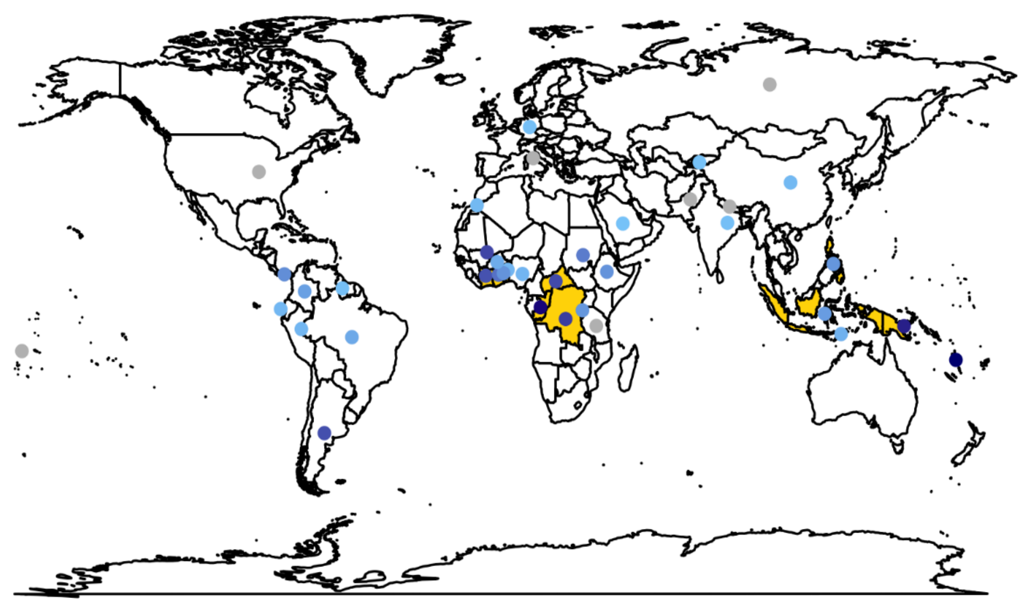


Figure 2.6 Prevalence of yaws and syphilis inferred from the 89 peer-reviewed articles. When a country was represented by more than one population sample for yaws or syphilis, the median of the inferred prevalence was used. The prevalence of yaws is shown by filled countries, with colours ranging from yellow (low) to red (high). The prevalence of syphilis is shown by the points appearing within their respective countries, with colours ranging from light blue (low) to dark blue (high). Grey points show countries where the prevalence of syphilis could not be inferred (due to those countries not being represented in the 61 syphilis articles). This shows that most countries where yaws occur lack peer-review studies about the prevalence of syphilis, except for four countries, i.e. Ecuador, Ghana, Liberia and Nigeria. Interestingly, two of these, Ecuador and Nigeria, are not currently among the list of countries where yaws is endemic. Furthermore, despite yaws also occurring in Wallis and Futuna, this country is not either in the list of countries where yaws is currently known to be endemic. Figure was generated on R version 4.1.2. with ggplot2, rworldmap, countrycode, maps , dplyr and viridis packages with data shown on Tables A1, A2 and A4 in the appendices 1.1 and 1.2



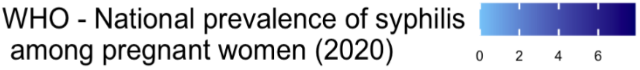


Figure 2.7 WHO national prevalence of syphilis among pregnant women in WHO's 2020 records (%) for the 39 countries covered by the 89 peer-reviewed articles. Yaws endemic countries are filled with yellow. Note that two countries (Cameroon and Liberia) where yaws is currently known to be endemic were not represented in peer-reviewed articles that fitted our search criteria to infer the prevalence of yaws or syphilis. Therefore, Cameroon and Liberia have been left blank here. The prevalence of syphilis among pregnant women are shown by the points appearing within their respective countries, with colours ranging from light blue (low) to dark blue (high). Grey points show countries where the prevalence of syphilis among pregnant women are not available in WHO data. This shows that in countries where yaws is endemic (yellow) the national prevalence among pregnant women in WHO's 2020 record tends to be high (darker blue). Figure was generated on R version 4.1.2. with ggplot2, rworldmap, countrycode, maps , dplyr and viridis packages with data from Tables A1, A2 and A4 in the appendices 1.1 and 1.2.

2.2.4 Some identifiable groups seem to be more affected by syphilis, but these groups are not equally investigated between WHO regions

The prevalence of syphilis in the published records showed a significant difference between the following pairs of groups: blood donors (BD) and female sex workers (FSW) (Dunn procedure adjusted p-value=0.00353), BD and inmates (Dunn procedure adjusted p-value=0.00766), BD and men who have sex with men (MSM) (Dunn procedure adjusted p-value=0.00002) (Figure 2.8A), where in all three pairs, BD is the group showing a significantly lower prevalence of syphilis in comparison to FSW, inmates and MSM. It was also tested if the number of studies reporting the prevalence of syphilis in these specific groups differed between WHO regions. They differed significantly between WHO regions (Kruskal-Wallis chi-squared p-value = 0.0003124) (Figure 2.8B). This suggests the prevalence of syphilis is not investigated equally and/or among the same groups within the WHO regions. Interestingly, MSM is the only group where the prevalence of syphilis has been investigated consistently in the six WHO regions. The prevalence of syphilis in BD is mostly investigated in Africa and not at all in the Americas, and, in inmates, it is mostly reported in the Americas and not at all in South-East Asia or in the Western Pacific (Figure 2.8B).

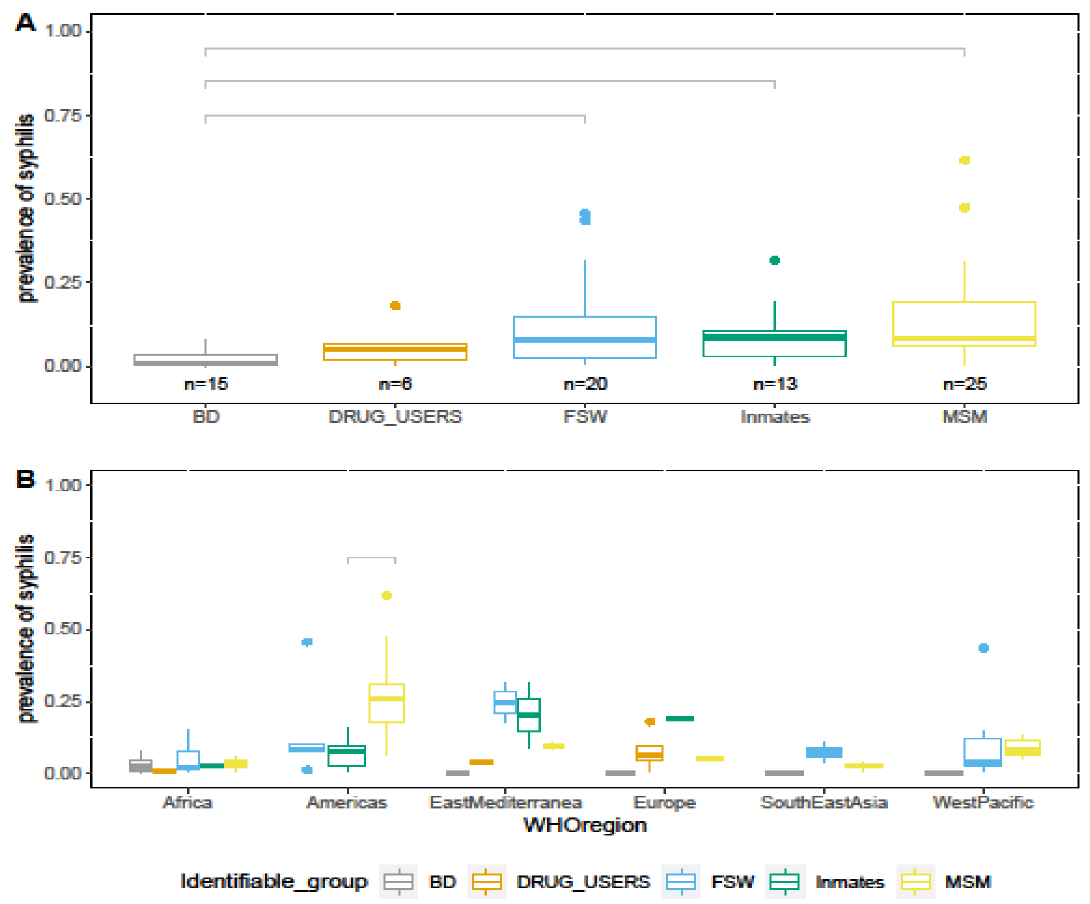


Figure 2.8. Prevalence of syphilis among identifiable groups in the published record. (A) Prevalence of syphilis among the identifiable groups investigated: BD - blood donors, DRUG_USERS - drug users, FSW - female sex workers, Inmates - prison inmates, MSM - men who have sex with men. Grey bars at the top show pairs of groups where the prevalence of syphilis is significantly different after adjusting for multiple comparisons (Dunn procedure): BD and FSW (adjusted p-value=0.00353), BD and inmates (adjusted p-value=0.00766), BD and MSM (adjusted p-value=0.00002). BD is the group showing a significantly lower prevalence of syphilis in comparison to FSW, inmates and MSM. (B) Prevalence of syphilis among identifiable groups in the six WHO regions. The grey bar shows the only significant difference in the prevalence of syphilis after adjusting for multiple comparisons (Dunn procedure): in the Americas syphilis prevalence is significantly lower in inmates in comparison to MSM (adjusted p-value=0.0085). There is no information on the prevalence of syphilis among drug users in South East Asia or in the West Pacific.

2.3 Factors affecting the eradication of yaws and the elimination of syphilis

As per today, congenital syphilis remains a significant global public health concern and is considered the most common infection associated with foetal loss or still birth in low-income settings (WHO, 2008, 2020a), while a new eradication programme targeting yaws has been devised by WHO (WHO, 2021a). This analysis and comparison of published research and data reported to the WHO highlights multiple caveats in our understanding of the distribution of these diseases. It also suggests that despite campaigns designed towards syphilis elimination and yaws eradication, the number of cases of both treponematoses still fluctuates with non-negligible prevalence. The comparison of published research and of data reported to WHO has highlighted that (i) more cohesion may be needed between regions so that sensitive groups that tend to be more affected by syphilis can be investigated consistently across various regions of the world, since this disease occurs worldwide; and (ii) continuous and consistent reports of yaws cases from all countries where yaws is endemic are necessary in order to establish current needs to achieving eradication of this disease in specific regions.

The success of eradicating or eliminating any disease also depends on the knowledge of the etiological agents of the disease including diagnosis, treatment, mode of transmission and the distribution of the disease. However, because treponemes remain challenging to grow in traditional laboratory conditions (Edmondson, Hu and Norris, 2018) our understanding of their biology is limited. Therefore, the biological relationship between TPA and TPE has mostly been investigated through molecular biology techniques and evolutionary analysis tools. These have recently evidenced that recombination within and between species can occur (Noda *et al.*, 2022). How these recombination events occur remains to be established, but they suggest that an individual may be co-infected by distinct strains of the same or of distinct *Treponema* species, and therefore the symptoms caused by yaws and syphilis may not be as independent as they have appeared. The analysis of the published research and of WHO repository data shows that syphilis and yaws are considered independently, which may contribute to slowing down our understanding of treponemes biology and hinder the elimination of syphilis and the eradication of yaws. The epidemiology of yaws has been further complicated by the presence of

other organisms which cause similar skin-lesions (Marks *et al.*, 2014; Mitjà *et al.*, 2014; Abdulai *et al.*, 2018).

Another aspect related to the biology of treponemes complicates the understanding of the transmission and spread of syphilis and yaws. There has been no evidence of zoonotic transmission of these treponemes (Peeling and Hook III, 2006) and it has consequently been assumed that humans are the only reservoir of human treponematoses. Knauf *et al.*, (2011) showed that the high prevalence of nonhuman primate infection in areas of tropical Africa where yaws is common in human suggests that cross-species infection may be possible. This suggests that nonhuman primates may be wild reservoirs for human treponemes. Further samples of yaws-like lesions from non-human primates and investigation of the evolutionary relationship of the strains present in these lesions to human treponeme strains are needed in order to tackle this question.

2.4 Syphilis and yaws in Ghana and significance of this PhD research.

Various eradication campaigns including the Morges strategy in 2012 and two large control programs between 1950s-60s and 1980s have been undertaken in Ghana but yaws still exists in this country. Ghana recorded an average of 9,269 cases of yaws annually (WHO, 2022b) with some communities recording more than 10% prevalence (Abdulai *et al.*, 2018). Likewise, the WHO initiative for global elimination of congenital syphilis through antenatal screening and treatment has also been implemented in Ghana since 2008 but the current national prevalence of syphilis among antenatal attendees (1.43%) is still higher than the acceptable range (<1%) (WHO, 2022a).

The main challenges to yaws eradication and syphilis elimination as highlighted in the previous sections may be due to diagnostic challenges, the existence of other organisms that causes similar lesions and probably antibiotics resistance. The routine serological tests (treponemal and non-treponemal test), although very useful in *T. pallidum* diagnosis, cannot detect most of the early infection and other pathogens in lesions leading to misdiagnosis and possibly false epidemiological data record. Combination of early detection of *T. pallidum* infection and

detection of other microorganisms that cause similar lesions can only be done by NAAT (PCR and sequencing). A combination of serological, PCR and sequencing is probably the most reliable way to get accurate epidemiological data for these diseases.

Probably because it is widely accepted that yaws and syphilis affect distinct cohorts (children and adolescents for yaws and adults for syphilis), they have been studied and recorded independently. Recently, evidence of inter-species recombination (Noda *et al.*, 2022) underscore the need to investigate these two diseases together, especially in places where they co -occur, since variants resulting from recombination are more likely to pose diagnostic challenges there. Indeed, recombination events can involve changes of conserved regions of DNA/RNA or protein targets, which tend to be the molecular targets of both serological and molecular diagnoses. In such situations, non recombinant antigens may not react properly with recombinant antibodies, which may lead to false negative diagnoses (Noda *et al.*, 2022).

The following chapters present the fieldwork and subsequent molecular analyses undertaken to investigate the prevalence of yaws and syphilis in Ghana. This fieldwork research applied serological test (DPP), multiplex PCR and 16S rRNA gene sequencing to identify *T. pallidum* and other microorganisms that may be present in skin-lesions attributed to those caused by *T. pallidum* in cases of yaws or syphilis from the same geographical location (Ashanti region of Ghana). Additionally, the microbiota in yaws-like and syphilis like lesions were described to identify other medically important bacteria which may prevent these lesions from healing. This is the first-time microbiomes of yaws-like and syphilis-like lesions have been described in Ghana. This will inform health practitioners on the aetiological agents of these lesions, which will improve health management, especially in places where state of the art molecular biology techniques is not readily available.

2.5 Aim of PhD work

This study aims to assess the prevalence of the treponematoses syphilis and yaws and determine the co-occurrence of pathogens causing similar skin lesions in Ghana.

2.6 Objectives of PhD work

- To determine the prevalence of treponematoses yaws and syphilis in Ghana (DPP test).
- To identify other microorganisms causing skin lesions similar to yaws and syphilis (multiplex PCR and 16S rRNA gene sequencing).
- To compare the microbiota of yaws-like lesions to that of syphilis-like lesions using molecular techniques (multiplex PCR and 16S rRNA gene sequencing).

CHAPTER 3

3 Methods

3.1 Study Location

Ghana, officially known as the Republic of Ghana, is in West Africa. Ghana is bordered by Cote d' Ivoire to the west, Togo to the east, Burkina Faso to the north and the Gulf of Guinea to the south (Figure 3.1). Ghana is found between latitudes 4°45'N and 11°N and longitudes 1°15'E and 3°15'W. The total land area of Ghana is about 227540 km^2 and has a total landmass of 238535 km (UN, 2020). The Prime Meridian passes through the port town of Tema, in Ghana. Ghana is divided into 16 regions, with Accra as its capital. Each of the sixteen regions has its own capital town. The population of Ghana is estimated at 30.8 million according to population and housing census conducted in 2021 (Ghana Statistical Serivce, 2021), which is about 0.4% of the world's population (UN, 2020).

The median age in Ghana is about 21.4 years, with approximately 57% of the population under the age of 25 years, 37.44% are below 15 years, and a population growth rate of 2.15%. The birth rate and death rate are 29.6/1,000 population and 6.6 / 1,000 population, respectively (CIA Factbook, 2018). Maternal mortality and infant mortality rates are 308 deaths per 100,000 live births and 32.1 deaths/1,000 live births, respectively. The current life expectancy at birth is approximately 68.2years. About 43% of the population lives in rural areas (CIA Factbook, 2018). The country's income per capita as of 2021 was $2363.3 (World Bank, 2023).

Ghana's health care is provided by both government and private sector There are about 1300 private and 1800 government health facilities. The health facilities are divided into health posts, health centres and clinics, district hospitals, regional hospitals, and tertiary hospitals. These categories are based on the type of service they can provide. The health post is the basic level (Ghana Statistical Serivce and Ministry of Health Ghana, 2003). Ghana is one of the countries where yaws is endemic and prevalence of syphilis among pregnant women is also above the acceptable range (> 1%).

Figure 3.1. Map of Africa showing the location of Ghana in red. Map was generated in R with rworldmap package.

3.2 Study Area

The study areas for both yaws and syphilis were in the Ashanti region of Ghana (Figure 3.2). However, yaws participants were recruited from six deprived rural communities (Brofoyedu, Bebu, Adweratia, Atia, Mpobi, and Kotei), and syphilis participants were recruited from two government hospitals (the Komfo Anokye teaching hospital and Suntreso government hospital) in Kumasi, the capital of the Ashanti region (Figure 3.2).

3.2.1 Ashanti region

Ashanti region is in the middle belt of Ghana (Mutuku Ryan, 2020) located between longitude 0.15 W and 2.25 W and latitude 5.50 N and 7.46 N. This region is the third largest region in Ghana and the second most populous after Greater Accra with a total land area of 24, 389 Km^2 (Ghana Statistical Serivce, 2021). The population of Ashanti region is around 5,432,485 million representing 17.6% of the total population of Ghana (Ghana Statistical Serivce, 2021). Kumasi is the capital city of the Ashanti region which is the second largest city in Ghana. The Ashanti region is made up of 43 districts comprising of one metropolitan, 19 municipals and 23 districts (GhanaDistricts, 2022). Kumasi metropolis accounts for about one third of the region's population and about 48% of the population live in rural settings (ModernGhana, 2022).

The Ashanti region has a total of 1654 health facilities which are owned by the government, mission, private and quasi-government organisations (GhanaDistricts, 2022). Doctor to patient and nurse to patient ration stands at 1:7500 and 1: 450 respectively (Ghana Health Service, 2022)

3.2.2 Study sites for yaws

The study sites for yaws were Brofoyedu, Bebu, Adweratia, Atia, Mpobi and Kotei in the Ashanti region. Brofoyedu and Bebu are found in the Atwima Kwanwoma district (Figures 3.2 and 3.3) which is located in the central part of the Ashanti region with a total land area of about 251 Km^2 (GhanaDistricts, 2022). The district has a population of 234,846 with 114,123 males and 120,723 females (GhanaDistricts, 2022).

Adweratia and Mpobi are found in Afigya Kwabre South district (Figure 3. 2) which is located in the central part of Ashanti with a total land area of 409.4 Km^2. Afigya Kwabre South district has a total population of 234,667 with 115,067 males and 119,600 females (GhanaDistricts, 2022).

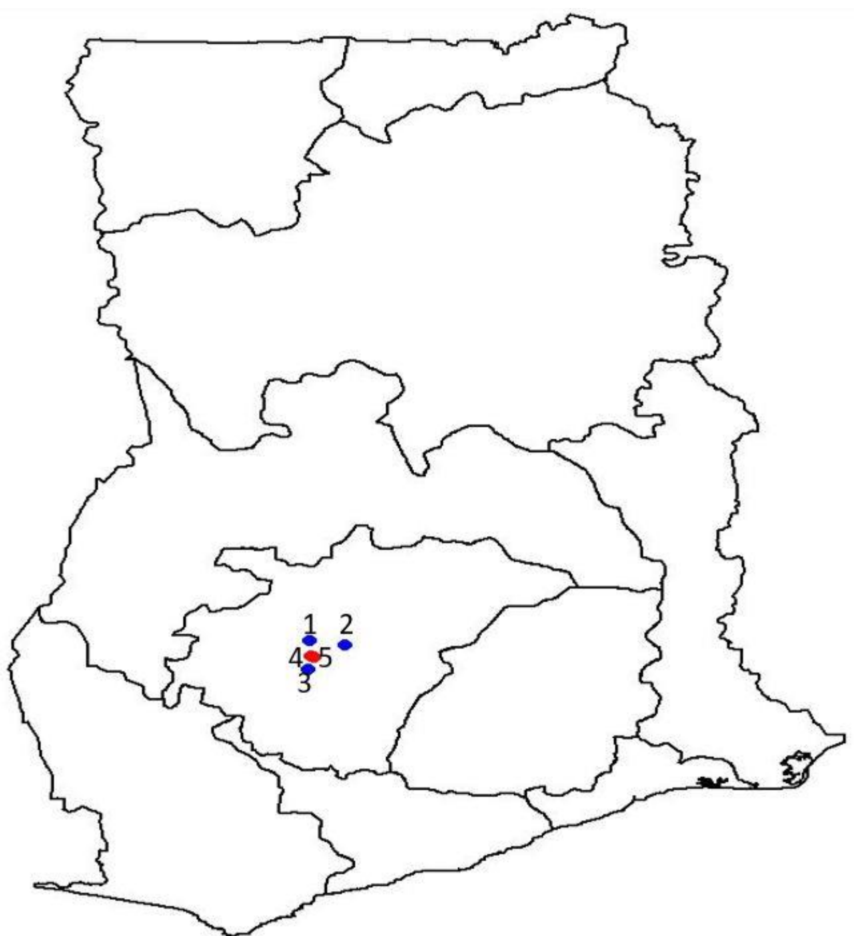


Figure 3.2 Map of Ghana showing the study area and sites. The study area was the Ashanti region. The locations in blue dots were the sites for yaws-like lesions sampling: Location 1 is Afigya Kwabre South municipality which had two study sites namely Adweratia and Mpobi, location 2 is Juaben municipality which had two study sites namely Kotei and Atia, location 3 is Atwima Kwanwoma municipality which had two study sites namely Brofoyedu and Bebu. The locations in red dots were the sites where syphilis-like lesions were sampled i.e., Kumasi metropolis, location 4 for KATH and location 5 for SSH. There is overlap of dots in red (4 and 5) because locations are close.

Kotei and Atia are found in the Juaben municipal (Figure 3.2) which has a total land area of 365 Km^2. Juaben municipal has a total population of 63, 929 with 31,203 males and 32,726 females (GhanaDistricts, 2022).

3.2.3 The study sites for syphilis

Komfo Anokye Teaching Hospital and Kumasi South Suntreso Hospital were the study sites for the syphilis study. The two hospitals are all located in Kumasi metropolis (Figures 3.2 and 3.3).

3.2.3.1 Komfo Anokye Teaching Hospital

Komfo Anokye Teaching Hospital is the second largest Hospital in Ghana, and it is in Kumasi in the Ashanti region. It has a 1200 bed capacity, and it serves as the referral hospital for most of the health facilities in the middle and northern part of Ghana (KATH, 2022). The geographical location of the hospital and the road network make it reachable to all parts of the country. The hospital has 15 directorates which are made up of 13 clinical and two non-clinical. The clinical directorate consists of emergency medicine, surgery, trauma and orthopaedics, medicine, obstetrics and gynaecology, child health and family medicine, oncology, eye, laboratory services, radiology, oral health, anaesthesia, intensive Care, ear, nose, and throat (EENT). The non-clinical consist of domestic and technical services (KATH, 2022).

The family medicine directorate and microbiology unit of the laboratory services were the places where participants for this study were recruited; the family medicine directorate provides specialist outpatient and inpatient services, medical emergency services, and training for various categories of clinical students. The laboratory services include microbiology, haematology, clinical chemistry, anatomic pathology, and mortuary, which provides various diagnostic services. The microbiology unit is further subdivided into bacteriology, parasitology and serology (KATH, 2022).

3.2.3.2 Suntreso Government Hospital

The Suntreso Government Hospital is located at North Suntreso and serves North and South Suntreso, Patasi Estate, Kwadaso, Adoato, Asuoyeboa, Breman and Suame communities in the Kumasi metropolis. It is within the Bantama Sub-metro, located at the West of Kumasi.
The facility has been divided into ten departments, namely Internal Medicine, Surgery, Paediatric, Obstetrics and Gynaecology, Eye, Ear Nose and Throat (ENT), Dental, Public health, Diagnostics, Theatre/Anaesthesia, Health Administration and Support Services (Suntreso Government Hospital, 2020). The departments have been further disaggregated into units.

Units are smaller management entities within the department. The units are mainly duty specific, and every unit performs one major function within the department. Syphilis-like lesions samples were taken from the sexually transmitted infection (STI) unit in the department of public health.

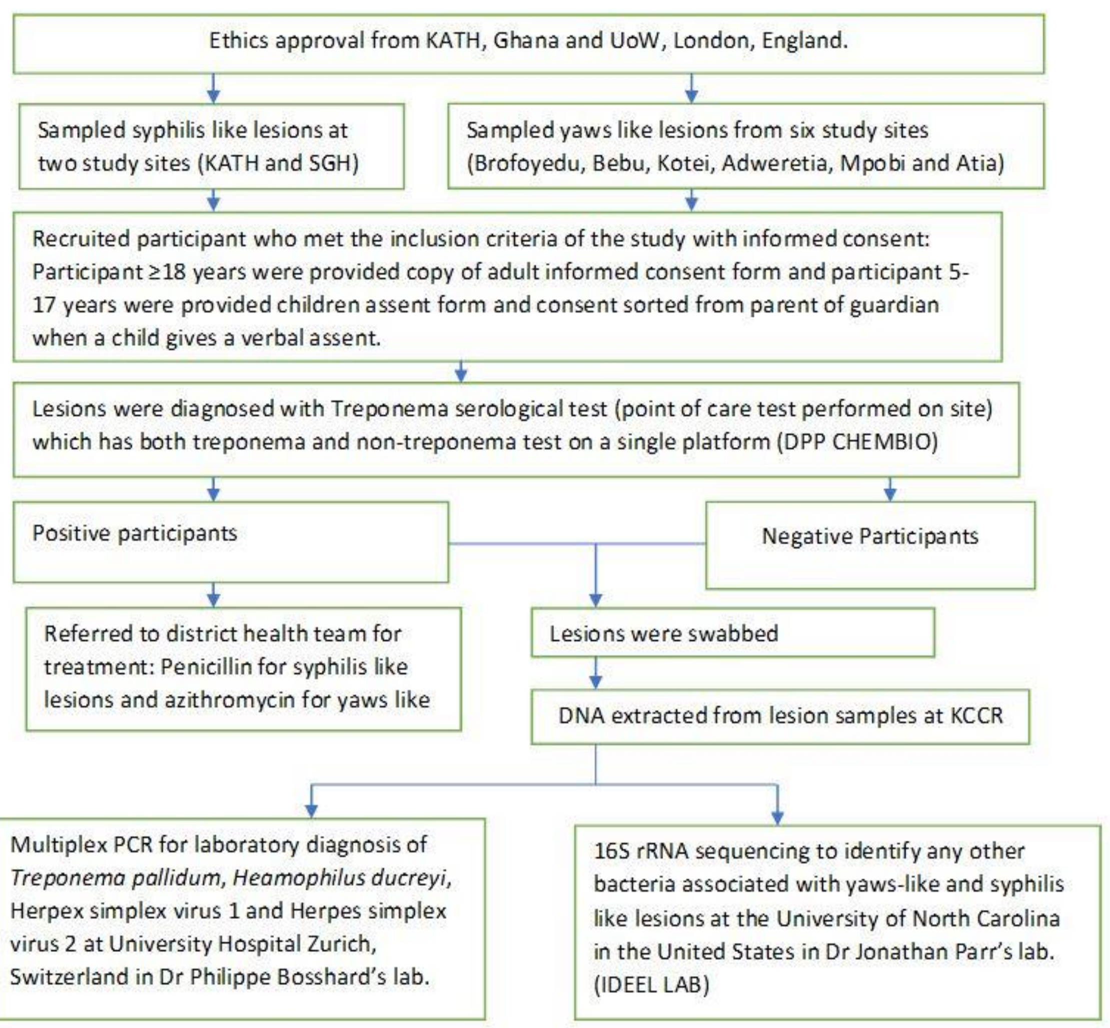


Figure 3.3 Conceptual framework showing summary of fieldwork and laboratory work of the study. KATH= Komfo Anokye Teaching Hospital, UoW= University of Westminster, KCCR= Kumasi Centre for Collaborative Research.

3.3 Ethics Approval

The study protocol was submitted to the ethical review committees of the Komfo Anokye Teaching Hospital, Ghana and the School of Life Sciences, University of Westminster, London, England, and it was approved with reference numbers KATH-IRB/AP/048/20 and ETH2122-0285 respectively (ethics approval can be found in the Appendices 2.1 and 2.2 respectively).

3.4 Community entering and sensitisation for yaws study sites

The various district health directorate, the assemblymen and chiefs in the communities were consulted first, the study was explained to them, and copies of the protocols were given to them before the recruitment of individual participants. Before recruitment of participant in this study, the assemblymen and the chiefs in the communities made an announcement in their various communities in appropriate local language (i.e., the Twi dialect) to sensitise their residents about the study. Knowledge and opinions on yaws were discussed. The study was explained to household heads, participating individuals, and their parents/guardians. Opportunities for questions to be answered were given at every stage of the process.

3.5 Inclusion criteria.

Participants who were from 5 to 17 years of age with a minimum of one clinical lesion consistent with primary or secondary treponemal (yaws) infection (WHO Morges Strategy, 2012) were enrolled for yaws study.

Participants who were from 18 years and above with a minimum of one clinical lesion consistent with primary or secondary treponemal infection (syphilis) were enrolled for syphilis.

3.6 Exclusion criteria

Individuals without clinical lesions or with clinical lesions not consistent with primary or secondary treponemal infection were excluded.

3.7 Sample size

Due to time and funding limitations, 100 individuals with yaws-like and 50 syphilis-like lesions respectively were targeted for sampling at the beginning of fieldwork. In fact, 110 yaws-like and 46 syphilis-like lesions were sampled by the end of the fieldwork.

3.8 Informed consent

Participants (adults) were provided with the participant information sheet (appendix 3.1), explaining the purpose of the study. Those willing to participate were asked to sign an informed consent form (appendix 3.3). A pictorial participant information sheet (appendix 3.2) explaining the study were given to participants from 5-17 years before they gave their assent (verbal). Their parent or guardian were asked to sign an informed consent form. The study was explained in the local language (Twi dialect) for participants who could not read and write.

3.9 Sampling and Data collection

The study fieldwork was conducted from February to December 2021. Participants (both genders) with lesions consistent with primary and secondary lesions of syphilis and yaws were actively selected from the recruitment sites (Hospitals and communities). Participant demographic data, including communities of residence, sex, ethnicity, and age were captured and all information recorded in the data capturing sheet (appendix 4) for each participant.

3.10 Serological Test (DPP® Syphilis Screen & Confirm Assay)

To determine the seroprevalence of treponematoses, the standard diagnostic method (both treponemal and non-treponemal serological test) for yaws and syphilis diagnosis were performed for participants who met the inclusion criteria of this study by finger pricking using the point of care test, i.e., Dual Path Platform (DPP) syphilis screen and confirm test (Chembio Diagnostic Systems Inc. Medford, New York). This test is capable of determine both treponemal and non-treponemal on a single platform.

Each participant with skin lesions consistent with the primary or secondary lesion of syphilis and yaws were screened using the DPP® Syphilis Screen & Confirm assay according to manufacturer's instructions (Chembio Diagnostic Systems Inc. Medford, NY) and as described previously (Marks *et al.*, 2014). The middle finger of each participant was disinfected with 70% ethanol. The finger was allowed to dry thoroughly before it was punctured with a sterile lancet. The first drop of blood was wiped off and squeezing of finger was also avoided to prevent blood being diluted with excess tissue fluid. 10 µL of the second drop of blood was collected with disposable microsafe tube (Chembio Diagnostic Systems Inc. Medford, NY). The blood was

transferred to the sample and buffer well-1 of the DPP test kits shown below (Figure 3.4). Two drops (about 50 µL) of running buffer were added to the sample and buffer well-1. After 5 minutes, 5 drops of the running buffer were added to the buffer well-2 (Figure 3.4). Test result was read after 15 minutes. A test was declared positive when both the treponemal (test line 1) and the non-treponemal (test line 2) were positive (Figure 3.4A).

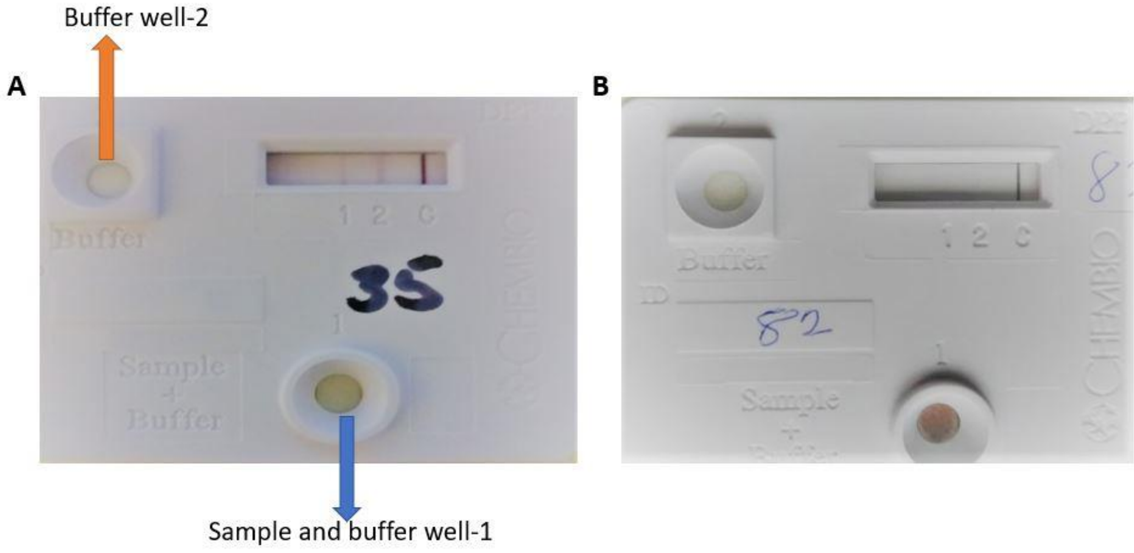


Figure 3.4: **DPP® Syphilis Screen & Confirm assay showing positive (A) and negative (B) test results of two participants in this study.**

3.11 Collection of Swab

Skin lesions of all the participants were collected by sterile dacron swabs (Thermo Fisher Scientific, UK) using a standard protocol (Rod James, 2018). Each lesion was sampled by pressing the head of the swab into the centre of the lesion and rotating the swab over the area. If a lesion or papilloma is dry, the area was first moistened using sterile saline (0.9% sodium chloride) and the outermost epithelial lining gradually removed using sterile forceps before swabbing. Lesions with the most exudate were identified first for swabbing. Only one lesion

was swabbed if patient had multiple lesions. Swabs were transported in containers without transport medium as described by Munson *et al.*, (2019) in a cool box and stored at 20 ^{o}C in phosphate buffer saline (PBS) at Kumasi Centre for Collaboration and Research (KCCR) in Kumasi, Ghana.

3.12 Extraction of Bacterial DNA

Bacterial DNA was extracted from lesion swabs using the QIAmp DNA mini kits (Qiagen, Germany) according to the manufacturer's instructions. Sample lysate was obtained by adding 200 µL lysis buffer (AL) (Qiagen, Germany) and 20 µL Proteinase K into a 1.5 mL microcentrifuge tube containing 200 µL of phosphate buffer saline (PBS) from the swab. This was vortexed immediately for 30 seconds, incubated at 56 ^{o}C for 10 minutes and centrifuge briefly at 8000 rpm in a microcentrifuge (Megafuge 8, ThermoFischer^{TM}) to remove drops from the inside lid. Next, 200 µL of 96% ethanol was added, mixed up by vortexing and centrifuged briefly at 8000 rpm in a microcentrifuge (Megafuge 8, ThermoFischer^{TM}) to remove drops from inside the lid. QIAamp Mini spin column (Qiagen, Germany) was placed in 2 mL microcentrifuge tube (collecting tube) and 620 µL of the resulting mixture was loaded into the QIAamp Mini spin column without wetting the rim and centrifuged at 8000 rpm for 1 minute. The flow-through in the 2 mL microcentrifuge tube (collecting tube) was discarded. The QIAamp Mini spin column was placed in a new 2 mL microcentrifuge tube (collecting tube) and 500 µL wash buffer (AW1) (Qiagen, Germany) was added to the QIAamp Mini spin column and centrifuged again at 8000 rpm for 1 minute. The 2 mL microcentrifuge tube with the flow-through was discarded. The QIAamp Mini spin column was placed in another new 2 mL microcentrifuge tube (collecting tube), and 500 µL of buffer (AW2) (Qiagen, Germany) was added to the QIAamp Mini spin column and centrifuged at 14000 rpm for 3 minutes. The collection tube and the flow-through was discarded. To eliminate the chance of carryover of buffer (AW2), the QIAamp Mini spin column was placed in a new 2 mL microcentrifuge tube and centrifuged for 1 minute. The 2 mL microcentrifuge tube was discarded. Finally, DNA was eluted by placing the QIAamp Mini spin column in a new 1.5 microcentrifuge tube, 200 µL elute buffer (AE) (Qiagen, Germany) was added and incubated at room temperature for one minute. This was centrifuged for 1 minute and the flow-through which contained the DNA extract was stored at -30 ^{o}C to -15 ^{o}C.

3.13 Multiplex PCR for the detection of *T. pallidum*, *Haemophilus ducreyi*, *Herpes simplex virus 1* (*HSV*-1) and *Herpes simplex virus 2* (*HSV*-2)

Identification of common pathogens causing yaws-like and syphilis-like lesions was achieved with multiplex PCR for laboratory diagnosis of *Treponema pallidum*, *Herpes simplex virus 1* (HSV-1), *Herpes simplex virus 2* (HSV-2) and *Heamophilus ducreyi* at the University Hospital Zurich, Switzerland at Dr Philipp Bosshard's laboratory. The multiplex PCR was performed as described by Glatz *et al.*, (2014) with the Roche LightCycler 2.0 (Roche Diagnosis) targeting the 16S ribosomal RNA gene (Glatz *et al*., 2014) for both *T. pallidum* and *H. ducreyi* , and glycoprotein B (*gB*) region of *HSV* 1 and 2 (Corey *et al.*, 1983). Briefly, the assay was split into two reactions (i.e., tube 1 for *T. pallidum* and the internal control (inhibition control) and tube 2 for *H. ducreyi*, *HVS-1*, *HSV-2*, and inhibition control). The purpose of the inhibition control was to make sure the PCR reaction was not inhibited. When sample reaction was negative and the PCR reaction was not inhibited, the inhibition control (which can bind to the primers) was amplified (positive) with cycle threshold (Ct) values between 32-35. However, when sample is positive the inhibition control will be negative since the primers will be used for the amplification of the positive sample. Microbial community standard was used as positive control and DNA free water was used as negative control.

The 20 µL reaction mixture consisted of 4 µL 5X LightCycler TagMan mastermix, 0.25 µL LightCycler Uracil-DNA Glycosylase, 0.5 µM of each primer, 0.1 µM probe, 1 µL internal control and 5 µL of extracted DNA. Reaction conditions were activation of uracil-N- glycosylase at 40 ^{o}C for 10 minutes, pre-denaturation at 95 °C for 10 minutes, 45 cycles of denaturation at 95 ^{o}C for 10 seconds and annealing and amplification at 66 ^{o}C for 1 minute. Cycle threshold (Ct) value less than 40 cycles was taken as positive. Below are the primers and probes used for the assay.

Table 3.1 Primer and Probes that were used in the multiplex PCR assay for detection of *T. pallidum*, *Haemophilus ducreyi*, Herpes simplex virus *1* (HSV-1) and Herpes simplex virus *2* (HSV-2)

Organism/primers and probes	Sequence
T. pallidum/	
TP-Zh-131-f	GCCTTTGAGATGGGGATAGC
TP-Zh-245-r	GTCGCCAGGCTCATCTCTGA
TP-Zh-220-p	FAM-CCGCAGCCCCTTTCCTCTCA-BHQ-1
Internal Control/	
IC-Zh-p	Yakima Yellow-TCGTGCCTCAGTGCCAGTCAC-BHQ-1
Haemophilus ducreyi/	
HD-Zh-992-f	ACATCCATAGAAGAACTCAGAGATGA
HD-Zh-1150-r	TTGAGTTCCCATCAYTACATGCT
HD-Zh-1022-p	Yakima Yellow-GTGCCTTCGGGAACTATGTGACAGGT-BHQ-1
Herpes Simplex 1 and 2	
GbTypF	CGCATCAAGACCACCTCCTC
GbTypR	GCTCGCACCACGCGA
GbTyp1	FAM-TGGCAACGCGGCCCAAC-BHQ-1
GbTyp2	ROX-CGGCGATGCGCCCCAG-BHQ-2

Probes, primers and inhibition control were adapted from Glatz *et al*., (2014).

3.14 16S ribosomal RNA gene library preparation for illumina MiSeq sequencing.

Other organisms (mainly bacteria) that may be present in these yaws like and syphilis like lesions were identified with 16S rRNA gene sequencing, performed at the University of North Carolina, Chapel hill, USA at Dr Jonathan Parr's laboratory. The variable regions (V1-V2 region) of the 16 ribosomal RNA gene (made up of about 300 base pairs) (Figure 3.5) were amplified using the quick 16S Next Generation library preparation kits (Zymo research, USA) (Figure 3.6) according to manufacturer's instructions. The Quick-16S^{TM} Primer set V1-V2 (Zymo research,

USA) was used because it provides excellent coverage for human associate microbes in comparison to other commonly used primer set (Zymo, 2022).

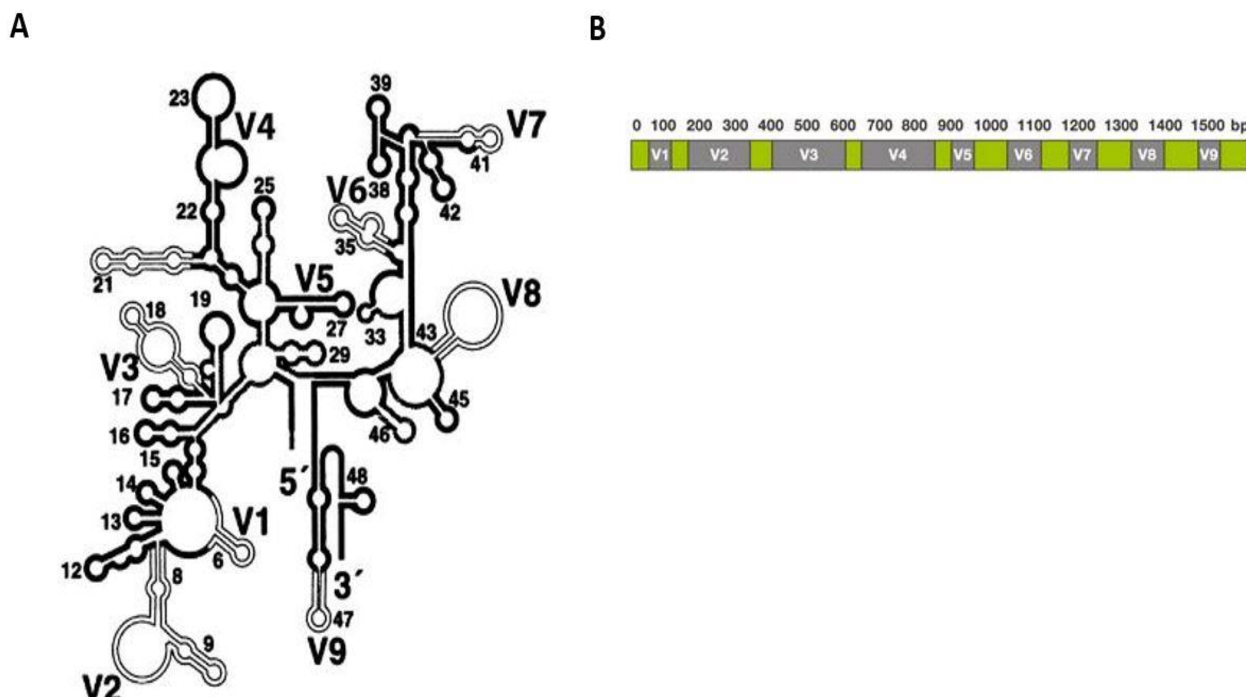


Figure 3.5. A) Secondary structure of the 16S ribosomal RNA showing the 9 variable regions. B) Basic structure of the 16S ribosomal RNA showing the 9 variable regions in grey and the conserved regions in light green (Van de Peer, Chapelle and De Wachter, 1996). The combination of both the conserved and the variable regions are made up of a total of 1500 base pairs (bp). The V1-V2 regions used in this study are made up of a total of 300 bp.

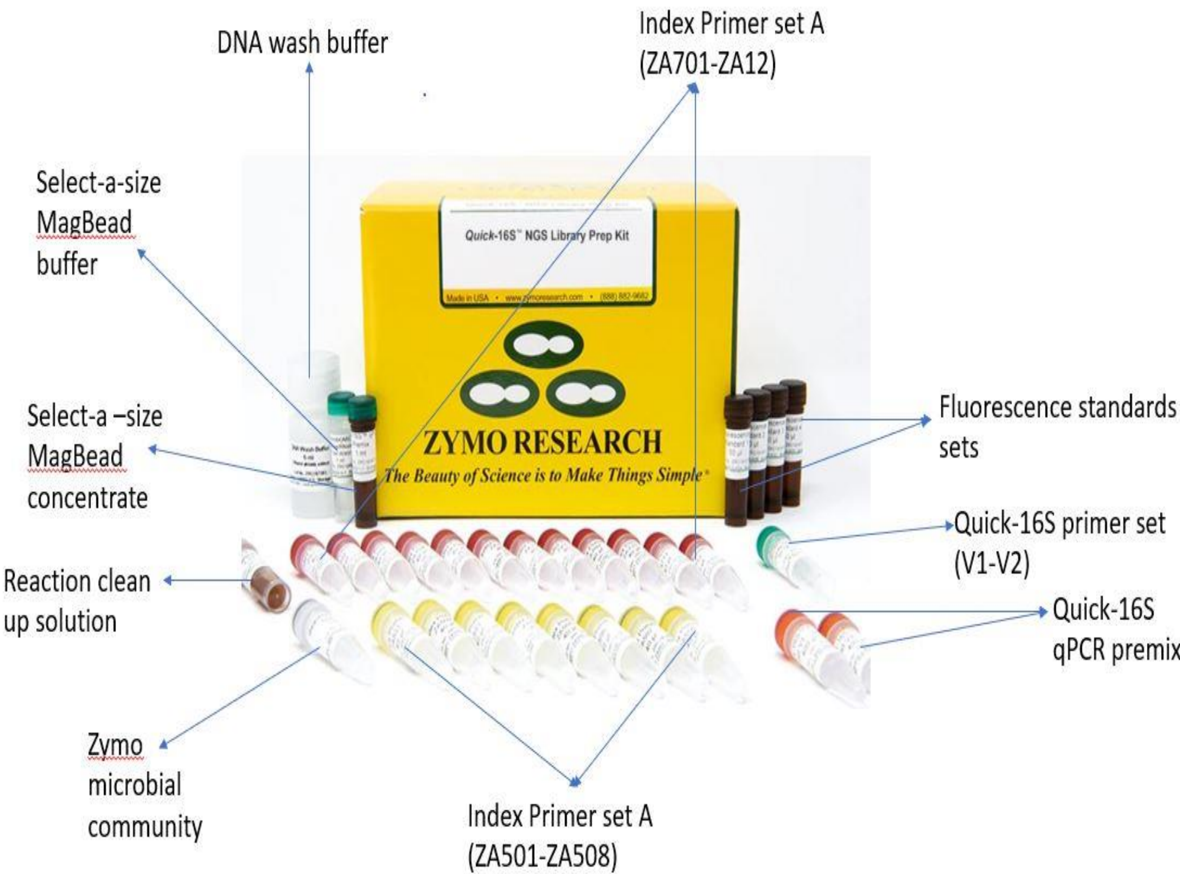


Figure 3.6. The 16S Next Generation library preparation kits (Zymo research, USA) used in this study. The reagents include Quick-16S^{TM} qPCR premix, Quick-16S^{TM} primer set V1-V2, ZymoBIOMICS DNase/RNase free water, ZymoBIOMICS Microbial community, reaction clean up solution, Index primer set, fluorescence standards set, select-a-size MagBead buffer and DNA wash buffer.

The library preparations involved five main setups, namely: i. targeted sequence amplification ii. enzymatic clean up iii. barcode addition iv. Library quantification and pooling and v. final library clean-up (Figure 3.7).

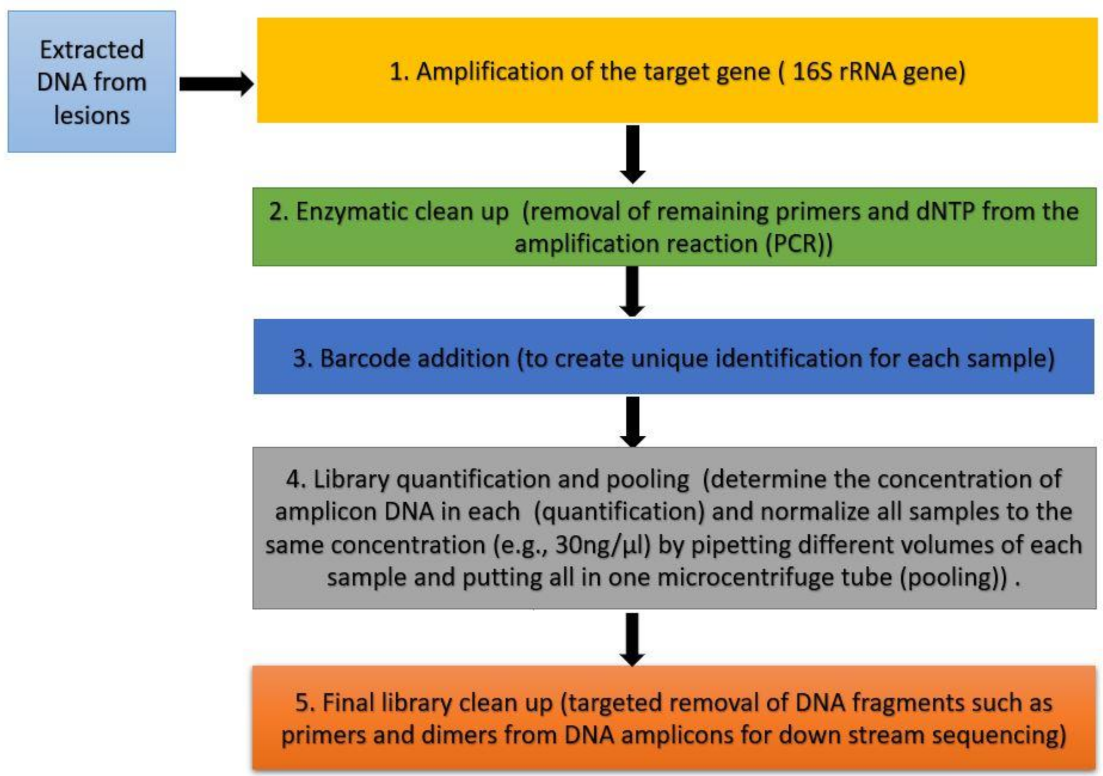


Figure 3.7. Summary of steps involved in the library preparation for the 16S rRNA gene sequencing.

3.14.1 Targeted sequence amplification

The targeted sequence amplification was performed by using 20 µL reaction mixture comprising of 10 µL of Quick-16S qPCR Premix, 4 µL of Quick-16S Primer set V1-V2, 4 µL of ZymoBIOMICS DNase/RNase free water and 2 µL of DNA. The ZymoBIOMICS microbial community standard was used as positive control and DNA free water was used as negative control. The following PCR conditions were used in the BioRad CFX96 real time PCR: initial denaturation at 95 ^{o}C for 10 minutes; 20 cycles: 95 ^{o}C for 30 seconds, 55 ^{o}C for 30 seconds, 72 ^{o}C for 3 minutes and plate read. This was followed by 4 ^{o}C holding temperature. The fluorescence (from SYBR green fluorophore) from each sample was read after the 20 cycles. Any sample with fluorescence reading above the fluorescent threshold (1200) (Figure 3.8) of the BioRad CFX96 real time PCR

machine was removed and placed in the same position as it was in the target plate in a new PCR plate labelled collection plate.

The remaining samples that did not pass the fluorescent threshold (1200) were put back into the BioRad CFX96 real time PCR for further 5 cycles using the same conditions. After the 5 cycles those samples that passed the fluorescent threshold (1200) were also removed and place in the same position in collection plate as it was in the target PCR plate. This 5-cycle program was repeated for samples that did not pass the threshold until 40 cycles were reached (Figure 3.8).

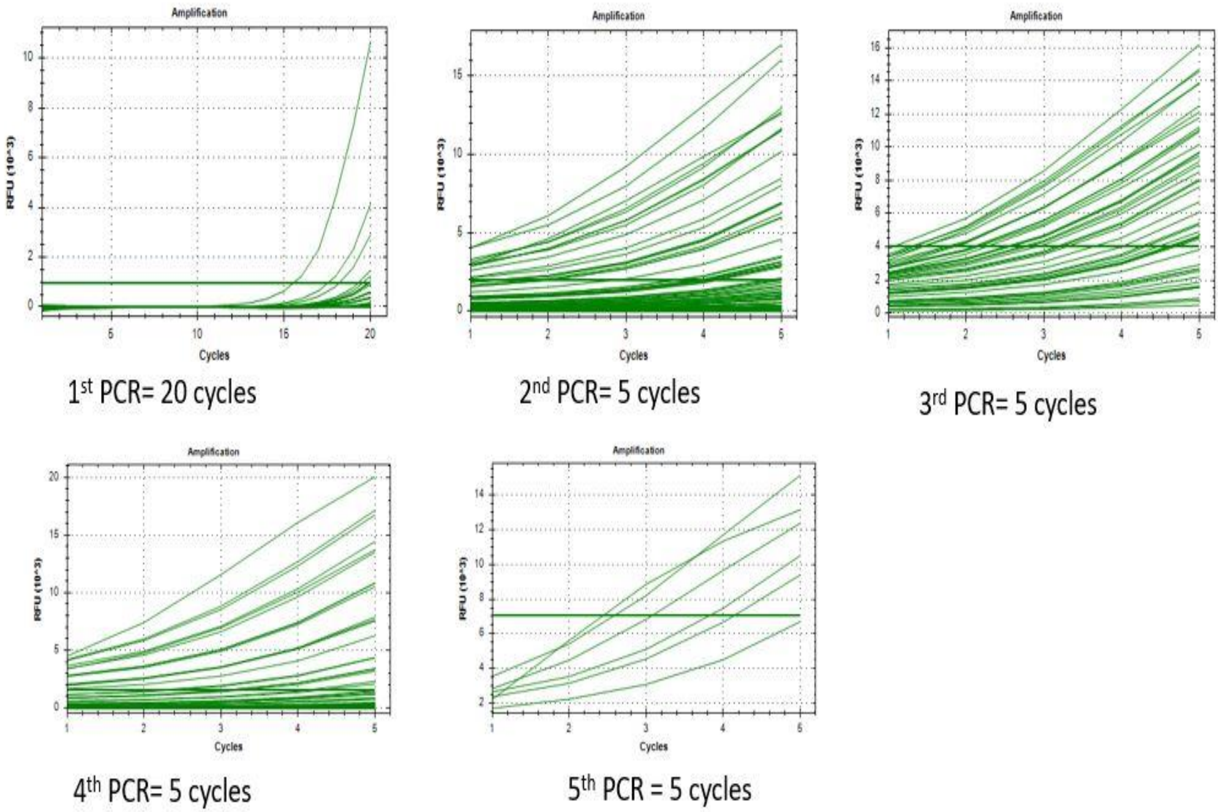


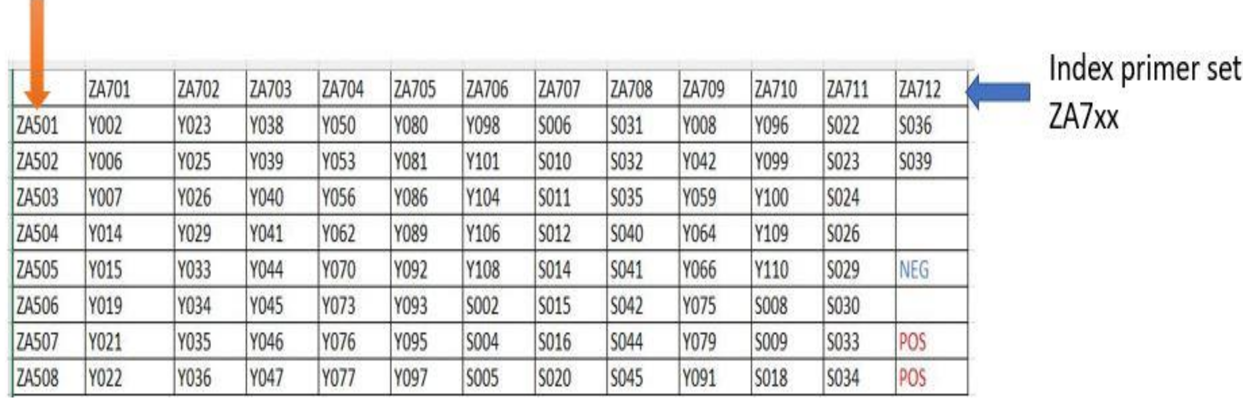
Figure 3.8. The target sequence amplification of the first 90 samples showing the amplifications of samples in first 20 cycles and each subsequent 5 cycles until the total 40 cycles (at the 5th PCR) were reached.

3.14.2 Enzymatic clean up

The enzymatic cleanup was done by adding 1 µL of reaction clean up solution into each well in the collection plate (containing the amplicons from the targeted sequence amplification step) and the following PCR program were used to run the enzymatic clean up set up in the same real time PCR machine (BioRad CFX96): 37°C for 15mins, 95°C for 10mins and 4°C holding time. After the enzymatic clean-up program, the collection plate was placed on ice for barcode addition.

3.14.3 Barcode addition

The purpose of the barcoding is to give unique identity to each sample before pooling all samples together for the sequencing. The barcode addition was done with new PCR plate labelled barcoded plate (Figure 3.9). This was achieved by using 20 µL reaction mixture comprising of 10 µL of Quick-16S qPCR Premix, 4 µL of ZymoBIOMICS DNase/RNase free water, 2 µL of index primer set ZA7xx (ZA701-ZA712) or ZB7xx (ZB701-ZB712), 2 µL of index primer set ZA5xx (ZA501-ZA508) and 2 µL of DNA sample from the collection plate at the end of enzymatic clean program. The following PCR program were used to run the barcode setup in the same real time PCR machine (BioRad CFX96): initial denaturation at 95°C for 10min; 5cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 3 minutes, plate read and 4°C holding temperature. While the plate was still in the PCR machine, additional program of 72°C for 2mins, plate read and 4°C holding was performed. The fluorescence recordings of each sample well were recorded for library quantification.



	ZA701	ZA702	ZA703	ZA704	ZA705	ZA706	ZA707	ZA708	ZA709	ZA710	ZA711	ZA712
ZA501	Y002	Y023	Y038	Y050	Y080	Y098	S006	S031	Y008	Y096	S022	S036
ZA502	Y006	Y025	Y039	Y053	Y081	Y101	S010	S032	Y042	Y099	S023	S039
ZA503	Y007	Y026	Y040	Y056	Y086	Y104	S011	S035	Y059	Y100	S024	
ZA504	Y014	Y029	Y041	Y062	Y089	Y106	S012	S040	Y064	Y109	S026	
ZA505	Y015	Y033	Y044	Y070	Y092	Y108	S014	S041	Y066	Y110	S029	NEG
ZA506	Y019	Y034	Y045	Y073	Y093	S002	S015	S042	Y075	S008	S030	
ZA507	Y021	Y035	Y046	Y076	Y095	S004	S016	S044	Y079	S009	S033	POS
ZA508	Y022	Y036	Y047	Y077	Y097	S005	S020	S045	Y091	S018	S034	POS

Figure 3.9. **PCR plate set up showing how the index primer sets were added**. Each index primer set ZA5xx was added to each well in the row it was positioned (e.g., ZA501 was added to each well from Y002-S036). Each Index primer set ZA7xx was also added to each well in the column it is positioned (e.g., ZA701 was added to each well from Y002-Y022)

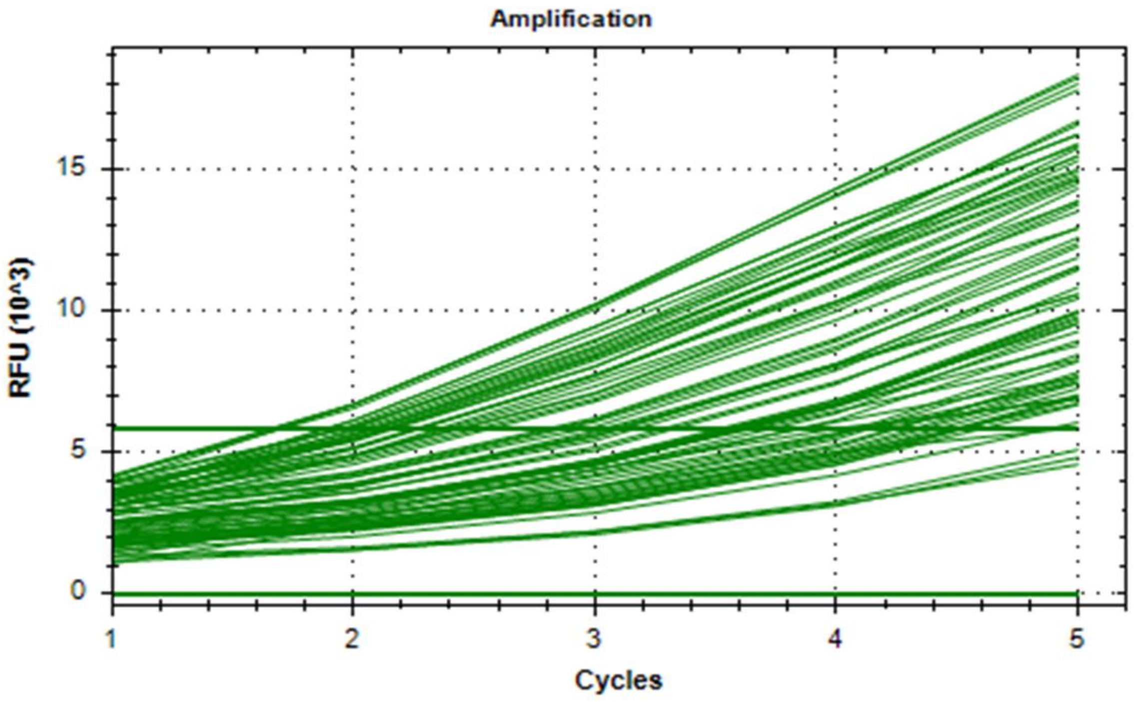


Figure 3.10. **Amplification of 90 samples after addition of barcodes in five cycle PCR reaction.**

3.14.4 Library quantification and pooling

Library quantification and pooling was performed by first recording the fluorescence from the four-fluorescence standard (Zymo research, USA) after running the following program on 20 µL triplicate of each fluorescence standard: 72 °C for 2 mins, plate read and 1 min holding time on same PCR machine. Using the fluorescence reading and the known concentrations (0 ng/ µL, 8ng/ µL, 16ng/ µL and 24ng/ µL) of these standards provided by the manufacturer in addition to the fluorescence readings of each sample obtained from the previous setup (barcoding step), the concentration and volume of each sample needed for the library quantification and pooling were automatically calculated using template spreadsheets provided by the manufacturer (Zymo research USA) (Figure 3.11 to 3.15). First, the fluorescence readings of the four standards were recorded on a spreadsheet provided by the manufacture (Zymo research, USA) (Figure 3.11). The average of the standard fluorescence readings and their standard concentrations were used to create a standard curve equation automatically on a separate spreadsheet (Figure 3.12). Afterwards the fluorescence readings of each sample obtained from the previous setup (barcoding step) were recorded on another spreadsheet (Figure 3.13A) and their concentrations were automatically calculated using the standard curve equation on a separate spreadsheet (Figure 3.13B). The concentration (30ng/µL) required for normalization was entered on another spreadsheet (Figure 3.13C) and the volume of each sample for normalization was also automatically calculated on another spreadsheet (Figure 3.14A). After this, the final volume of the pooled library as well as the volume of the select-a-size MagBead buffer needed for the final library clean up were automatically calculated on a different spreadsheet (Figure 3.14B).

Enter the fluorescence reading (F) values from the "Fluorescence Standards Plate Read" program in the appropriate cells below.				
Standard #	1	2	3	4
F (Replicate 1)	2355.66	7103.76	7579.89	14345.67
F (Replicate 2)	2194.21	6998.19	7688.79	14329.21
F (Replicate 3)	2199.75	7063.37	7542.62	14301.72

Figure 3.11 Spreadsheet used to record the fluorescence standard (Zymo Research, USA). Each standard fluorescence was prepared and read in triplicate and recorded.

The average fluorescence reading values will be calculated below and then are used to created a standard curve equation, represented by Values A, B, and C. The R-sqaured value is also calculated and shown.					
Average		2250	7055	7604	14326
Concentration (ng/µl)		0	8	16	24
Value A:	-5.73E-08	Value B:	2.96E-03	Value C:	-6.59301
R-squared:			0.927		

Array 1 (Used only for R-squared calculation)	
2250	0
7055	8
7604	16
14326	24

Figure 3.12. Spreadsheet used to automatically generate the standard curve equation (Zymo Research, USA). The average of each fluorescence standard was automatically calculated by this spreadsheet, and they were used to create a standard curve equatio**n.** The standard equation was used to calculate the unknown DNA concentration for each sample using the fluorescence reading of the sample. The R-squared value indicate how well the standard equation can predict the unknown DNA concentration of the samples. The R-squared value ranges from 0-1 or 0-100%. The higher the value the better. Here 0.927 was obtain.

A

Fluorescence Reading (F)

Enter the fluorescence reading (F) values from the "Plate Read" program below.												
	1	2	3	4	5	6	7	8	9	10	11	12
A	7118.54969	11564.83652	8247.505865	15791.82322	10858.45229	10029.12305	10259.93982	12785.88489	16493.38266	16022.29129	10462.96077	11067.01662
B	17536.30736	11136.06727	8532.127789	13804.74618	11033.83972	16749.77095	12848.08707	18277.14397	15573.97267	23793.11024	27756.23509	14341.94762
C	20476.93657	12208.22938	12931.86516	21968.7588	8680.556543	21171.57393	18655.01795	9112.345911	13871.62291	14276.02008	10962.36211	18152.80915
D	14850.61686	6769.288321	13619.58922	10285.95128	18951.02288	20373.5911	21549.39913	11188.57014	9118.589053	12739.40907	27532.71122	11338.75801
E	9067.789386	11011.48439	22047.19217	2904.493609	16806.95813	18666.88578	10983.47512	11179.01033	15649.26009	18888.8108	28253.02894	
F	6580.126967	17444.51756	24220.90404	21732.7173	8787.215749	11230.7533	2400.770806	12081.97842	6797.861914	16195.99004	10819.35912	
G		9957.64102	12638.60722	18220.82498	18096.62156	19245.39874	12924.81236	13159.26479	12233.01271	24826.16433	14514.25825	
H		8861.436954	16158.36102	15781.95418	8294.388936	8858.860683	8539.46153	10773.15634	11168.07215	17237.43627	19006.4803	12631.94709

B

DNA Concentration (ng/µl)

The DNA concentration of each sample will be calculated below in ng/µl.												
	1	2	3	4	5	6	7	8	9	10	11	12
A	11.60	20.02	13.96	25.93	18.84	17.37	17.79	21.94	26.71	26.19	18.15	19.19
B	27.77	19.31	14.53	23.41	19.14	26.98	22.03	28.44	25.67	31.50	31.54	24.13
C	30.08	21.06	22.16	30.87	14.82	30.48	28.77	15.66	23.50	24.05	19.02	28.34
D	24.79	10.85	23.15	17.83	29.00	30.02	30.68	19.40	15.67	21.87	31.59	19.65
E	15.57	19.10	30.91	1.53	27.04	28.77	19.05	19.38	25.76	28.96	31.42	No Sample
F	10.43	27.68	31.59	30.77	15.03	19.47	0.19	20.86	10.91	26.39	18.77	No Sample
G	No Sample	17.24	21.72	28.40	28.29	29.23	22.15	22.49	21.09	31.68	24.36	No Sample
H	No Sample	15.17	26.34	25.92	14.05	15.17	14.54	18.69	19.37	27.48	29.05	21.71

C

Enter the amount of product you would like to use (in ng) for normalization below:	
Desired amount of product (ng):	30

Figure 3.13 The spreadsheets used to record the fluorescence readings of the samples (A), calculated the concentrations of the samples(B) and used to enter the desired concentration of DNA for normalization (C) (Zymo Research, USA). The fluorescence readings of the first 90 samples were entered onto the spreadsheet (A) and the concentrations were automatically calculated on the spreadsheet (B). The concentration of DNA (30 ng/µl) for normalisation was also entered on spreadsheet (c).

A

The volume that should be used for normalization will be calculated below in µl.

Volume to Normalize (µl)

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.6	1.5	2.1	1.2	1.6	1.7	1.7	1.4	1.1	1.1	1.7	1.6
B	1.1	1.6	2.1	1.3	1.6	1.1	1.4	1.1	1.2	1.0	1.0	1.2
C	1.0	1.4	1.4	1.0	2.0	1.0	1.0	1.9	1.3	1.2	1.6	1.1
D	1.2	2.8	1.3	1.7	1.0	1.0	1.0	1.5	1.9	1.4	0.9	1.5
E	1.9	1.6	1.0	19.6	1.1	1.0	1.6	1.5	1.2	1.0	1.0	No Sample
F	2.9	1.1	0.9	1.0	2.0	1.5	20.0	1.4	2.7	1.1	1.6	No Sample
G	No Sample	1.7	1.4	1.1	1.1	1.0	1.4	1.3	1.4	0.9	1.2	No Sample
H	No Sample	2.0	1.1	1.2	2.1	2.0	2.1	1.6	1.5	1.1	1.0	1.4

Troubleshooting: Samples highlighted in red do not have enough DNA to meet the desired amount of product. For samples highlighted in red, pool the entire volume (20 µl). Alternatively, decrease the value in Table 5 (cell R28) until there are no remaining highlighted samples.

B

Obtain Select-a-Size MagBead Buffer Volume Here

Do not edit.	Volume of Pooled Library (µl)	166.26
	Volume of Select-a-Size MagBead Buffer (µl)	133.01

Figure 3.14 The spreadsheets used to automatically calculate the required volume of each sample for normalization (A) and the total volume of pool library as well as the volume of Select-a-Size MagBead buffer required for final library clean-up (B). These volumes were automatically generated after entering the normalization volume (30ng/µl) in Figure 3.13C. The concentration of the volume highlighted in red was very low so the total volume (20 µl) in the well was used for the normalization.

3.14.5 Final library clean up

The final library clean-up was performed by adding 265.34 µL (obtained by picking the volume of each of the 156 samlpes required for normalization together) of library pool to 212.28 µL (0.8 × library pool volume) of Select-a-Size MagBead buffer (mixture of 30 µL of Select-a-Size MagBead Concentrate and 1 mL of Select-a-Size MagBead concentrate). This was mixed thoroughly by vortexing and pipetting until it was uniform. The mixture was incubated at 25°C for 5mins. The resulting homogeneous mixture was placed in a magnetic rack and incubated at room temperature until all the beads had separated from the solution. The supernatant was removed and discarded. 200 µl of DNA wash buffer was added whiles the tube containing the

beads and pool library were still on the magnetic rack. The supernatant was removed and discarded. All residual buffer was aspirated with the 10µl pipette. The tube containing the pooled library and the beads was then removed from the magnetic rack and incubated for 3mins at room temperature to remove traces of buffer. 20µl of ZymoBIOMICS DNase/RNase free water was added to the beads and mixed thoroughly by pipetting. This was placed back into the magnetic rack and incubated at room temperature until all the magnetic beads were separated completely from the elute. The supernatant was then transferred into a clean tube as the final library.

3.14.6 Quality check for the final library

The final DNA amplicon size after addition of barcodes is approximately 486 base pairs according to the manufacturer's library preparation instructions (Zymo research, USA). The tape station was used to measure if the correct expected library length of the DNA amplicons were obtained for sequencing (Figure 3.15). The library was then diluted to a final concentration of 20 µL and was sequenced using the illumina MiSeq 2 x 250 paired end reads sequencing pipeline.

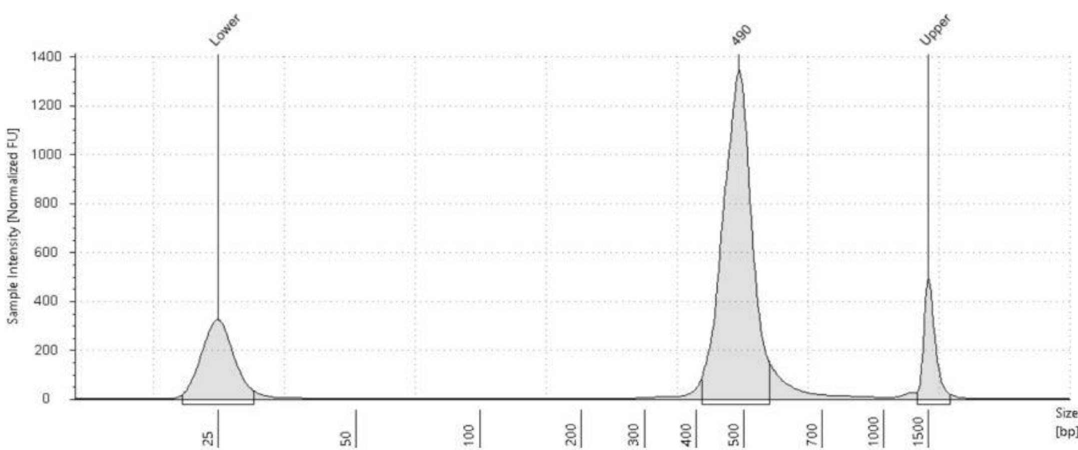


Figure 3.15. The final average amplicons size (490) in this study (156 samples) after addition of barcodes. The expected amplicon size after addition of barcode according to the manufacturer of the library preparation kits (Zymo Research, USA) is 486.

3.15 Processing of sequence output

Sequencing output from the Illumina MiSeq platform were converted to fastq format and demultiplexed using Illumina Bcl2Fastq 2.20.0. The resulting paired-end reads were processed by the R statistical software (version 4.2.2) with dada2 package (version 1.26.0) with various functions (Figure 3.16) as follows:

1. The dada2 package was first loaded in R with the function library (dada2).
2. The fastq files (both the forward and reverse reads) were imported onto the R environment.
3. The profiles of both the forward and reverse reads were inspected for their quality with the function plotQualityProfile().
4. After inspection of the quality of the reads, they were filtered and trimmed with the function filterAndTrim (). The forward and the reverse reads were truncated at position 220 and 160 respectively because the quality of reads dropped significantly at these positions (Figure 3.17). The maximum error threshold allowed was 2 for both the forward and reverse reads.
5. The error rate of the amplicons were estimated by the function learnErrors() and this was visualised by the function plotErrors() to see if the observed error rate was a good fit of the estimated error rate.

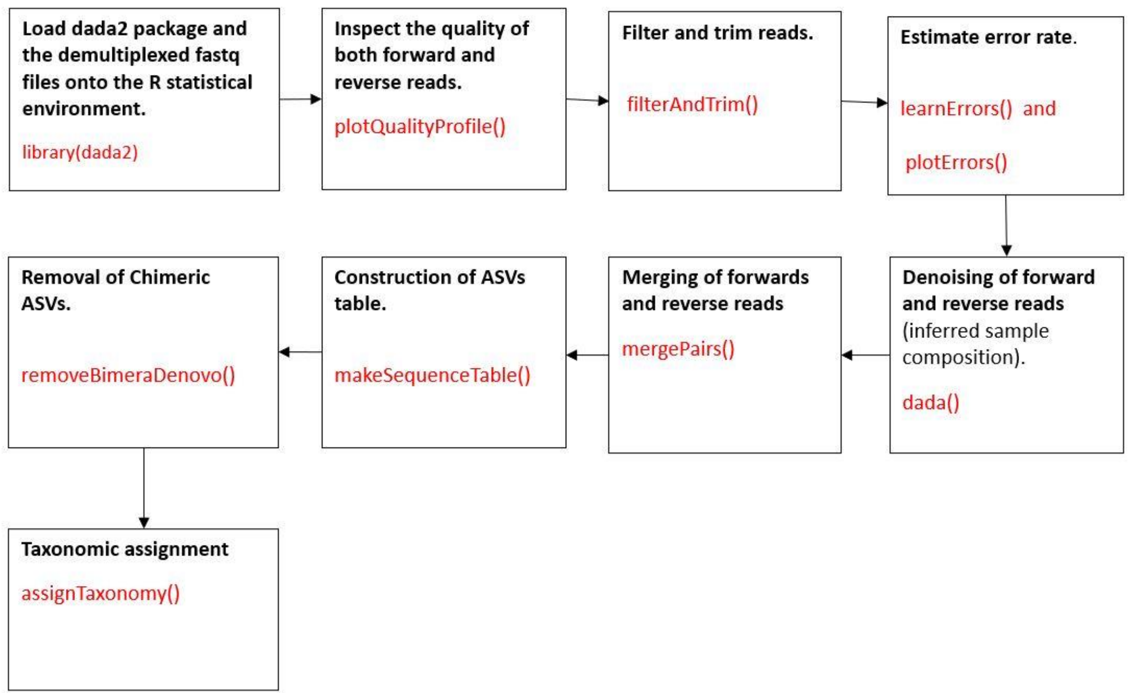


Figure 3.16. Summary of steps involved in the process of the sequence output. The R functions in the dada2 package used at each step are in red text. ASVs= Amplicon sequence variants.

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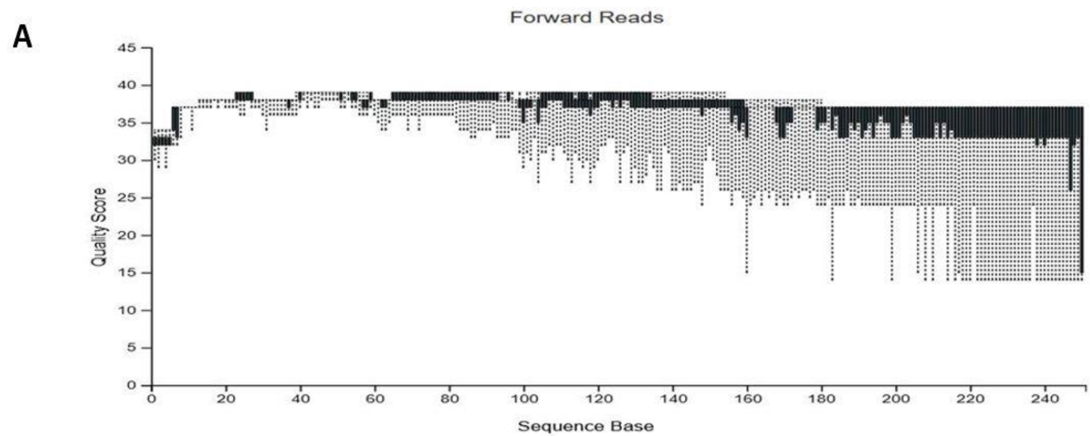
6. The sequence variants in both the forward and reverse reads in each sample were estimated (inferred sample composition) with the function dada(). This step is also known as denoising. At this step, sequences that differ by a single nucleotide are assigned as different amplicon sequence variant (ASV).

7. The denoised forward and reverse reads were merged to obtain the full denoised sequences with the function mergePairs(). Merging was performed by aligning the denoised forward reads with the corresponding denoised reverse read. By default, merged sequences were only output if the forward and reverse reads overlap by at least 12 bases (Quast *et al.*, 2013; Yilmaz *et al.*, 2014).

8.The amplicon sequence variant (ASV) table was constructed with function makeSequenceTable(). The sequence table was a matrix with rows corresponding to sample names and columns corresponding to the ASVs.

9. Chimeric ASVs were then removed with the function removeBimeraDenovo().
10. Taxonomic assignment to resulting ASVs table was performed with respect to the Silva reference database (version 138.1) (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) with the function assignTaxonomy().



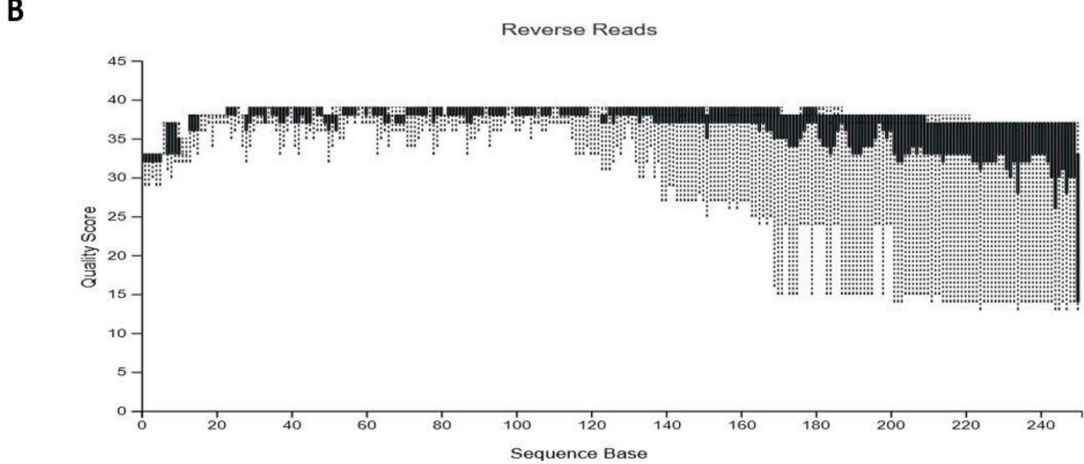


Figure 3.17 Average quality scores for A) forward and B) Reverse reads. The forward and the reverse reads were truncated at position 220 and 160 respectively because the quality of reads significantly dropped (below 20) continuously at these positions.

3.16 Statistical analysis

3.16.1 Statistical analysis for participants' demographics, DPP and multiplex PCR results

The Fisher's exact test (when lowest sample size was below 5) or Chi-squared tests (when lowest sample size was 5 or above) were used to compare categorical characteristics between groups. The Bonferroni method was used for pairwise comparison between groups. Statistical analyses were performed with R (version 4.2.2) on R Studio version 2022.07.1 Build 554, which were also used to produce Figures with the ggplot2 package (version 3.4.0).

3.16.2 Statistical analysis for 16S rRNA gene sequence data.

The ASV table and the taxonomic table generated by the dada2 as well as the metadata were used as input for subsequent analysis in R (version 4.2.2) using the Phyloseq (version 1.42.0), vegan (2.6.4) and DESeq2 (version 1.38.2) packages. The relative abundances of taxa were estimated and plotted with the transform_sample_counts and plot_bar function available in the phyloseq package. Both intra-sample (alpha diversity) and inter-sample (beta diversity) were computed. The alpha diversity was estimated by the Shannon and Simpson metrics because both metrics consider richness (number of different taxa observed in a sample) and evenness (abundance of taxa) in their computation as compared to other metrics that use only richness. Pairwise comparison of alpha diversity for significant difference between groups (e.g., yaws-like and syphilis-like lesions) was estimated by Wilcoxon rank test with the function pairwise.wilcox.test() in the Phyloseq package.

Beta-diversity were visualised using non metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) on Jaccard and and Bray-Curtis dissimilarity measures. The Jaccard and Bray-Curtis distances were used for the beta diversity analysis because micriobial data contain null (absent of taxa) values and these methods can tolerate missing pairwise distances well, while other distances cannot (Taguchi and Oono, 2005; Kim *et al.*, 2018). Additionally, NMDS makes few assumptions about the nature of the data, and both NMDS and PCoA allow the use of any distance measure which are two advantages over other ordination methods (Taguchi and Oono, 2005; Kim *et al.*, 2018). For both distance measures (Jaccard and Bray-Curtis), the data were transformed to proportions by the function transform_samples_counts

before the ordination function (ordinate()) was applied to estimate the stress value (i.e how well the ordination summarizes the observed distance among the samples in reduced dimensions (2D or 3D dimensions)) (Dexter, Rollwagen-Bollens and Bollens, 2018) for each distance measure.

A permutational multivariate analysis of variance (PERMANOVA) and test of homogeneity of dispersion among lesions were performed with the adonis and betadisper functions available in vegan package. Significant differential abundance testing was also performed with the function phyloseq_to_deseq2() available in the DESeq2 package. Figures were generated with the help of R package ggplot2 (version 3.4.0).

Chapter 4

4 Results and analysis

4.1 Distribution of participants by age, sex, and study sites

The fieldwork of this study was conducted between February and December 2021. A total of 156 individuals with skin lesion (children) or genital lesions (adults) consistent with primary and secondary yaws and syphilis were recruited from eight study sites from one metropolitan area and three districts in the Ashanti region of Ghana. A total of 110 individuals with yaws-like lesions were recruited from six rural communities (Adweratia, Mpobi, Bebu, Brofoyedu, Kotei and Atia) and 46 individuals with syphilis-like lesions were recruited from two study sites, Komfo Anokye teaching hospital (KATH) and South Suntreso hospital (SSH) in an urban area (Figure 4.1).

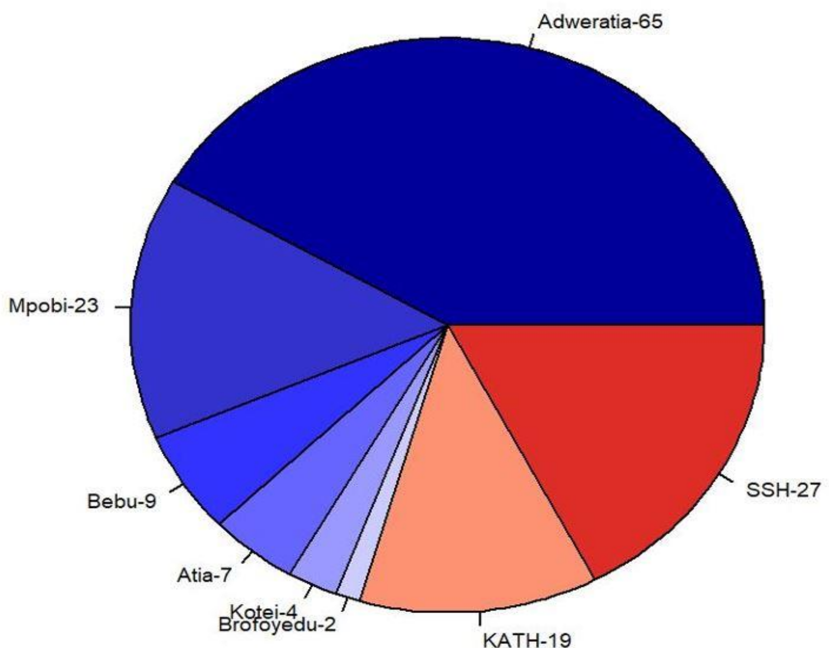


Figure 4.1 Number of participants and associated skin-lesions sampled from the different study sites. The sites in blue shades show where the yaws-like lesions were sampled from and the sites in red shades show where the syphilis-like lesions were sampled from.

The median age of individuals with yaws-like lesions was 8 years with a minimum and maximum age of 5 and 17 years-old respectively (Figure 4.2). There was no significant difference between the number of male (65) and female (45) participants for yaws-like lesions (Chi square = 3.6364, p-value= 0.06). Participants recruited for syphilis-like lesions had a median age of 29 years, and a minimum and maximum age of 19 and 75 years respectively (Figure 4.2). There was no significant difference between the number of male (17) and female (29) participants for syphilis-like lesions (Chi square = 3.1304, p-value= 0.08).

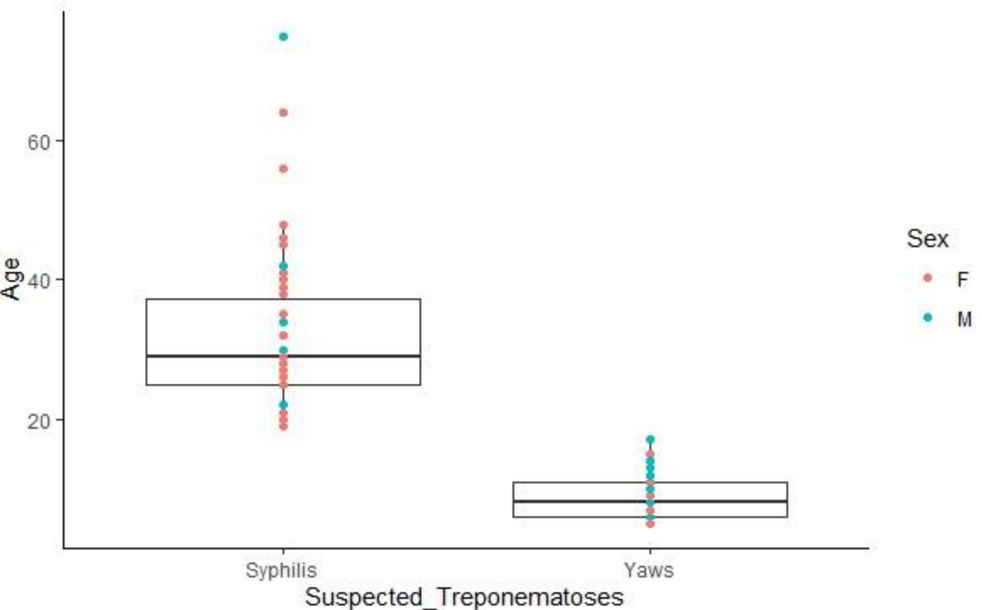


Figure 4.2 Age and sex distributions of participants with yaws-like and syphilis-like lesions. The median age of yaws-like and syphilis-like lesions were 8 and 29 years respectively. There was no significant difference between the number of male (65) and female (45) participants with yaws-like lesions (Chi square = 3.6364, p-value= 0.06). Likewise, there was no significant difference between the number of male (17) and female (29) participants with syphilis-like lesions (Chi square = 3.1304, p-value= 0.08). F= female, M=male

Yaws-like lesions were significantly associated with study sites (Figure 4.1) (Chi-square=73.726, p-value= 1.716×10^{-14}) however, this was not the case for syphilis-like lesions (Chi-square= 1.1066, p-value= 0.2928). Pairwise comparison showed that yaws-like lesions were significantly higher in Adweretia compared to the other study sites where yaws-lesions had been sampled from (p-value adjusted for all comparison were ≤ 0.00011, method: Bonferroni). Additionally, pairwise comparison showed that yaws-like lesions were significantly more common in Atia (23) than in Brofoyedu (2) (p-value adjusted = 0.0004, method: Bonferroni) and Kotei (4) (p-value adjusted = 0.003, method: Bonferroni). For syphilis-like lesions, more participants (27) were sampled in the South Suntreso Hospital (SSH) than in Komfo Anokye Teaching Hospital (KAHT) (19), but this difference was not significant (Chi-square= 1.1066, p-value= 0.2928).

4.2 Distribution of lesions on body parts

Lesions were found on different parts of the body (Table 4.1). There were 75 participants (68%) with yaws-like lesions on the legs only or legs in combination with either one or two other parts of the body such as the arm, belly, and skull (Table 4.1). There were 41 participants (37.3%) who had lesions on arms only or in combination with other parts of the body such as belly, buttocks, and skull (Table 4.1). Most of the syphilis-like lesions (42 [91.3%]) were found in the genital area (Table 1).

Table 4.1 Distribution of skin lesions associated to yaws and syphilis on body parts sampled

Location of lesions	Yaws-like lesions (n=110)	Syphilis-like lesions (n=46)
Genital	0	42
Arms only	10	0
Arms and legs	28	0
Arms and belly	0	1

Location of lesions	Yaws-like lesions (n=110)	Syphilis-like lesions (n=46)
Arms, legs and belly	2	0
Arms, legs and skull	1	0
Arms, Buttocks and Belly	0	2
Belly only	3	0
Belly and chest	1	0
Buttocks only	4	0
Chin	2	0
Legs	44	0
Oral	7	1
Skull only	7	0
Ear	1	0

4.3 Distribution of Participants by stage of lesions and ethnicity

The number of lesions that were consistent with primary and secondary yaws lesions were 79 (71.8%) and 31 (28.2%) respectively, and those consistent with primary and secondary syphilis were 41 (89.1%) and five (10.9%) respectively (Figure 4.3).

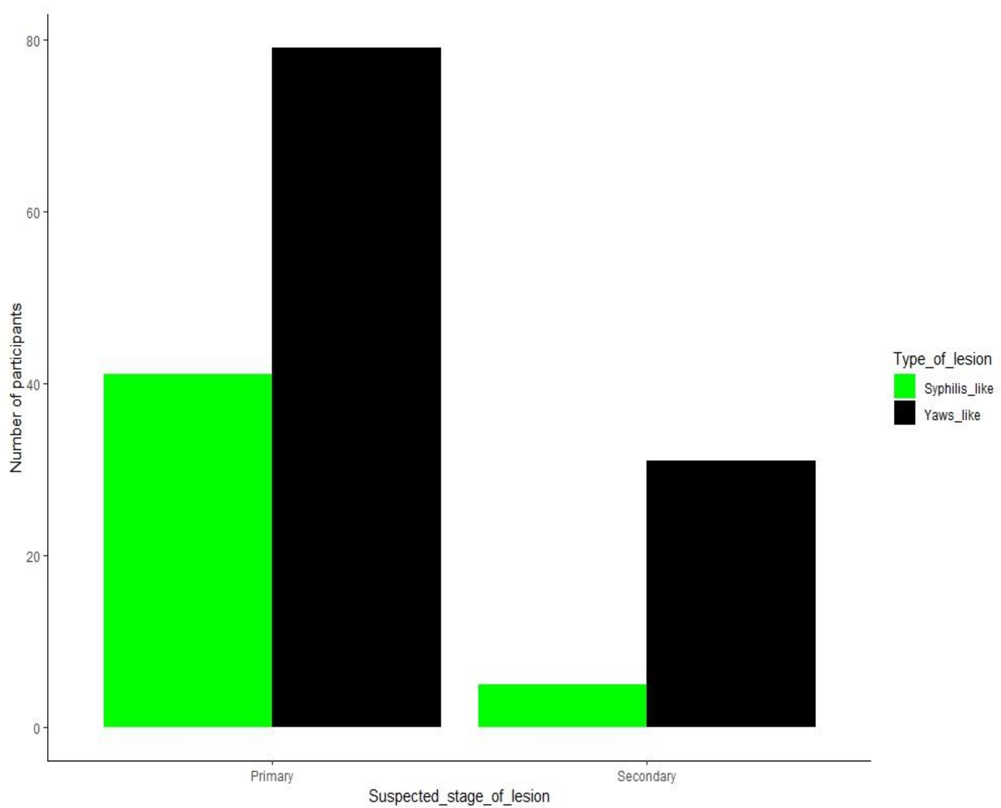


Figure 4.3 Number of participants with lesions consistent with primary and secondary treponematoses. Lesions consistent with primary and secondary yaws (black) were 71.8% (79/110) and 28.2% (31/100) respectively. Lesions consistent with primary and secondary syphilis (green) were 89.1% (41/46) and 10.9 (5/46) respectively.

The participants from this study belonged to four ethnic groups in Ghana, namely the Ashantis (the dominant ethnic group at the study area), the Akwapims, the Ewes, the Fantis and the Northerners (Figure 4.4). Most participants were either Northerners (54.5%) or Ashantis (41.7%).

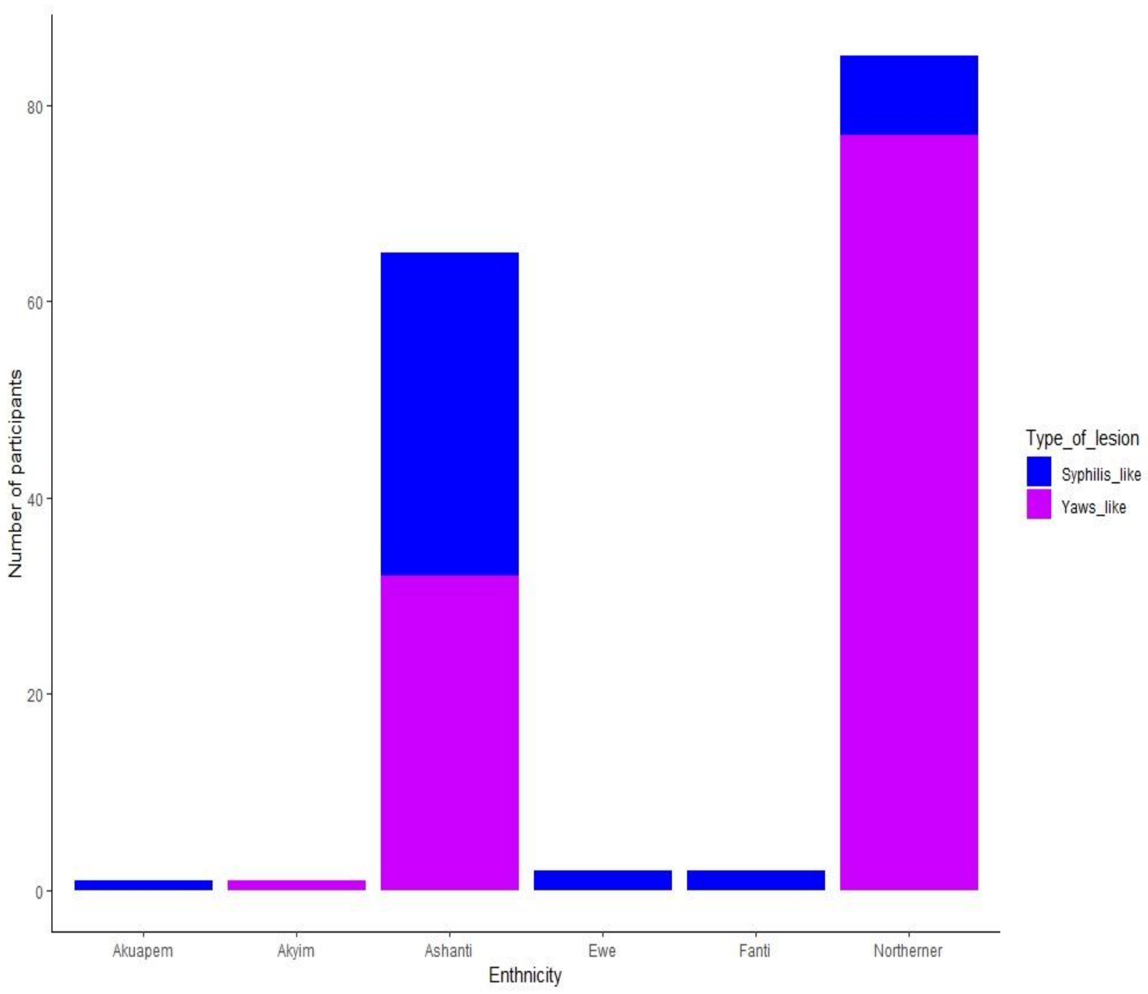


Figure 4.4 Distribution of the participants recruited for either yaws-like or syphilis-like lesions by ethnicity. When yaws-like lesions (purple) were considered alone, majority of the participants were Northerners (70% [77/110]) followed by the Ashantis (32 [29%]) with the number of Northerners significantly higher than the Ashantis (p-value adjusted = 9×10^{-5}, method: Bonferroni). For syphilis-like lesions (blue), the Ashantis (71.7% [33/46]) were significantly more than the Northerners (17.4% [8/46]) (p-value adjusted =0.00094, method: Bonferroni).

When the yaws-like lesions were considered alone there was significantly more Northerners (77) than Ashantis (32) (p-value adjusted = 9×10^{-5}, method: Bonferroni) or Akyim (1) (p-value adjusted = 2×10^{-16}, method: Bonferroni). However, when syphilis -like lesions were considered

alone there were significantly more Ashantis (32) than Northerners (8) (p-value adjusted =0.00094, method: Bonferroni).

The Northerners in Ghana consist of people from the Upper East, Upper West, Savanah, and Northern regions of Ghana. Most of the poorest communities in Ghana are found in these regions (Dittoh, Awuni and Akuriba, 2013; Ghana Statistical Serivce, 2015), where basic social amenities such as drinking water, toilet and good shelter are not readily available. People from these regions migrate and settle in rural communities in the Southern regions, such as the Ashanti region, in search for jobs, but where their living environment may still be precarious. This leads to poor hygiene, exposing these people to several infectious diseases (Bhutta *et al.*, 2014). It is hence not surprising that most of the yaws-like lesions were sampled from these deprived communities of Northerners living in the Ashanti region, since yaws is known to be associated with poverty and poor hygiene (Mushayabasa *et al.*, 2012; Fitzpatrick, Asiedu and Jannin, 2014).

4.4 Seroprevalence of treponematoses

The seroprevalence of the treponematoses was determined by the DPP serological test (Chembio Diagnostic Systems Inc. Medford, NY). Individuals who tested positive for both treponemal and nontreponemal test lines were considered as seropositive. Seroprevalence was determined by dividing the number of DPP positive participants by the total number of study participants. Seroprevalence of treponematoses (both yaws and syphilis combined) in this study was 15.4% (24/156) (CI=10.6%-21.9%) (Figure 4.5A). When yaws-like and syphilis-like lesions were considered separately, the seroprevalence of treponematoses were 17.3% (19/110) (CI=11.4%-25.4%) and 10.8% (5/46) (CI-4.7%-23%) respectively (Figure 4.5A).

When study sites for yaws-like lesions were considered, Adweratia had the highest treponematoses (yaws) seroprevalence, followed by Mpobi and Bebu, with 10% (11/110 [CI-5.7-17.0]), 6.3% (7/110 [CI-3.1-12.5]) and 0.9% (1/110 [CI-0.1-4.9]) seroprevalence, respectively (Figure 4.5B).

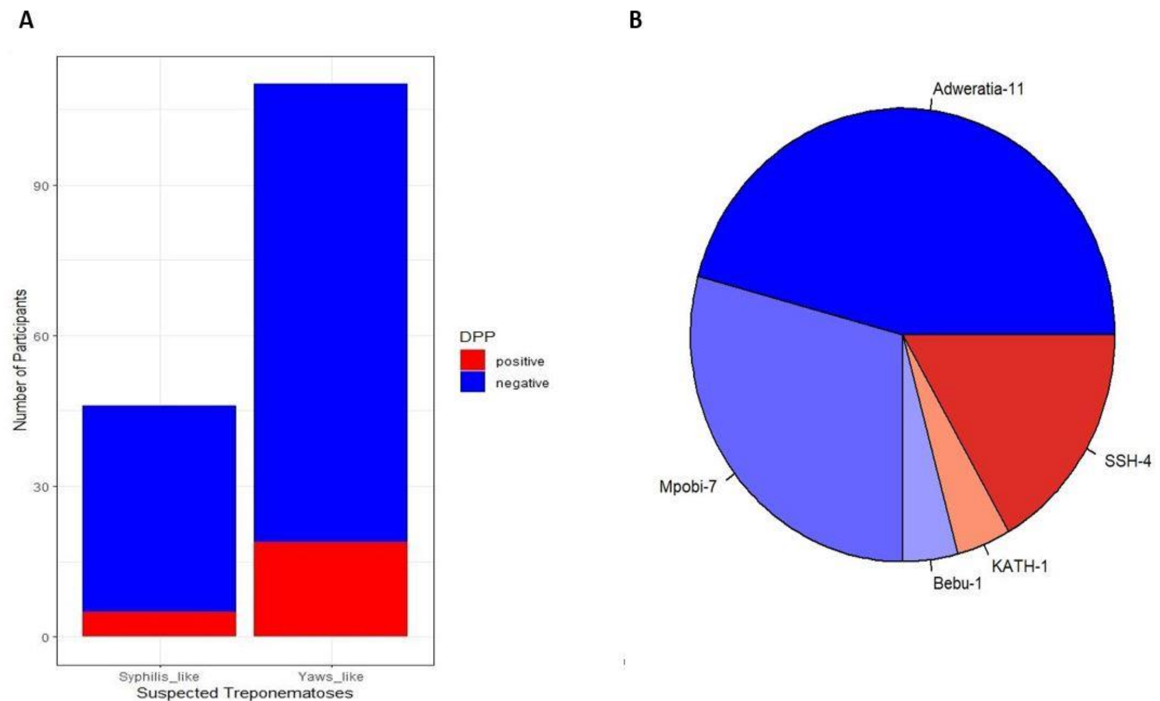


Figure 4.5 A) Number of seropositive participants for yaws-like and syphilis-like lesions. The number of participants with positive and negative DPP test is shown in red and blue respectively. Seroprevalence of treponematoses for yaws-like lesions was 17.3% (19/110) and that of syphilis-like lesions was 10.8% (5/46). **B) Number of seropositive participants according to the study site**. Seroprevalence of treponematoses at yaws-like lesions sites were: Adweratia (10% [11/110]), Mpobi (6.3% [7/110]) and Bebu (0.9% [1/110]). Brofeyedu and Kotei recorded no seropositives, so they were not included in the pie chat. Seroprevalence of treponematoses at syphilis -like lesions sites were: KATH (2.2% [1/46]) and SSH (8.6% [4/46]). The sites in blue shades show where the yaws-like lesions were sampled from and the sites in red shades show where the syphilis-like lesions were sampled from.

Interestingly, the pairwise comparison between the seroprevalence of Adweratia (10%) and Mpobi (6.3%), located in the same district (Afigya Kwabre South district), did not show any significant difference (p-value adjusted= 0.46, method: Bonferroni). However, the seroprevalence of Bebu (0.9%), located in the Atwima Kwanwoma district, and of Adweratia (10%) was significantly different (p-value adjusted= 0.007, method: Bonferroni). The seroprevalence of Mpobi (6%) and of Bebu (0.9%) was not significantly different (p-value

adjusted= 0.07, method: Bonferroni), even though Mpobi seroprevalence is 6 times higher than Bebu's. None of the remaining three sites (Brofoyedu, Atia and Kotei) recorded any seroprevalence of treponematoses (yaws). This suggests that yaws was more prevalent in the Afigya Kwabre South district (Adweratia and Mpobi) in the Ashanti region of Ghana compared to the other sites sampled. Even though the seroprevalence of syphilis was about 4 times higher from the SSH (8.6% [4/46]) in comparison to that of KATH (2.2% [1/46]), this difference was not statistically significant (Figure 4.5B).

The major disadvantage of the serological test (DPP) is that it cannot determine the very early stage of *T. pallidum* infection. This is associated to individuals at this stage not producing sufficient antibodies in serum for detection by the serological test (Marks, Mitjà, *et al.*, 2015). To address this, two molecular techniques (Multiplex PCR and the 16S rRNA gene sequencing), which can detect *Treponema* DNA in lesions during early stage of the disease, were performed. The multiplex PCR was used to target *T. pallidum*, as well as other organisms that are commonly known to cause lesions similar to yaws and syphilis (i.e. *H.ducreyi*, Herpes simplex-1 (HSV-1) and Herpes simplex-2 (HSV-2)) (Abdulai *et al.*, 2018). 16S rRNA gene sequencing was also performed to identify *Treponema*, determine the microbiota of yaws-like and syphilis-like lesions, and compare the diversity between these yaws-like and syphilis-like lesions.

4.5 The common microorganisms known to cause yaws-like, and syphilis-like lesions identified by the multiplex polymerase chain reaction (PCR)

Multiplex PCR confirmed the presence of *T. pallidum* and the presence of other common microorganisms that cause lesions similar to yaws and syphilis in some of the lesions sampled. Out of the 156 lesions sampled, 10 showed the presence of *H. ducreyi*, one of *T. pallidum*, two of HSV-1 and 13 of HSV-2 (Figure 4.6). All the *H. ducreyi*, *T. pallidum* and HSV-1 that were identified were found from lesions associated to yaws (Figure 4.6).

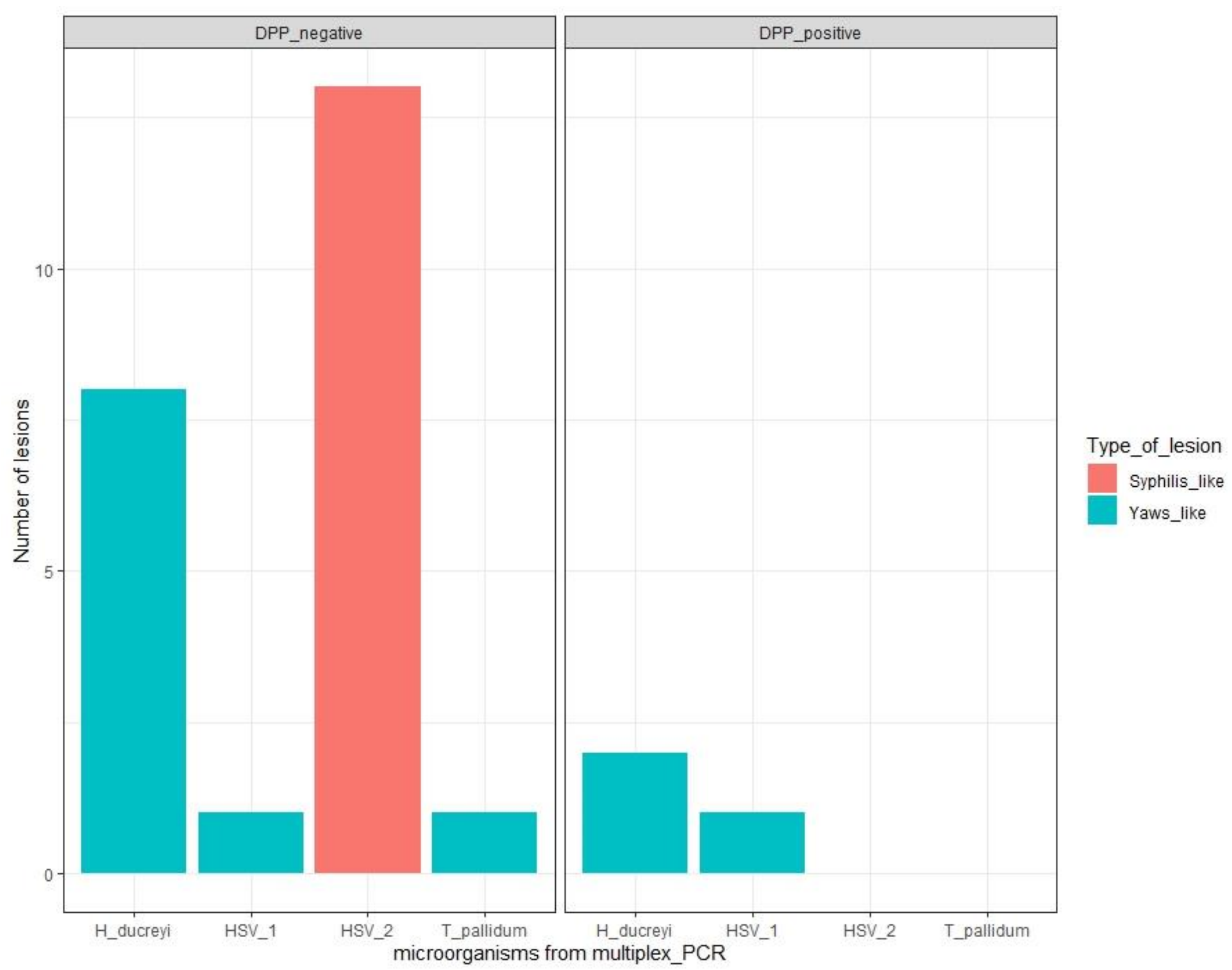


Figure 4.6 Distribution of the microorganisms targeted by the multiplex PCR according to the associated treponematose and the DPP result. *T. pallidum* was identified in a single skin-lesion sample associated with yaws from a participant whose DPP test was negative. All the *H.ducreyi* -positive samples were identified from yaws-like lesions, while all the HSV-2 -positive samples were identified in syphilis-like lesions.

The one sample that was *T. pallidum* -positive from the multiplex PCR was identified from the lesion of a participant who had been DPP-negative. All the HSV-2 -positive samples were identified from syphilis-like lesions (Figure 4.6). The *H. ducreyi and* the HSV-1 -positive samples were associated with skin lesions of participants who had been DPP-positive and DPP-negative samples. The multiplex PCR results indicate that 28.2% (13/46) of the syphilis-like lesions were

positive for HSV-2. Additionally, 9% (10/110), 1.8% (2/110) and 0.9% (1/110) of yaws-like lesions were positive for *H. ducreyi*, HSV_1 and *T. pallidum* respectively (Figure 4.6).

4.6 Microbiota of yaws-like and syphilis-like lesions

Recent advances in next generation sequencing now allow to identify bacteria at various taxonomic levels. Here, the 16S rRNA, the 'fingerprint gene' present in all bacteria, was used in order to identify and quantify individual taxa present in each lesion. The data produced here consisted of overlapping Illumina Miseq 2×250 amplicon sequences from the V1-V2 variable regions of the 16S rRNA gene for all 156 skin lesions collected. In addition, a positive control and a negative control were included in the samples' library preparation. The negative control was included to ensure that amplicons generated from the PCR were not due to reagent contaminations. When the negative control yields no amplicon, as was the case here, the data produced is not due to reagent contamination and the data can be processed further. The positive control (which contains a known bacterial community) also ensured that the PCR and the sequencer reagents worked as expected. The amplicons and sequences expected from the positive control are shown in Figure A1 in Appendix 6.

After quality control of the sequence reads, which includes inspection of read quality, filtering out and trimming of low-quality sequence , estimation of error rate (the probability that a given base is called incorrectly by the sequencer) (Figures 4.7 and 4.8) and merging of paired reads (forward and reverse reads) by DADA2 package (version 1.26.0) in R (version 4.2.2), a total of 3,490,650 sequence reads, with mean reads of 22,375.96 per sample, remained.

A rarefaction curve was plotted using the alpha diversity metric Shannon index to determine if these lesion samples (yaws-like and syphilis-like lesions) were sequenced to a sufficient depth. The rarefaction curve showed the expected plateau indicative of all samples having been sequenced to a sufficient depth (Figure 4.9). The plateau implies that no new species would be observed in these lesions if samples were sequenced beyond the maximum sequence depth (5000) shown in the plot (Figure 4.9).

The amplicon sequence variant (ASV) table was constructed using the DADA2 package (version 1.26.0) in R in order to identify each unique sequence based on single nucleotide differences.

Out of 136,666 ASV identified, 23% (31,433) were chimeras (artifact sequences formed by two biological sequences).

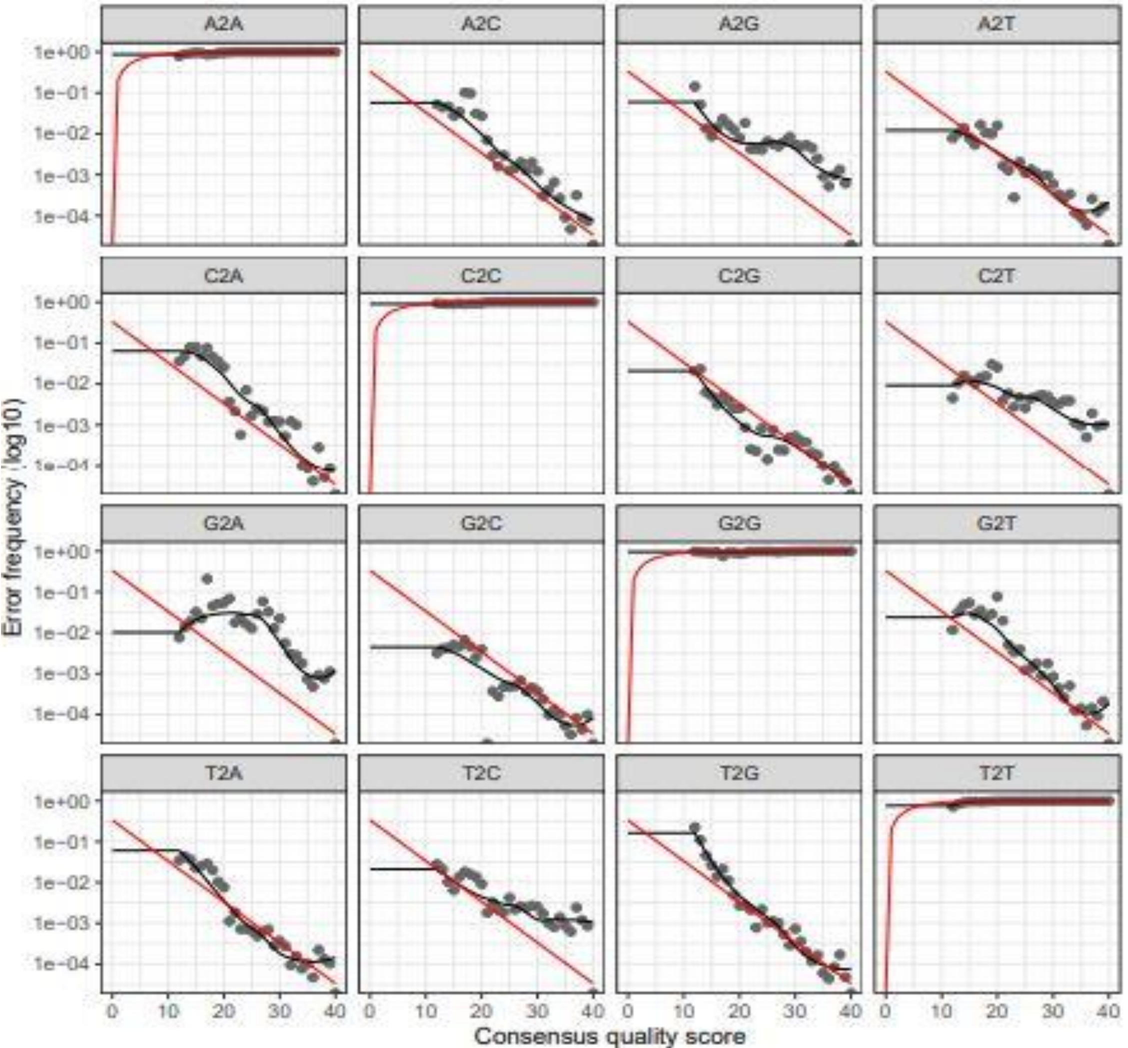


Figure 4.7 Error rates of each possible transition of the four nucleotide bases (A->C, A->G, A->T…) for the forward reads of the 16S rRNA genes equencing. Points are the observed error rates for each consensus quality score (Q-score). The Q-score indicates the probability that a given base is called incorrectly by the sequencer. The black line shows the estimated error rates. The red line is the error rates expected under the nominal definition of Q-score. Here the estimated error rates (black lines) were good fit to the observed error rates (points). This implies the data analysis can proceed with confidence. A=Adenine, C=Cytosine, G= Guanine, T= Thymine

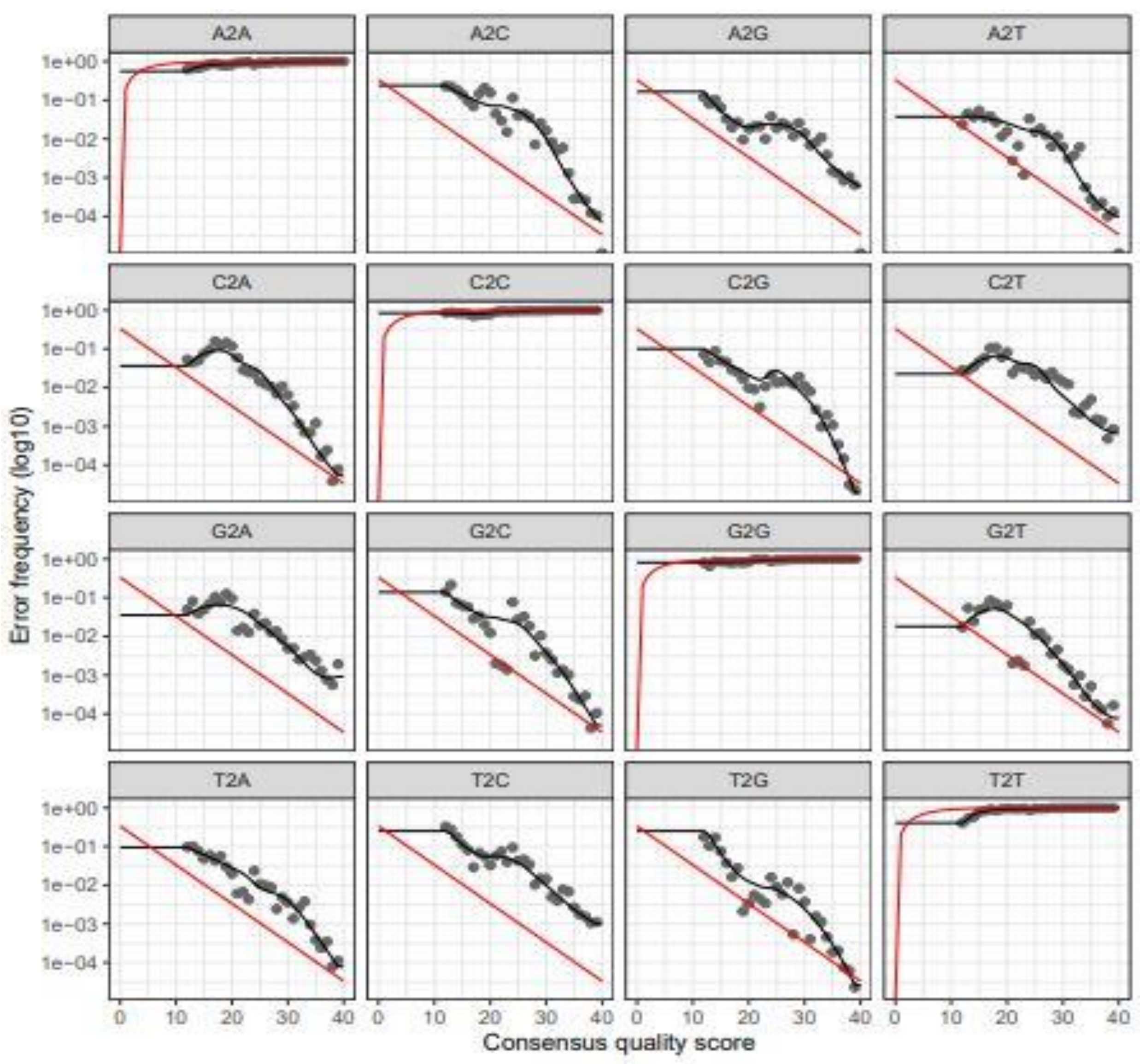


Figure 4.8 Error rates of each possible transition of the four nucleotide bases (A->C, A->G, A->T…) for the reverse reads of the 16S rRNA gene sequencing. Points are the observed error rates for each consensus quality score (Q-score). The Q-score indicates the probability that a given base is called incorrectly by the sequencer. The black line shows the estimated error rates. The red line is the error rates expected under the nominal definition of Q-score. Here the estimated error rates (black lines) were good fit to the observed error rates (points). This implies the data analysis can proceed with confidence. A=Adenine, C=Cytosine, G= Guanine, T= Thymine

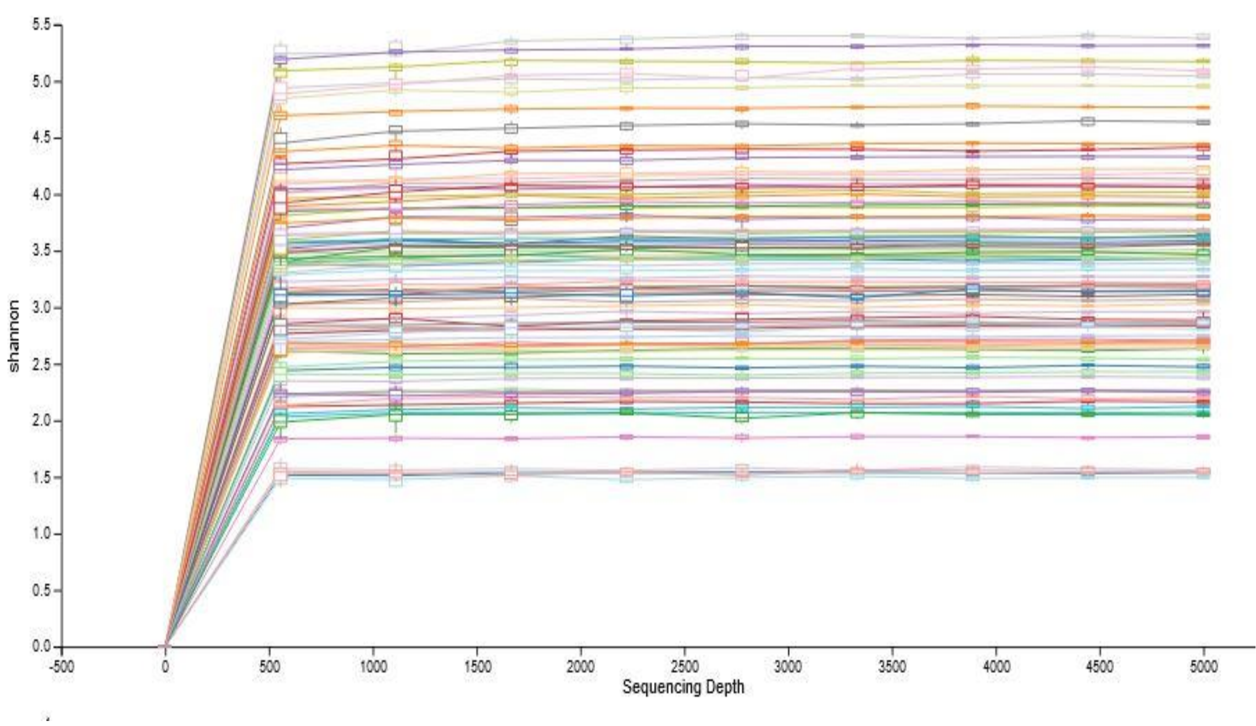


Figure 4.9 Rarefaction curve showing Shannon diversity index for all samples (one curve per sample). A plateau indicates that no new species will be observed if the sample was sequenced further. This shows each sample was sequenced to a sufficient depth.

4.7 The different phyla identified in yaws-like and syphilis-like lesions.

The silva database (version 138.1) (Quast *et al.*, 2013; Yilmaz *et al.*, 2014) was used to assign taxonomic identifiers to the ASV. Across 156 samples, 24 phyla, 146 families and a total of 408 different genera were identified. Overall, the same phyla were identified in yaws-like and syphilis-like lesions. The phyla consist of 23 bacteria phyla and one archaea phylum (Figure 4.10). These phyla were visualised and analysed with the statistical computing software R (estimation of alpha diversity, beta diversity and differentially abundant taxa) using the phyloseq (version 1.42.0), DEseq2 (version 1.38.2) and the ggplot2 packages (version 3.4.0).

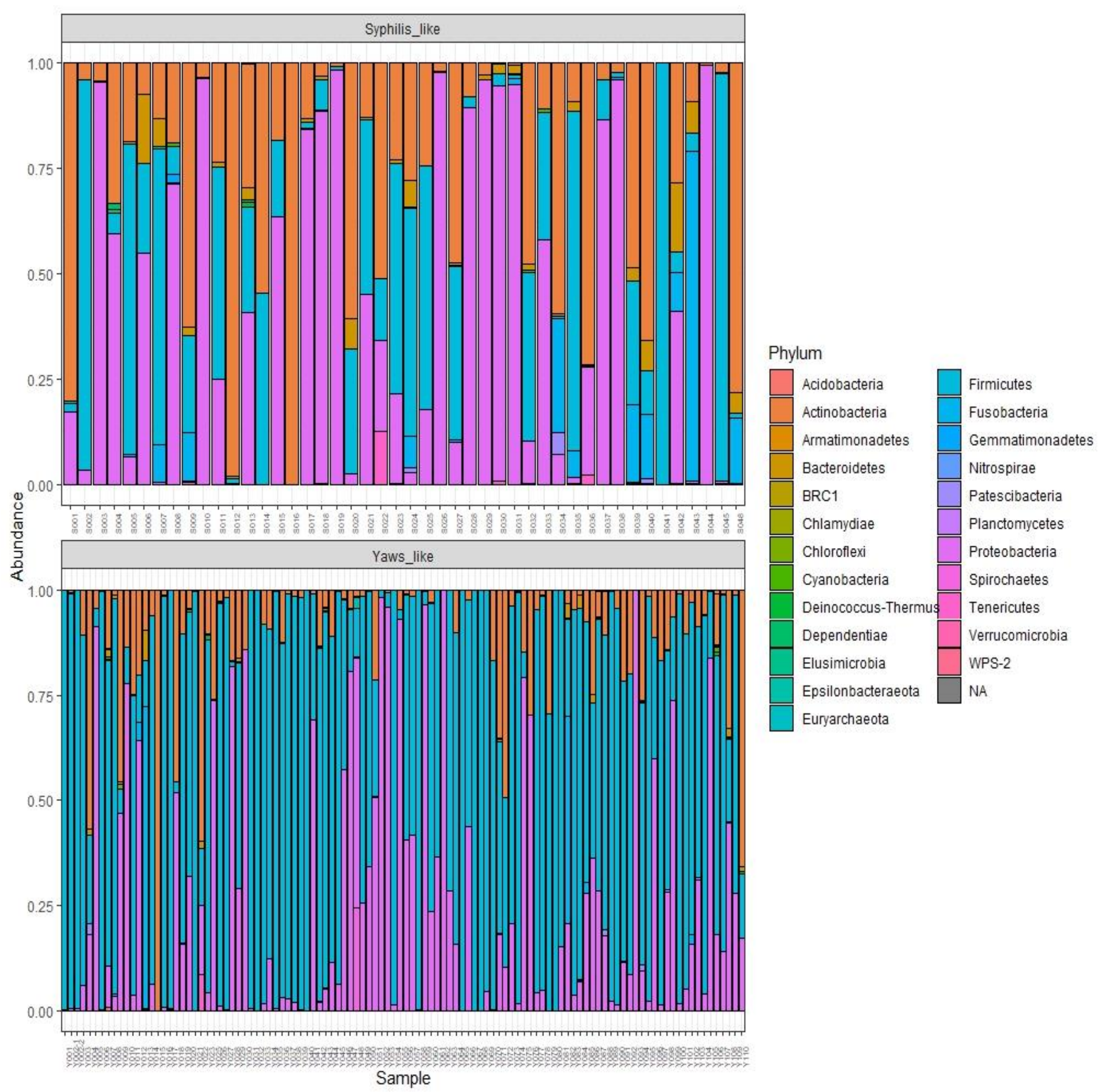


Figure 4.10 Relative abundance of the 24 different phyla identified in the syphilis-like (46 samples) and yaws-like lesions (110 samples). The 23 phyla belonged to bacteria and one to the archaea (Euryarchaeota). The same 24 phyla were identified in both syphilis-like and yaws-like lesions.

4.7.1 The phyla that represent at least 1% of reads in at least one sample

When the phyla that represent at least 1% of reads in terms of abundance in at least one sample were considered, *Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, *Fusobacteria*, *Patescibacteria*, *Proteobacteria* and *Tenericutes* were identified in both yaws-like and syphilis-like lesions (Figures 11 and 12). However, *Gemmatimonadetes*, *Elusimicrobia* and *Dependentiae* were present in syphilis-like lesions but not in yaws-like lesions (Figure 4.11).

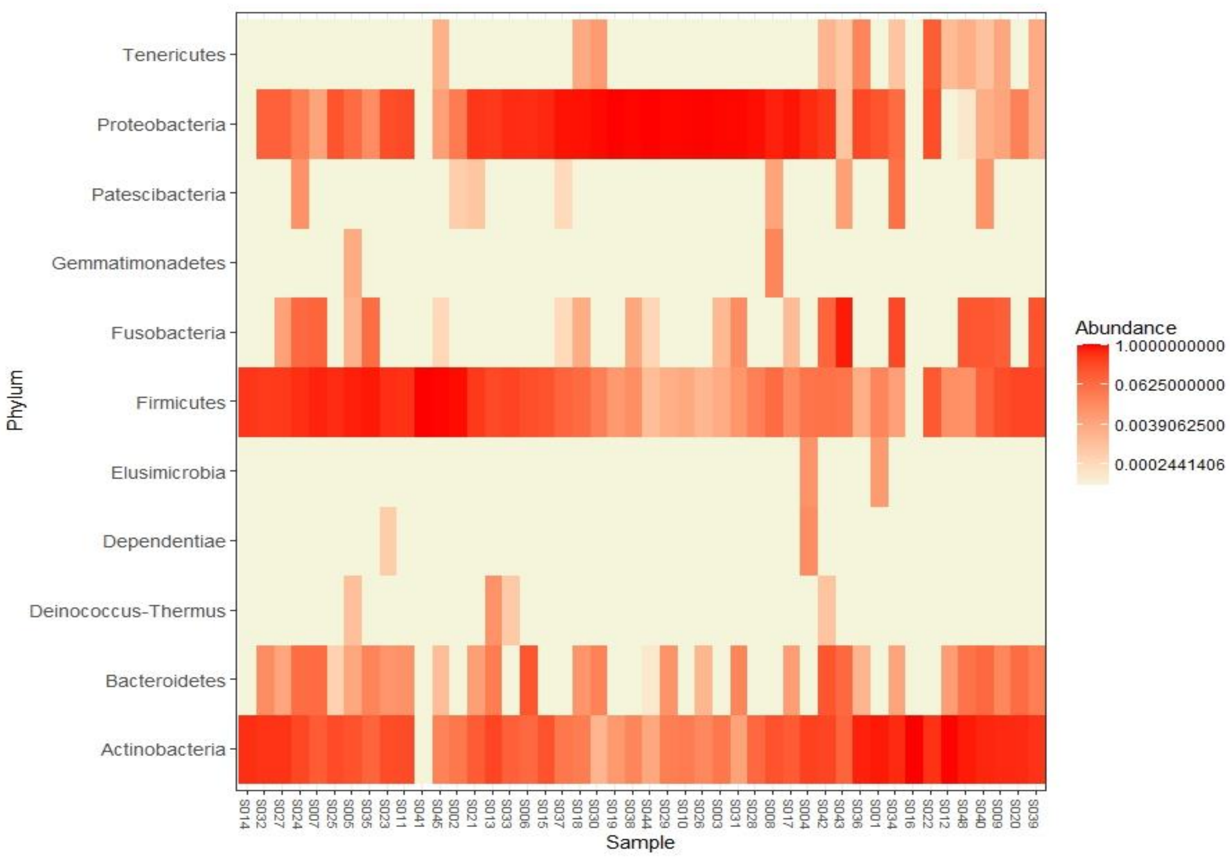


Figure 4.11 Heatmap of the abundance of phyla that represent at least 1% of reads in at least one sample of the 46 syphilis-like lesions. The phyla on the y-axis are those that represent at least 1% of reads in at least one sample. The phyla that did not represent 1% of reads in at least one sample are not present on the y-axis. The colour scale showing the abundance represent the relative abundance of the phyla on the y-axis across all samples. The phylum with the highest abundance was *Proteobacteria*, followed by *Actinobacteria* and *Firmicutes*.

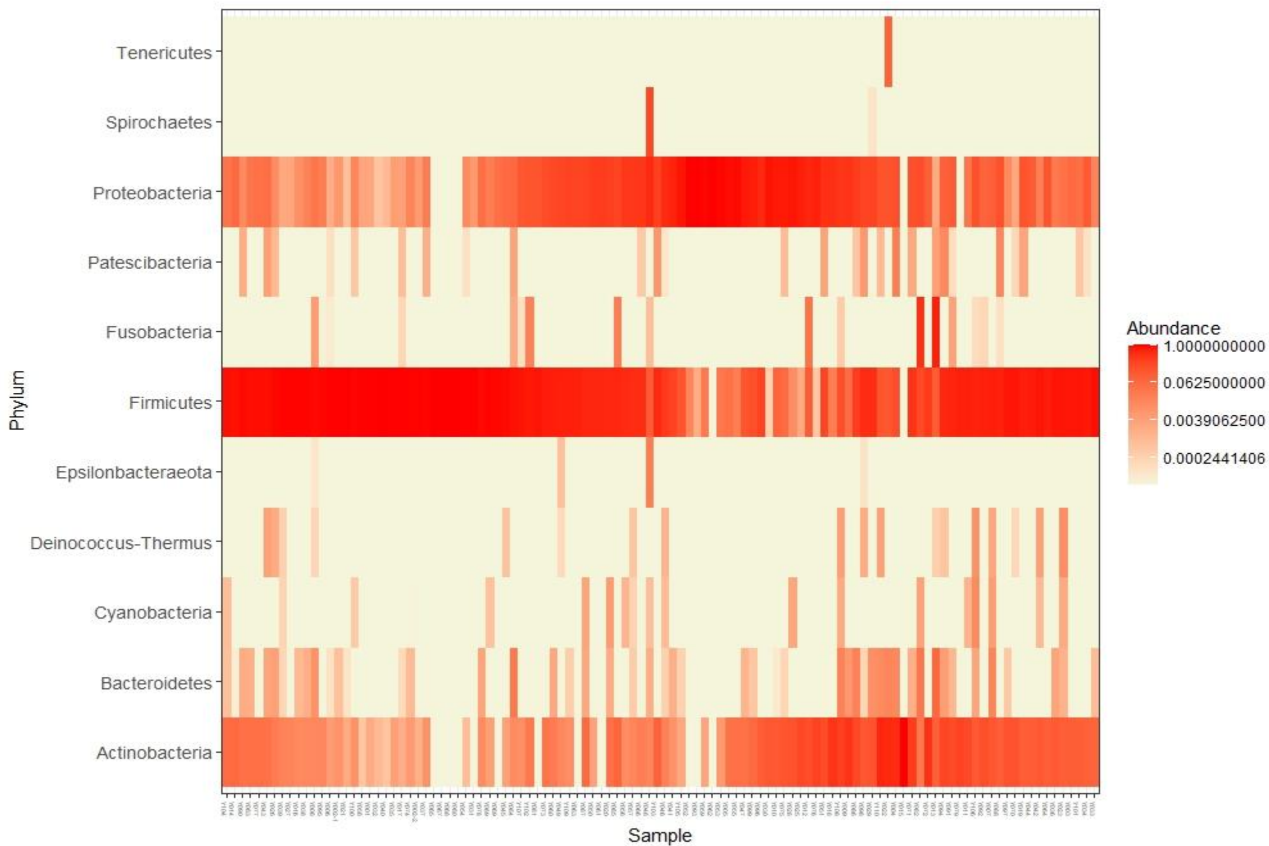


Figure 4.12 Heatmap of the abundance of the phyla that represent at least 1% of reads in at least one sample of the 110 yaws-like lesions. The phyla on the y-axis are those that represent at least 1% of reads in at least one sample. The phyla that did not represent 1% of reads in at least one sample are not present on the y-axis. The colour scale showing the abundance represent the relative abundance of the phyla on the y-axis across all samples. The phylum with the highest abundance was *Firmicutes* followed by *Proteobacteria* and *Actinobacteria*.

Likewise, *Epsilonbacteraeota*, *Cyanobacteria* and *Spirochaetes* were present in yaws-like lesions but not in syphilis-like lesions when phyla that represent at least 1% of reads in at least one sample were considered (Figure 4.12). *Spirochaetes* were however present in one syphilis-like lesion, but it represented less than 1% of reads in that sample (Figure 4.13).

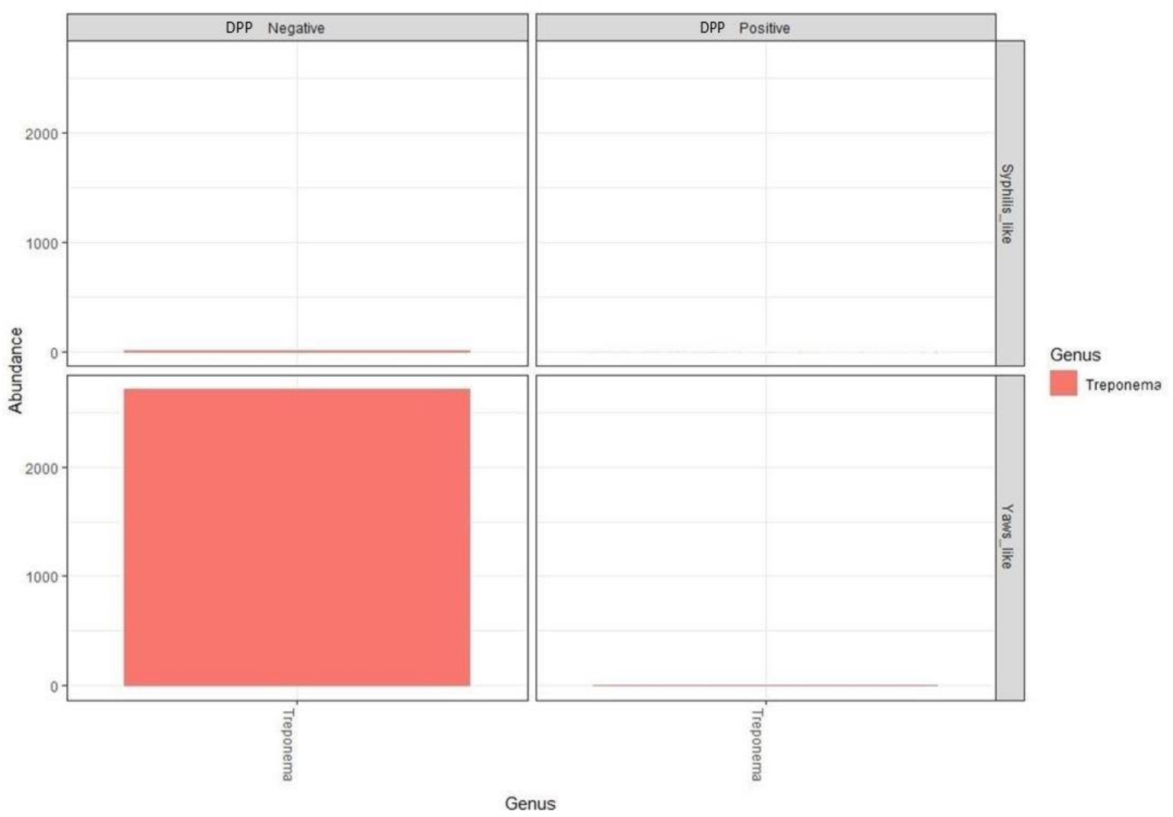


Figure 4.13 Abundance (in number of reads) of *Treponema* (genus belonging to the *Spirochaetes* phyla) in both yaws-like and syphilis-like lesions according to the corresponding DPP result. The *Treponema* (*a* Spirochaetes) found in syphilis-like lesions had low abundance (9 reads) which represented less than 1% (187) of reads in that sample.

4.7.2 The top five phyla and differentially abundant taxa

The top five phyla in syphilis-like lesions in terms of relative abundance were *Proteobacteria* (39.7%), followed by *Actinobacteria* (27.9%), *Firmicutes* (25.1%), *Fusobacteria* (4.4%) and *Bacteroidetes* (2%) (Figures 4.14). In yaws-like lesions, while the same top five phyla were observed, their order differed substantially, where *Firmicutes* (62.1%) were the most abundant, followed by *Proteobacteria* (25.5%), *Actinobacteria* (10%), *Fusobacteria* (1.1%) and *Bacteroidetes* (0.2%) (Figures 4.14). The differential abundance analysis showed that the genera *Gardnerella* (*Actinobacteria*) and *Raoultella* (*Proteobacteria*) were significantly more abundant

in syphilis-like lesions than yaws-like lesions (Wald test, Benjamin-Hochberg (BH) p-value adjusted= 0.002 for *Gardnerella* and BH p-value adjusted= 0.0004 for *Raoultella*). However, the genera *Staphylococcus*, *Streptococcus* and *Bradyrhizobium* were significantly more abundant in yaws- like lesions than syphilis-like lesions (Figure 4.15) (Wald test, BH p-value adjusted= 1.2×10^{-29} for *Streptococcus*, BH p-value adjusted= 1.3×10^{-5} for *Staphylococcus* and BH p-value adjusted= 6.4×10^{-4} for *Bradyrhizobium*).

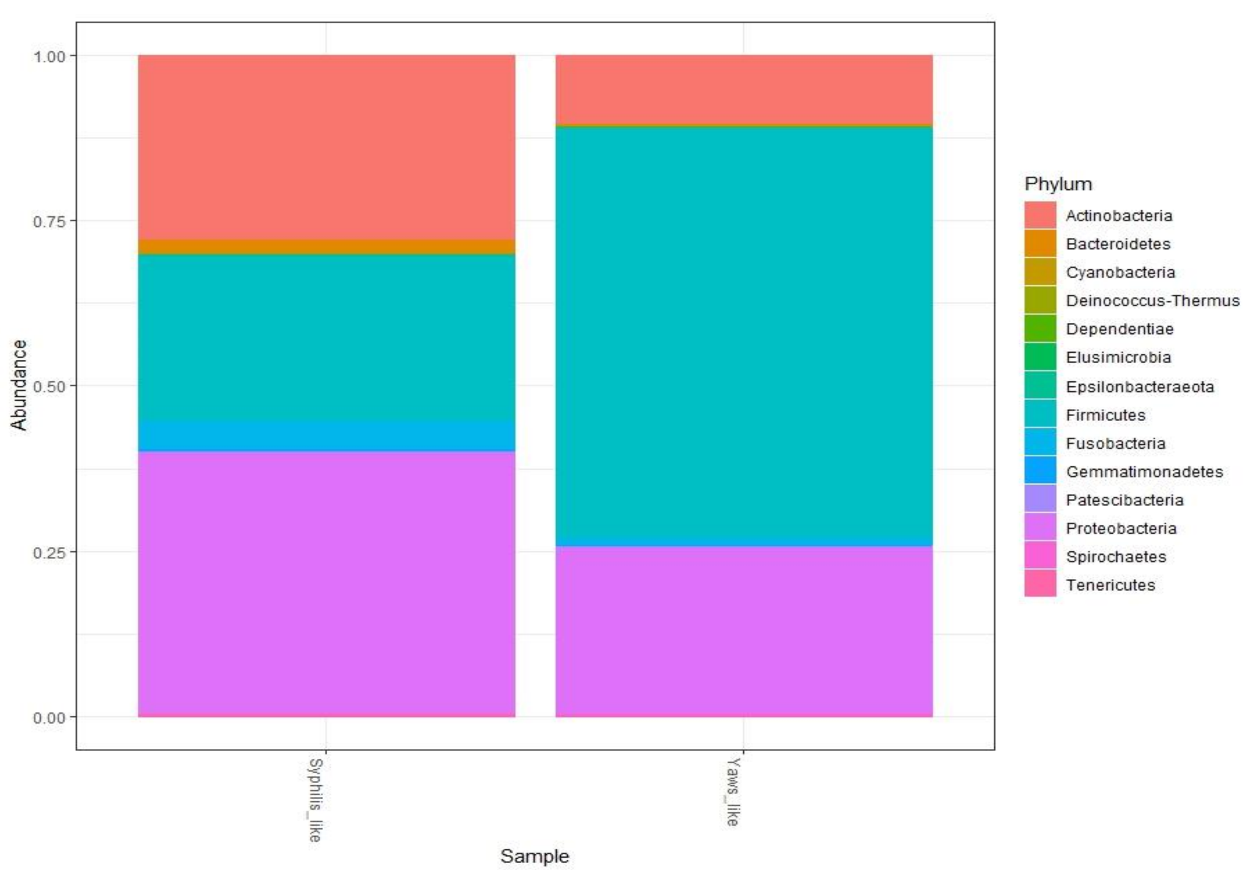


Figure 4.14 Relative abundance of phyla that represent at least 1% of reads in at least one sample in yaws-like and syphilis-like lesions. The top five phyla in syphilis-like lesions in terms of relative abundance were *Proteobacteria* (39.7%), followed by *Actinobacteria* (27.9%), *Firmicutes* (25.1%), *Fusobacteria* (4.4%) and *Bacteroidetes* (2%). In yaws-like lesions, while the same top five phyla were observed, their order differed substantially, where *Firmicutes* (62.1%) were the most abundant, followed by *Proteobacteria* (25.5%), *Actinobacteria* (10%), *Fusobacteria* (1.1%) and *Bacteroidetes* (0.2%) (Figures 4.14).

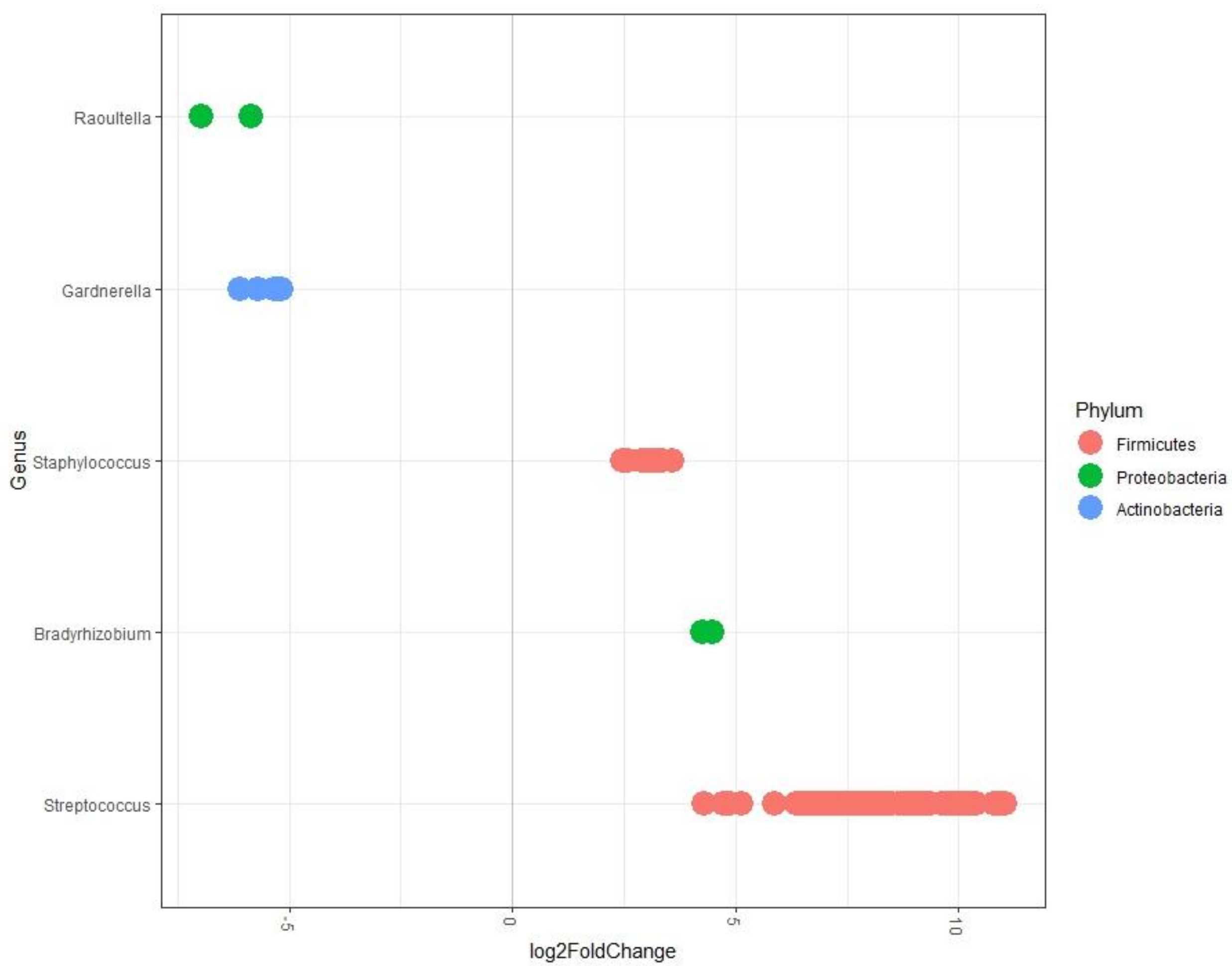


Figure 4.15 Taxa significantly differentially abundant in either yaws-like or syphilis-like lesions. The points represent different ASV for the same genus. The "fold change" is the change in abundance (in folds) of a particular genus in syphilis-like lesions (represented here as A) compared to yaws-like lesions (represented here as B). Statistically, fold change from A to B is B/A. When B is bigger than A, fold change is greater than one and when A is bigger than B, fold change is less than one. The log2FoldChange is used to change fold changes lower than 1 to negatives, while those greater than 1 become positive. Hence dots on the negative side of the x-axis (left) are those genera that are significantly more abundant in syphilis-like lesions compared to yaws-like lesions and those on the positive side (right) are those that are significantly more abundant in yaws-like lesions.

4.8 Number of lesions that were *Treponema*-positive with the 16S rRNA gene sequencing

As 24 individuals tested positive on the serological test (DPP), it could be expected that the corresponding 24 skin-lesion samples would be *Treponema*-positive with the 16S rRNA gene sequencing. However, out of these 24, only one was *Treponema*-positive with the 16S rRNA gene sequencing. In fact, two skin lesions sampled from DPP-negative individuals (one yaws-like lesion and one syphilis-like lesion) were *Treponema*-positive for the 16S rRNA gene sequencing (Table 4.2).

Table 4.2 Comparison between DDP positive lesions and the number of lesions that were positive for *Treponema* in the 16S rRNA gene sequencing

DPP test (n)	Number of lesions positive for *Treponema* with 16S rRNA gene sequencing
Yaws-like lesions positive (19)	1
Yaws-like lesions negative (91)	1
Syphilis -like lesions positive (5)	0
Syphilis -like lesions negative (41)	1
Total (156)	3

4.9 Other medically important genera identified in yaws-like and syphilis-like lesions

Other common medically important genera were identified in these lesions (Table 4.3). More than 50% of syphilis-like lesions were positive for *Pseudomonas spp* (69.5%), *Gadnerella spp* (60.9%) and *Cutibacterium spp* (54.3%). More than 50% of yaws-like lesions were also positive

for *Pseudomonas spp* (80.9%) and *Cutibacterium spp* (79.1%) (Table 4.3). *Klebsiella spp*, *Escherichia-Shigella spp*, *Acinetobacter spp*, *Anaerococcus spp*, *Parvimonas spp*, *Atopobium spp* and *Sneathia spp* were identified in more than 20% of syphilis-like lesions. Likewise, *Corynebacterium spp, Escherichia-Shigella spp* and *Anaerococcus spp* were identified in more than 20% of yaws-like lesions (Table 4.3).

Table 4.3 The common medically important bacteria genera that were identified in either yaws-like or syphilis-like lesions in the 16S rRNA gene sequencing

Pylum	**Genus**	**Number of lesions that were positive in the 16S rRNA gene sequencing**	
		Syphilis-like (%) n=46	Yaws-like (%) n=110
Proteobacteria	*Haemophilus spp*	8 (17.4)	7 (6.3)
	Pseudomonas spp	32 (69.5)	89 (80.9)
	Klebsiella spp	15 (32.6)	3 (2.7)
	Escherichia-Shigella spp	13 (28.2)	46 (41.8)
	Proteous spp	4 (8.6)	2 (1.8)
	Actinobacillus spp	0 (0)	10 (9)
	Acinetobacter spp	17 (36.9)	44 (40)
	Salmonella spp	2 (4.3)	16 (14.5)
	Neisseria spp	2 (4.3)	5 (4.5)

Pylum	**Genus**	**Number of lesions that were positive in the 16S rRNA gene sequencing**	
		Syphilis-like (%) n=46	Yaws-like (%) n=110
	Enterobacilli spp	3 (6.5)	8 (2.7)
	Morganella spp	2 (4.3)	0 (0)
Actinobacteria	*Mycobacterium spp*	0 (0)	4 (3.6)
	Cutibacterium spp	25 (54.3)	87 (79.1)
	Corynebacterim spp	6 (13.0)	28 (25.5)
	Atopobium spp	14 (30.4)	2 (1.8)
	Anaerococcus spp	24 (52.2)	23 (20.9)
	Lactobacillus spp	5 (10.8)	5 (4.5)
	Peptostreptococcus spp	13 (28.2)	6 (5.4)
	Parvimonas spp	10 (21.7)	3 (2.7)
Fusobacteria	*Sneathia spp*	17 (36.9)	9 (8.1)
	Fusobacterium spp	9 (19.6)	5 (4.5)
Tenericutes	*Ureaplasma spp*	8 (17.4)	1 (0.9)
	Mycoplasma spp	7 (15.2)	0 (0)

Fusobacterium spp, *Ureaplasma spp* and *Heamophilus spp* were identified in 19.6%, 17.4%, and 17.4% of syphilis-like lesions, respectively*. Salmonella spp* was identified in 14.5% of yaws-like lesions. The rest of the genera in Table 4.3 infected less than 10% of both yaws-like and syphilis-like lesions. *Mycobacterium spp* was found in 3.4% of yaws-like lesions but none is syphilis-like lesions. Likewise, *Morganella spp* and *Mycoplasma spp* were found in 4.3% and 15.2% of syphilis-like lesions respectively, but neither were in yaws-like lesions (Table 4.3).

4.10 Comparison of the diversity between the microbiota of the yaws-like and syphilis-like lesions

Both the alpha (intra sample variation) and beta diversity (inter sample variation) analyses were performed to highlight significant differences between the community richness or dissimilarity, respectively, of yaws-like and syphilis-like lesions. The alpha diversity was measured with the Shannon and Simpson metrics (Figure 4.16). Both metrics showed that the syphilis-like lesions had a significantly higher alpha diversity compared to yaws-like lesions (Figure 4.17) (Wilcoxon rank test for Shannon metric: p-value= 0.012, Wilcoxon rank test for Simpson metric: p-value=0.033). This implies that more taxonomic members tend to be observed in syphilis-like lesions compared to yaws-like lesions.

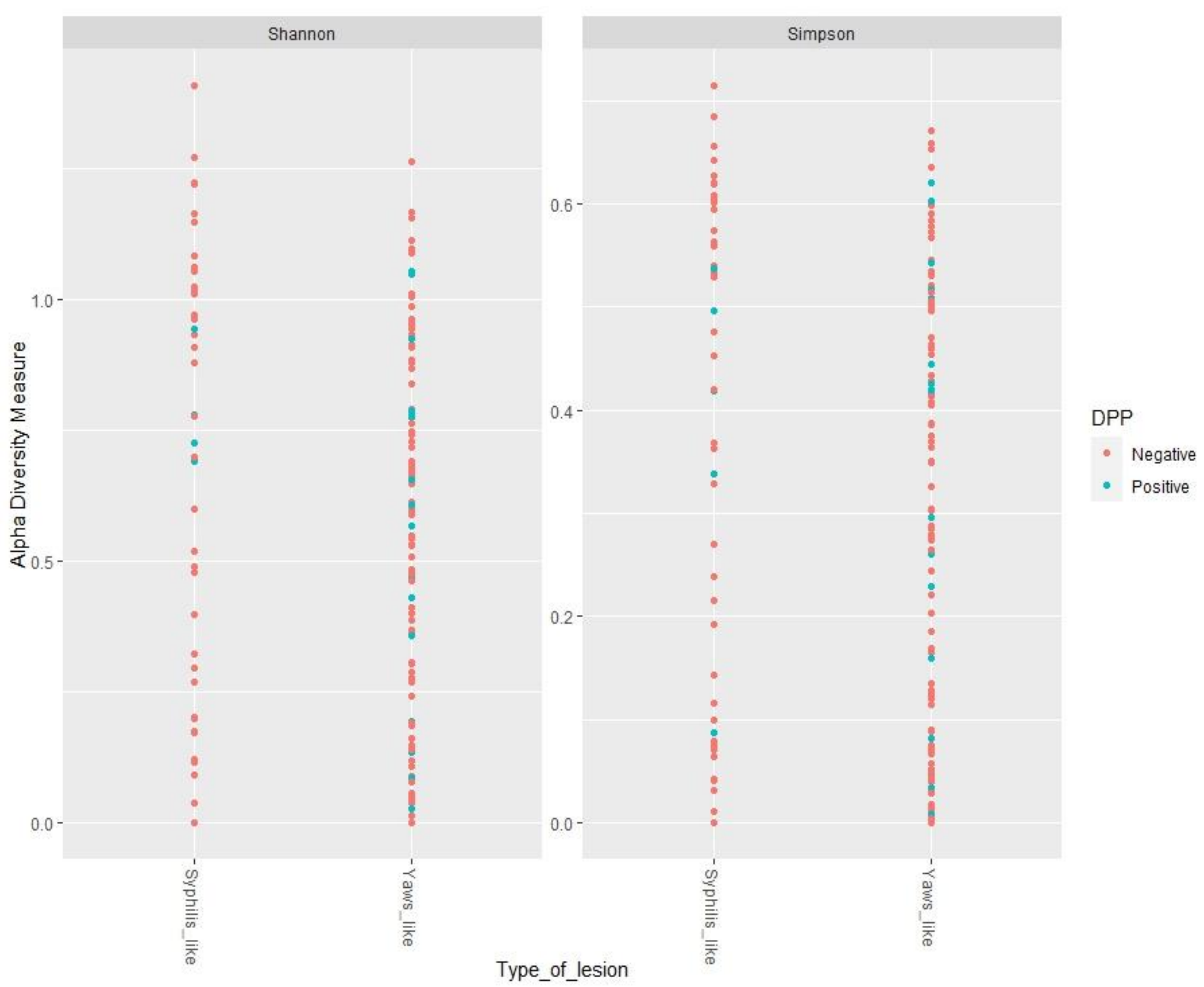


Figure 4.16 Shannon and Simpson alpha diversity measures of the microbiota of yaws-like and syphilis like lesions. The higher the index (alpha diversity measure), the more members (taxa) or diverse that sample. A value of zero implies only one member (taxa) is present**.**

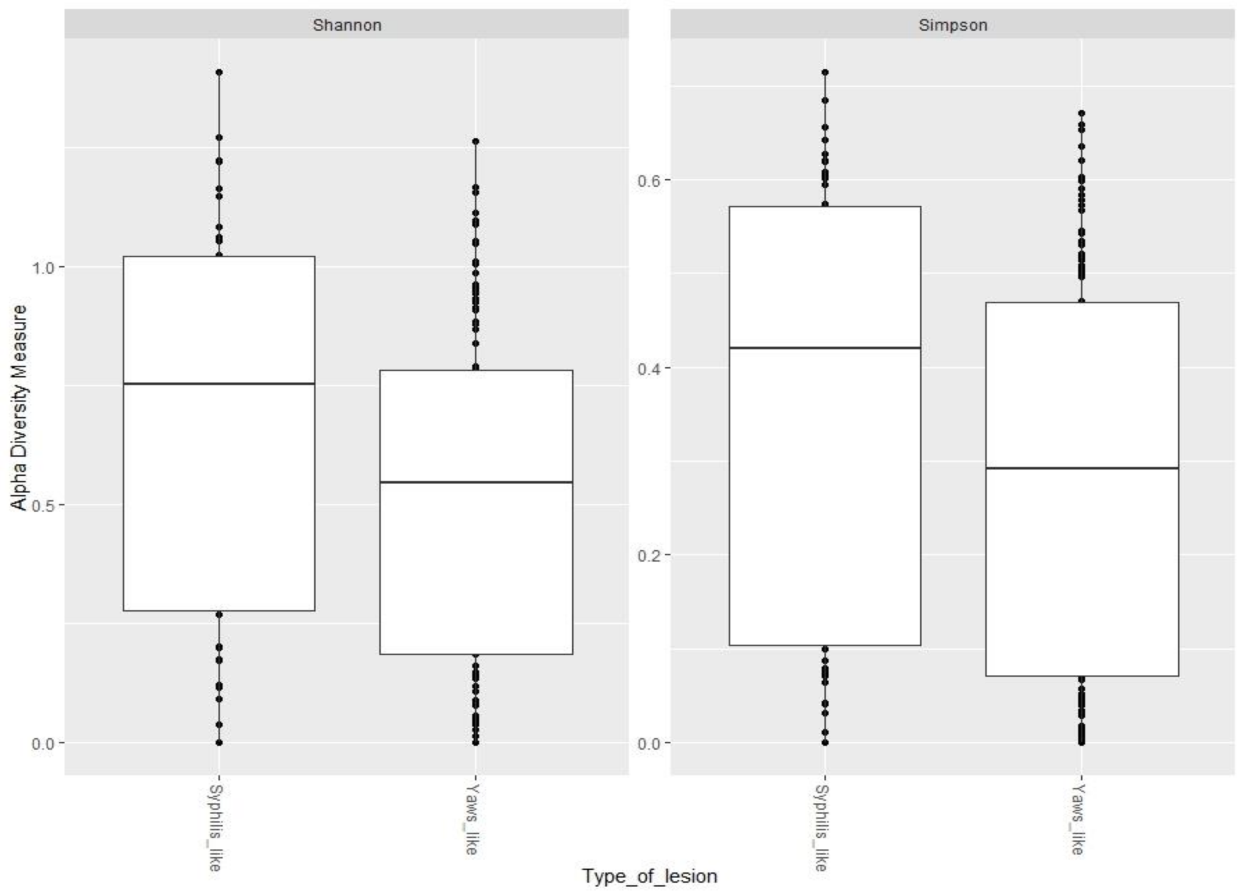


Figure 4.17 Boxplots of the microbiota alpha diversity of yaws-like and syphilis-like lesions measured with the Shannon and Simpson metrics. The syphilis-like lesions were significantly more diverse (intra sample variation) than the yaws-like lesions (Wilcoxon rank test for Shannon metric: p-value= 0.012, Wilcoxon rank test for Simpson metric: p-value=0.033).

In order to investigate potential significant clustering of syphilis-like lesions or yaws-like lesions that would enable distinguishing between the type of treponematose, pairwise distances were computed and plotted using the non-metric multidimensional scaling (NMDS) ordination method and principal coordinate analysis (PCoA). As a measure of fit of the graphical representation of these distances in two dimensions, the stress value of fifteen different distance metrics of beta diversity (inter sample variation) between the microbiota of the yaws-like and syphilis-like lesions was estimated by the NMDS ordination method (Figure 4.18). The

stress value reflects how well the ordination summarises the observed distance among the samples in reduced dimensions (2D or 3D dimensions) (Dexter, Rollwagen-Bollens and Bollens, 2018). As a rule of thumb, a stress value less than 0.05 provides an excellent representation, one that is less than 0.1 is good, greater than 0.2 is ok but a stress value greater than 0.3 provides a poor representation (Dexter, Rollwagen-Bollens and Bollens, 2018). A PCoA generating the first two to three axes capturing above 50% of variation of input data can be interpreted with more confidence (Buttigieg and Ramette, 2014).

Most of the distance metrics estimated here gave a good stress value, except for the mountford and binomial distances (Figure 4.18) and the PCoA plots (Figure 4.19) also captured 92.9% (for Bray-Curtis distance) and 74.3% (for Jaccard distance) of variation of the input data. Overall, the NMDS coordinate plot of all the 15-distance metrics (Figure 4.18) as well as PCoA using the Bray-Curtis and Jaccard distances (Figure 4.19) did not show any distinct separation between yaws-like lesions and syphilis-like lesions.

Since microbiota data are sparse and the Bray-Curtis and Jaccard distances are known to better deal with the presence of many double zero in a dataset (Clarke, Somerfield and Chapman, 2006; Ricotta and Podani, 2017), they were chosen in downstream analyses to investigate further the dissimilarity between yaws-like and syphilis-like lesions. Neither the Bray-Curtis nor the Jaccard distances showed a significant dissimilarity between the microbiota of yaws-like and syphilis-like lesions (Figure 4.20) (PERMANOVA, F=3.6444, p-value=0.061 for Jaccard and PERMANOVA, F=3.5958, p-value=0.062 for Bray-Curtis).

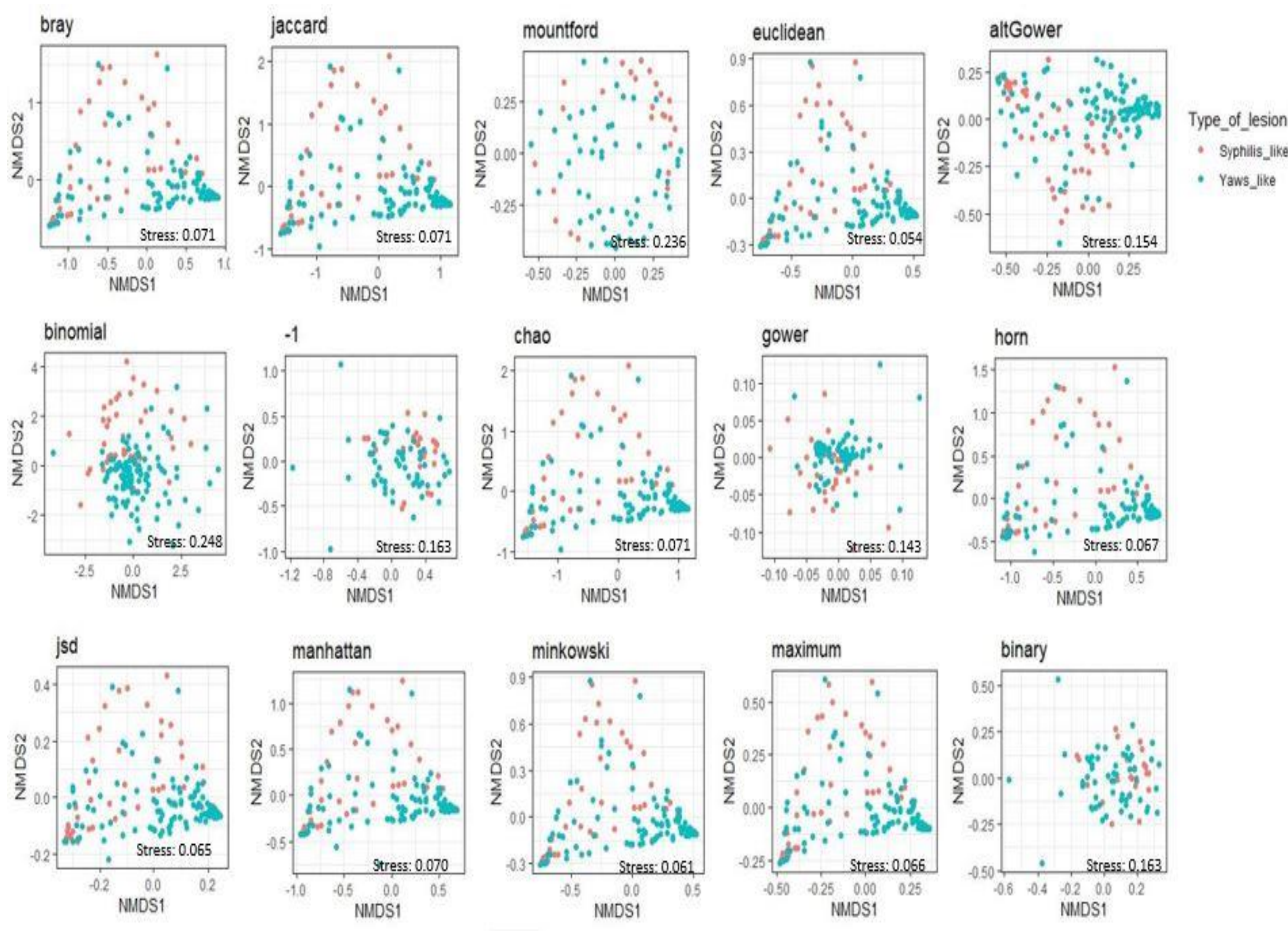


Figure 4.18 NMDS ordination plots using 15 different distances showed no distinct separation between the microbiota of yaws-like and syphilis-like lesions. Lesions that are closer to each other have similar microbiota. Samples from yaws-like and syphilis-like lesions tend to overlap, regardless of the distance metrics used.

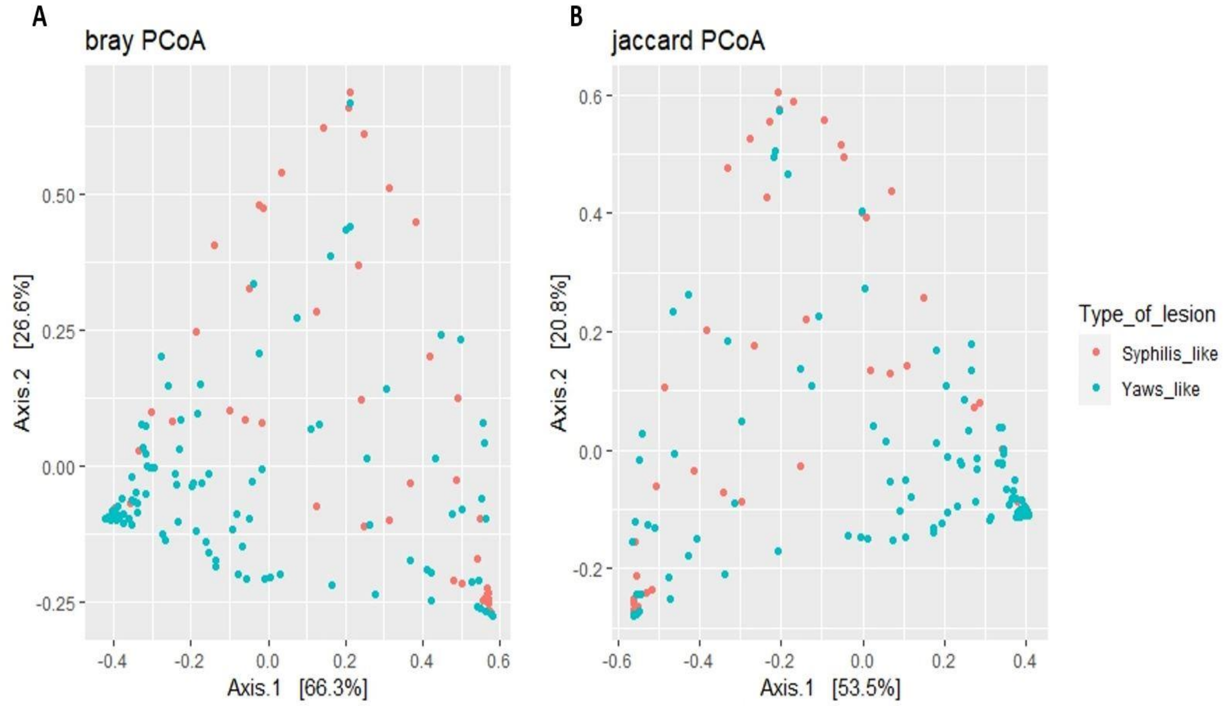


Figure 4.19 PCoA ordination plots using the Bray-Curtis (A) and the Jaccard (B) distances showed no distinct separation between the microbiota of yaws-like and syphilis-like lesions. The first two components of the Bray-Curtis and the Jaccard PCoA plots (represented here with axes 1 and 2) capture 92.9% and 74.3% of the variance of the corresponding distance matrices respectively. This implies that these plots are a good representation of the underlying distances.

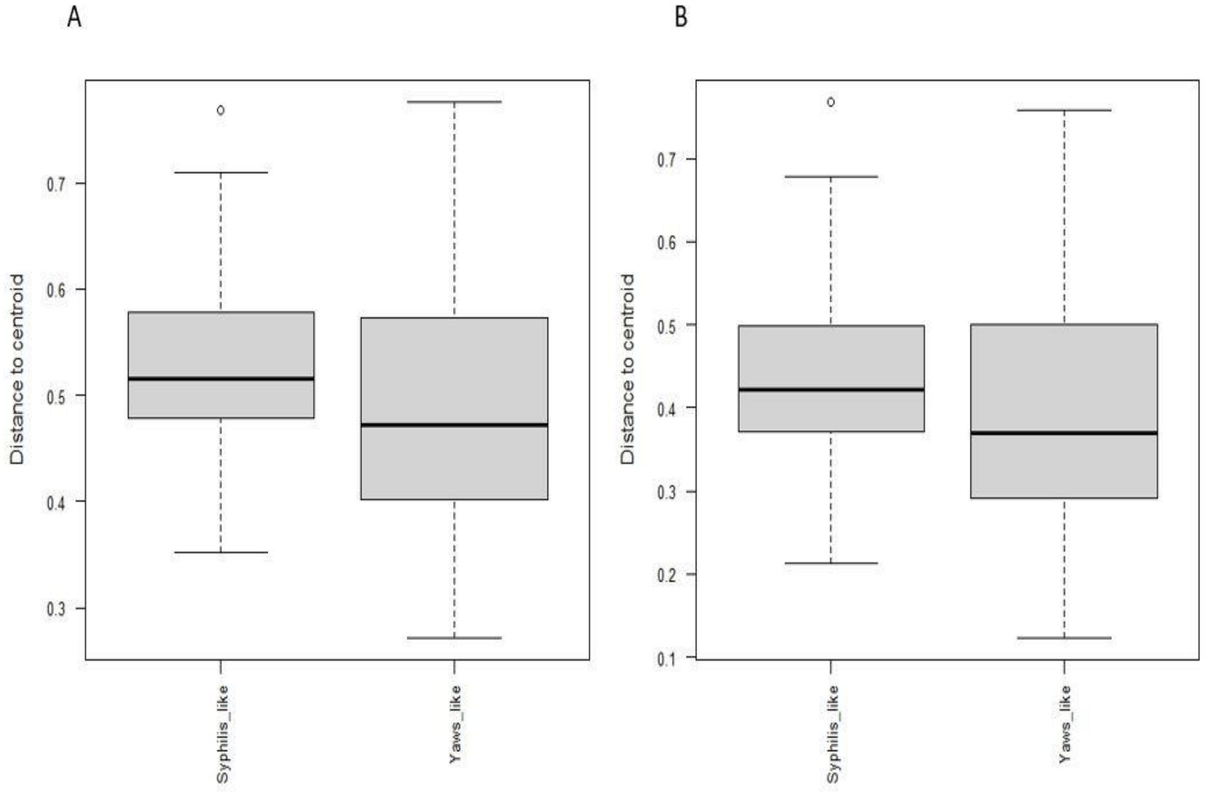


Figure 4.20 Boxplot showing the dissimilarity (beta diversity) between the microbiota of yaws-like and syphilis-like lesions measured with the Jaccard distance (A) and the Bray-Curtis distance (B). Both distance metrics did not show any significant dissimilarity between the yaws-like and syphilis like lesions (PERMONOVA, F=3.6444, p-value=0.061 for Jaccard and PERMANOVA, F=3.5958, p-value=0.062 for Bray Curtis).

4.11 16S rRNA gene sequence diversity in the microbiota of *H. ducreyi* -positive and HSV-2 -positive lesions from the multiplex PCR

The alpha and beta diversity were estimated to investigate whether significant differences existed between the communities of the lesions that had been positive for *H. ducreyi* and those positive for HSV-2 from the multiplex PCR (Figure 4.21).

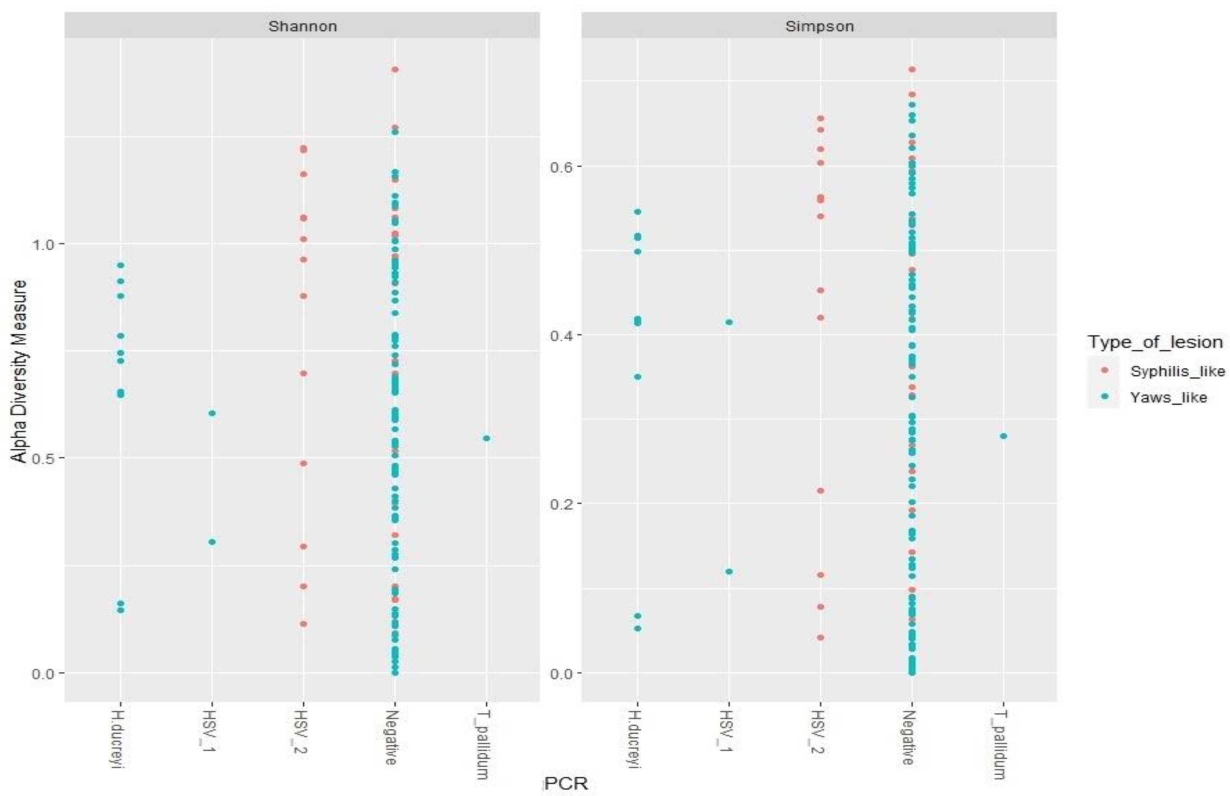


Figure 4.21 Shannon and Simpson alpha diversity measures of the lesion microbiota that were *H. ducreyi*-positive, HSV-1-positive, HSV-2-positive or *T. pallidum*-positive in the multiplex PCR. The higher the index (alpha diversity measure), the more members (taxa) or diverse that sample. A value of zero index means only one member (taxa) is present**.**

The alpha diversity based on Shannon and Simpson metric did not show any significant difference between the *H.ducreyi*-positive and HSV-2-positive lesions (Pairwise Wilcoxon test, p-value adjusted= 1.0) (Figure 4.22).

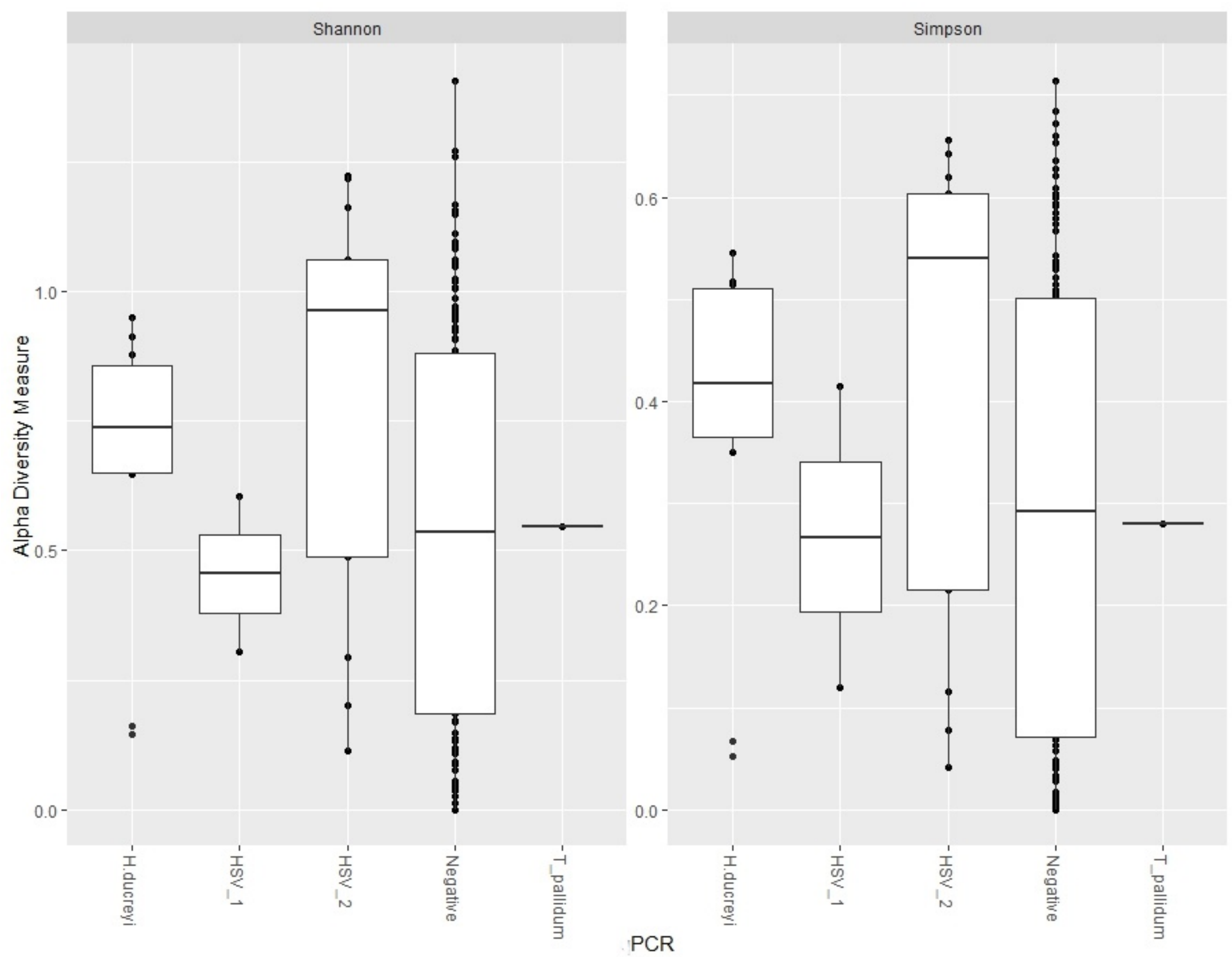


Figure 4.22 Boxplot of the alpha diversity (Shannon and Simpson metrics) of the microbiota of lesions that were positive for *H. ducreyi*, HSV-1, HSV-2 or *T. pallidum* in the multiplex PCR. Pairwise comparison did not show any significant differences between the *H. ducreyi*-positive lesions and the HSV-2 -positive lesions (Pairwise Wilcoxon test, p-value adjusted= 1.0)

Likewise, the beta diversity based on Bray-Curtis and Jaccard distances using NMDS method did not show any significant dissimilarity between the lesions that were *H. ducreyi*-positive and those that were HSV-2-positive in the multiplex qPCR (Tukey pairwise test, p-adjusted = 0.203 for Bray-Curtis distance and p-adjusted =0.145 for Jaccard distance) (Figure 4.23).

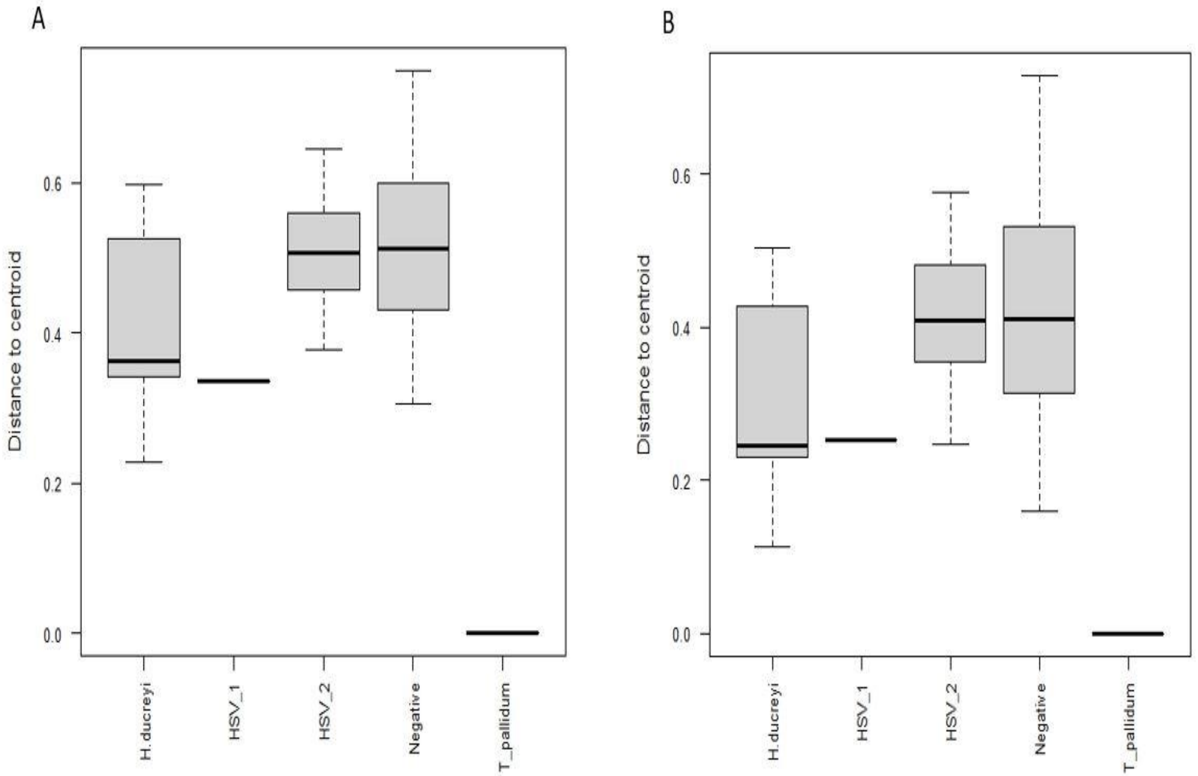


Figure 4.23 Dissimilarity (beta diversity, i.e. Jaccard distance (A) and Bray-Curtis distance metrics (B)) among the microbiota of lesions that were *HSV-1*-positive, *H.ducreyi*-positive, HSV-2-positive or *T. pallidum*-positive with the multiplex PCR. Statistical significance was only tested for *HSV-2*-positive and *H.ducreyi*-positive lesions. No significant dissimilarity between the HSV-2-positive and *H.ducreyi*-positive lesions (Tukey pairwise test, p-adjusted = 0.203 for Bray Curtis distance and p-adjusted =0.145 for Jaccard distance) were identified.

Additionally the NMDS coordinate plot for the Bray-Curtis distance and Jaccard distance metrics did not show any distinct separation of lesions associated to the presence of either *H.ducreyi* or HSV-2 in the lesions (Figure 4.24).

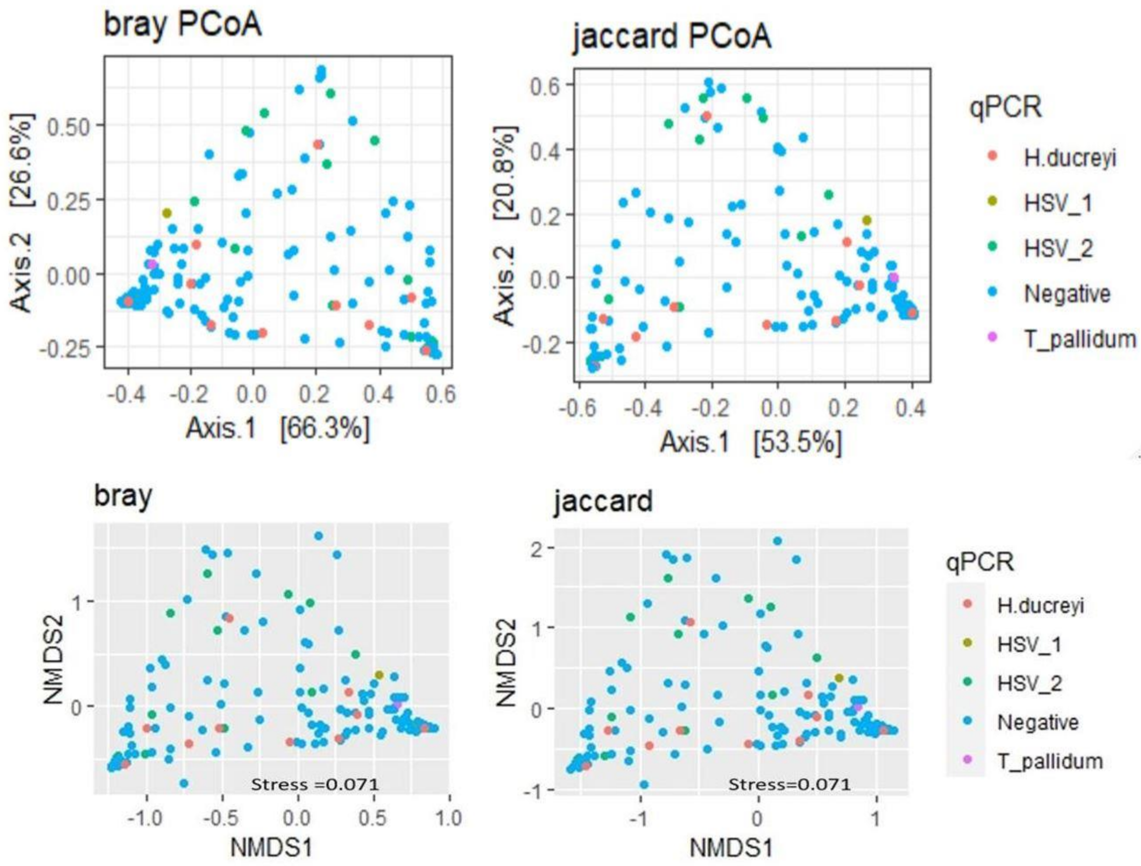


Figure 4.24 NMDS and PCoA ordination plot of Bray-Curtis and Jaccard distances of the microbiota of lesions that were *H. ducreyi*-positive, HSV-1-positive, HSV-2-positive and *T. pallidum*-positive with the multiplex PCR. There was no distinct separation between the *H. ducreyi-positive* lesions and HSV-2-*positive* lesions. Lesions that are closer to each other tend to have similar microbiota.

Chapter 5

5. Discussion

5.1 Prevalence of yaws and syphilis in Ashanti region of Ghana

This study used serological testing to assess the prevalence of treponematoses in the Ashanti region of Ghana. The seroprevalence of treponematoses in participants with yaw-like lesions and syphilis-like lesions were 17.3% (CI=11.4%-25.4%) and 10.8% (CI-4.7%-23%) respectively (Figure 4.5A). This reported seroprevalence of yaws is non-negligible when compared to the seroprevalence from other yaws endemic countries, where the lowest range (0.2%) has been reported from Benin (Ayelo *et al.*, 2019), and falls within other reported seroprevalences, including 10.9% from the Eastern region in Ghana (Abdulai *et al.*, 2018), 13.0% from Vanuatu (Fegan *et al.*, 2010) and 19.0% from the Solomon Islands (Marks, *et al.*, 2015). It is however lower than the 40.8% of yaws prevalence reported from Papua-New Guinea (Ayove *et al.*, 2014).

Likewise, the 10.8% seroprevalence of treponematoses from participants with syphilis-like lesions in this study falls within the range reported by Owusu-Ofori, Parry and Bates (2011), i.e., 3.5% and Ampofo *et al.*, (2002) i.e., 13.5%, from different parts of Ghana. The seroprevalence of syphilis reported here for Ghana is also consistent with other studies, including an African cohort study from Kenya, Tanzania, Ugandan and Nigeria, where a global syphilis seroprevalence of 5.3% was reported (Gilbert *et al.*, 2021), and a systemic review and meta-analysis on the prevalence of syphilis among men who have sex with men, where a worldwide prevalence of 7.5% was recorded (Tsuboi *et al.*, 2021). Interestingly, in their cross-sectional study of pregnant women from rural area in Ghana, Völker *et al.*, (2017) did not record any active case of syphilis, despite 5% of their participants testing positive for the treponemal test solely, indicating past or latent infection.

In addition to the serological test (DPP), multiplex PCR and 16S rRNA gene sequencing approaches were performed for two major purposes. The first purpose was to confirm serological testing, i.e., it was expected that DPP-positive individuals would show the presence/amplification of *Treponema* DNA from their lesions. This is because serological testing is known to miss the diagnosis of the very early stage of *T. pallidum* infection, when

treponemes are in skin lesions, but not enough antibodies are present yet for detection by the DPP test since detectable antibodies to *T. pallidum* infection is after two weeks to 45 days of infection (Mitjà, Šmajs and Bassat, 2013). The second purpose was to characterise the microbiota of these skin lesions and identify other microorganisms that may co-exist in these lesions (including *H. ducreyi*, HSV-1 and HSV-2). The multiplex PCR identified *Treponema* in one yaws-like lesion but not in any of the syphilis-like lesions. Hence, when multiplex PCR was considered alone, the estimated prevalence of treponematoses is 0.9% from yaws-like lesions (Figure 4.6), or about 0.7% from all lesions sampled. However, the participant whose yaws-like lesion was multiplex PCR positive for *T. pallidum* was negative on the DPP serological test.

The 16S rRNA gene sequencing identified *Treponema* in two yaws-like lesions and one syphilis-like lesions (Table 4.2). When 16S rRNA gene amplification approach is considered alone, the estimated prevalence of the treponematoses is 1.8%, 2.2% or about 2% in yaws-like, syphilis-like, or all lesions, respectively. One of the participants whose yaws-like lesion was *Treponema* positive with 16S rRNA gene sequencing was also positive on the DPP test (Table 4.2). However, this was not the case for the other yaws-like lesion and syphilis-like lesion that were 16S rRNA *Treponema* positive, i.e., the participants both showed negative DPP test results.

There were 23 lesions that showed a positive DPP test (serologically positive for *T. pallidum*), but *Treponema* DNA was not recovered with the multiplex PCR nor with 16S rRNA gene sequencing (Table A5 in appendix 6). Such situations are often encountered in studies involving yaws-lesions sampling fieldwork (Mitjà *et al.*, 2014; Abdulai *et al.*, 2018; Camila *et al.*, 2021). Mitjà *et al.*, (2014) and Abdulai *et al.*, (2018) explained that DPP positive individuals with negative *T.pallidum* DNA amplification may be in the latent stage of the disease where they can be positive for serological testing (both treponemal and non-treponemal tests), but the existing lesion may host other organisms at the time of sampling. In such situations, *Treponema* DNA may not be present in enough quantities, in comparison to other micro-organisms, to be detected by the molecular approaches used here (multiplex PCR and the 16S rRNA gene sequencing). It is widely known that the viability of the treponemes outside the host is very limited and that they are delicate to withstand any manipulation that can be used to preserve them (Edmondson, Hu and Norris, 2018). These features are associated to the challenges of

growing treponemes in traditional laboratory conditions. They may also facilitate the degradation of *Treponema* DNA in biological samples, especially in sampling locations where yaws is endemic and where the climate tends to be warm and humid.

Noteworthy, *Treponema* DNA recovery in the multiplex PCR and 16S rRNA gene sequencing in this study was low when compared to the seropositivity of treponemal antibodies. A basic local alignment search tool (BLAST) query showed the multiplex PCR primers targeting *Treponema pallidum* amplify the 16S rRNA gene. The results of the multiplex PCR and the 16S rRNA gene sequencing were expected to be the same in terms of detection of *Treponema* DNA. The differences between these molecular results can however be explained by distinct DNA conservation during courier service shipment of the samples to the University of Zurich for multiplex PCR and to the University of North Carolina for the 16S rRNA gene sequencing. The DNA reaching the University of Zurich for multiplex PCR was of better quality because the cold chain had not been broken during transportation between the University of Westminster, United Kingdom and the University of Zurich Switzerland.

Other factors may have affected the conservation of the DNA in skin lesion samples after they were collected. First, for funding reasons, one lesion per participant was swabbed, while swabbing distinct lesions may have increased the chances of *Treponema* DNA amplification. Second, lesion swabs were placed in phosphate buffer saline (PBS) (Cocks *et al.*, 2016; Handley *et al.*, 2019) rather than using specific DNA/RNA shield. DNA/RNA shield reagents have been shown to conserve DNA better when temperature fluctuates (Kazantseva *et al.*, 2021). This may be a major alternative for improving DNA conservation in sampling situations such as those encountered in Ghana, where warm and humid conditions, as well as unstable refrigeration (due to instability of electricity supply), can affect the stability of DNA molecules. There were further delays in the transportation of the DNA samples from Ghana to the United Kingdom. These various factors created suboptimal conditions for DNA conservation, which could also have facilitated degradation of DNA, and fragilised even further the already sensitive *Treponema* DNA. Nonetheless, when Dobay *et al.*, (2019) compared the taxonomic composition and clustering patterns of samples processed shortly after collection from the same samples exposed for 30 days at indoor environmental conditions (e.g., temperature of 25°C) prior to

analysing their sequencing data (to see the effect of environmental changes to 16S rRNA conservation), they showed that even exposed samples conserved a microbial signature that enabled distinguishing the bodily origin. This implies that, even if DNA conservation was not optimal in this study, the taxonomic distribution observed in the samples analysed should still be fair representatives of the original yaws-like and syphilis-like lesions.

The yaws eradication program, the Morges strategy, aimed at reducing the incidence of yaws to zero through total community treatment (TCT) with administration of single dose of azithromycin where yaws cases are present (WHO, 2012). The routine diagnosis of treponematoses (yaws and syphilis) and the Morges strategy state diagnosis of active treponematoses is when an individual with skin lesions is tested serologically positive for both treponemal and non-treponemal tests (WHO, 2012; Marks *et al.*, 2014). All participants who were DPP-positive in this study tested positive for both the treponemal and non-treponemal test, hence the finding of 19 (17.2%) seropositive cases of *T. pallidum*, one (0.9%) PCR *T. pallidum* positive and two (1.8%)*Treponema* positive in the 16S rRNA genefrom yaws-like lesions (Table 4.2) in this study confirms yaws is still present in these communities in Ashanti region of Ghana, especially in the Afigya Kwabre South district (Adweratia and Mpobi), where 18 out of the 19 seropositive cases were found (Figure 4.5B). Interestingly, out of the two yaws-like lesions that were positive for *Treponema* in the 16S rRNA gene sequencing, one was found at Adweratia and the other at Mpobi. These two communities are adjacent in the Afigya Kwabre South district. Additionally, the ethnic group that was disproportionately affected by yaws in this geographical region were the Northerners, leaving in deprived communities in Ashanti region of Ghana. This highlights specific areas for prioritising yaws-eradication efforts locally.

Furthermore, the WHO launched a campaign globally to eliminate congenital syphilis through antenatal screening and treatment (WHO, 2008). The seroprevalence of 10.8% of *T. pallidum* and the presence of one *Treponema* in syphilis-like lesions in the 16S rRNA gene sequencing in this study confirms active or latent syphilis in these communities in Ghana. To eliminate any sexually transmitted disease in specific group of people such as pregnant women, the disease needs to be eliminated from the general population as well. In this study, the median age of

participants with syphilis-like lesion was 29 with minimum age of 19 and maximum age of 75 (Figure 4.2). This implies most of these infected participants are sexually active and could transmit the *T. pallidum* infection to an unborn baby (Giacani and Lukehart, 2014). The prevalence of both yaws and syphilis in this study show both syphilis and yaws co-occur in the same geographical location (Ashanti region) of Ghana.

Yaws and syphilis are widely known to affect distinct cohorts (children and adolescents for yaws and adults for syphilis) so they have been studied and recorded independently. The results of this study emphasise they should be investigated together in places where they co-occur, or at least in areas where yaws is endemic. For a better understanding of *T. pallidum* biology, and ultimately reaching yaws eradication and syphilis elimination, more studies on the prevalence of syphilis and of its causative agent in yaws endemic countries are needed. This is strengthened further by recent evidence of inter-species recombination (Pětrošová *et al.*, 2012; Staudová *et al.*, 2014; Arora *et al.*, 2016; Grillová *et al.*, 2019; Pla-Díaz *et al.*, 2021; Noda *et al.*, 2022). This is likely to happen during co-infection of an individual by two different syphilis clades (Nichols-like or SS14-like) or by two different *Treponema* subspecies (Pětrošová *et al.*, 2012; Staudová *et al.*, 2014; Arora *et al.*, 2016; Grillová *et al.*, 2019; Pla-Díaz *et al.*, 2021; Noda *et al.*, 2022). Since *T. pallidum* can remain latent for years, it implies an individual in yaws endemic region can aquire *T. pallidum pertenue* (yaws treponemes) at childhood (which can remain latent for years) and also *T.pallidum pallidum* (syphilis-treponemes) as young adult, leading to a co-infection of both these treponemes. Such situation would enable recombination of yaws and syphilis treponemes.

Variants resulting from recombination can pose diagnostic challenges, because they can involve changes of conserved regions of DNA/RNA or proteins, which tend to be the molecular targets of both serological and molecular diagnoses (Marks *et al.*, 2018; Pla-Díaz *et al.*, 2021; Noda *et al.*, 2022). In those situations, non recombinant antigens (used in serological test reagents) may not react properly with recombinant antibodies (produced by infected individual), inducing false negative diagnosis (Marks *et al.*, 2018; Pla-Díaz *et al.*, 2021; Noda *et al.*, 2022). Marks *et al.*, 2018 reported that Solomons island strains of *T. pallidum subsp pertenue* were undetectable by the Centers for Disease Control and Prevention (CDC) diagnostic PCR assay

(2015 CDC real-time PCR assay) used in some studies (Chi *et al.*, 2015; Marks *et al.*, 2018). They reported this was due to recombinant regions in Solomon Island strains, where single nucleotide polymorphisms in the tp0858 gene region introduced sequence variation in the primer binding site of this CDC assay. This would in turn invalidate epidemiological data, which is essential to inform yaws eradication policies.

5.2 *H. ducreyi* and HSV-1 in yaws-like lesions

The multiplex PCR results from this study showed 9.1% (10/110), 1.8% (2/110) and 0.9% (1/110) of the yaws-like lesions were positive for *H. ducreyi*, HSV-1 and *T. Pallidum*, respectively (Figure 6). Additionally, 6.3% (7/110) of the yaws-like lesions were *Heamophilus*-positive with the 16S rRNA gene sequencing (Table 3). One yaws like lesion that was positive for *H. ducreyi* in the multiplex PCR was also *Heamophilus*-positive with 16S rRNA gene sequencing. The presence of *H. ducreyi* in yaws-like lesions in this study is consistent with previous studies on yaws conducted in Papua new Guinea (Mitjà *et al.*, 2014), Ghana (Ghinai *et al.*, 2015), Vanuatu (Pillay *et al.*, 2016), the Solomon Islands (Marks *et al.*, 2015) and Cameroon (Ngono *et al.*, 2021), where *H. ducreyi* was reported as an important cause of skin ulcers and also contributing to more than half of cutaneous ulcers in yaws endemic areas. A shotgun metagenomics sequencing study on DNA extracted from ulcers from children in Papua New Guinea showed that 23% of the specimen collected were infected with *H.ducreyi* and 16% were infected with *T. pallidum peternue* (yaws treponemes) (Noguera-Julian *et al.*, 2019). Lesions of *H. ducreyi* are extremely difficult to differentiate clinically from yaws and this bacterium can be found in individuals that are both seropositive and seronegative for yaws (Abdulai *et al.*, 2018). This is consistent with this study, where out of the 10 samples that were *H. ducreyi*-positive, two were seropositive and eight were seronegative on the DPP test.

Several studies have reported oral-facial (Westley, Seymour and Staines, 2011; Yildirim, Sengüven and Demir, 2011; Ramchandani *et al.*, 2016) and genital infections (Gupta, Warren and Wald, 2007; Biškup, Uršič and Petrovec, 2015; Parra-Sánchez, 2019) of HSV-1 worldwide but there seems to be paucity of data of HSV-I on other skin sites other than the oral-facial and genital site. In this study, HSV-1 was identified in two (1.8%) yaws-like lesions. This is the first

time HSV-1 has been reported from yaws-like lesions from yaws endemic area. Similar report of HSV-1 infection from other skin conditions has been documented; Tojo *et al.*, (2003) reported four (5.8%) cases of HSV-1 infection from 17 lesions of patients with Bechet's disease, Sweet's disease and erythema nodosum, Jevšnik *et al.*, (2020) also reported 23% of HSV-1 infection from suspected Herpes virus infected skin lesions using both real time PCR and isothermal amplification, and Sun *et al.*, (2003) also reported 66.7% of HSV-1 prevalence from patients with erythema multiform.

5.3 HSV-2 in syphilis-like lesions

All the syphilis-like lesions that showed amplification of DNA with the multiplex PCR in this study were HSV-2 (Figure 4.6). This finding was consistent with that of James *et al.*, (2020), who reported HVS-2 as the leading cause of genital ulcers worldwide. The prevalence of 28.26% (13/46) of HSV-2 recorded in this study was also consistent with the 52.7% and 38.5% prevalence reported by Magdaleno-Tapial *et al.*, (2020) in a sexually transmitted infection clinic in Valencia, Spain and Machiha *et al.*, (2018) in Zimbabwe respectively. Looker *et al.*, (2020) also reported 9.2 % prevalence of HVS-2 in Africa in a meta-analysis of HSV2-infections in genital ulcer disease (GUD). Additionally, Looker *et al.*, (2020) reported 4.8%, 2.6%, 2.6%, 1% and 4.8% of HSV-2 prevalence in GUD from Americas, South-East Asia, West Pacific, Eastern Mediterranean and globally, respectively. High seroprevalence of 23.6%, 46.0%, 62.2% of HSV-2 have also been reported among men who have sex with men (MSM), HIV positive patient and female sex workers (FSW) respectively (AlMukdad *et al.*, 2021). In contrast, low seroprevalence of 0.5% and 0.7% of HSV-2 have been recorded among Pakistanis and Yemenis, respectively (Dargham *et al.*, 2018). While Debrah *et al.*, (2022) reported 4.3% prevalence of *HSV-2* from vaginal and cervical swabs of asymptomatic women from Ghana, and seroprevalences of 78.4% of *HSV-2* among women attending routine cervical care clinic in two regions in Ghana, this study is the first to report the prevalence of *HSV-2* from genital lesions from the general population in Ghana.

5.4 The impact of other microorganisms other than *T.pallidum* in syphilis-like and yaws-like lesions

In this study, two participants who were DPP-positive had their lesions (yaws-like lesions) negative for *Treponema* with both PCR and 16S rRNA gene sequencing, but PCR positive for *H. duceyi.* Similarly, one participant who was DPP positive had his lesion (yaws-like lesion) negative for *Treponema* with both PCR and 16S rRNA gene sequencing, but PCR positive for HSV-1. Routinely, *T. pallidum* infection diagnosis is based on serological testing and clinical signs. In this situation, serological testing and clinical signs suggest *T. pallidum pertenue* (yaws) infection of these three individuals, but no *Treponema* DNA was identified in the corresponding lesion samples, while *H. ducreyi* or *HSV-1* DNA were, suggesting alternative causative agents of the current lesions, or - more likely co-infection of these treponemal lesions by these pathogens.

The presence of HSV-2 (Figure 4.6) as part of the microbiota of the syphilis-like lesions in this study is also a concern. HSV-2 is well known to enhance the spread of HIV by providing a favourable microenvironment that assists HIV infection in host (Crisci *et al.*, 2019). The dentritic cells (DCs) represent one of the first innate cell types that encounter HIV-1 and 2 in the genital mucosa. HSV-2 has been shown to modulate DCs, rendering them more receptive to HIV (Crisci *et al.*, 2019). In low-and middle-income countries, such as Ghana, resources for molecular diagnoses (PCR and sequencing) are not readily available and most diagnoses of genital ulcers, like syphilis, are based on clinical examination and serological diagnosis. Additionally, syndromic management of genital ulcer diseases (GUD) is adopted in most of these low-middle-income countries (Garrett *et al.*, 2018; Wi *et al.*, 2019). In syndromic management of GUD, all patients who present with genital ulcers receive a combined antibacterial and antiviral treatment targeting HSV-1 and 2, *T. pallidum pallidum* and *H. ducreyi* together, without any laboratory confirmation. Although the low-costs of syndromic management may outweigh that of specific treatment in low- middle-income countries, this can lead to unnecessary drug uses, possibly facilitating the emergence of antibiotic resistance strains, but also patient distress. Often, patients with low-income from these low-middle-income countries cannot afford these specific drugs to treat their GUD anyways. This contribute to the persistence of these diseases and to spreading other infections, such as HIV. Though targeted diagnosis may be expensive, it will

reduce cost for the patients and acquisition of antibiotic resistance. Precisely knowing the aetiological agent (or agents) for a particular disease will improve management and provide the correct epidemiological data, thereby informing health authorities and consequently disease elimination and eradication programs to target the group or communities that are specifically affected.

Even though taxonomic identification to species is not always possible with the 16S rRNA gene sequencing approach applied here, the presence of other medically important bacteria in these lesions (Table 4.3) is a concern because some of these bacteria identified could cause skin ulcers or infection of preexisting ulcers. Identification of these organisms to the species level matters to differentiate those species or subspecies that are pathogenic from those that are part of the expected flora. This also applies to the identification of *T. pallidum* subspecies, which is crucial, especially in places where both yaws and syphilis co-occur, such as Ghana. Although costly, alternative methods such as shotgun metagenomic sequencing (Noguera-Julian *et al.*, 2019) could enable distinguishing further taxonomic levels.

For example, four (3.6%) yaws-like lesions were positive for *Mycobacterium* in the 16S rDNA sequencing. *Mycobacterium ulcerans* is only one of the species belonging to the *Mycobacterium* genera that causes ulcerative lesions of the skin (buruli ulcer). It is one of the neglected tropical diseases (NTDs) which has been reported from at least 33 countries in Africa (including Ghana), South America and the West Pacific (WHO, 2023). The category 1 severity stage of buruli ulcer, where lesions are less than 5 cm, can also resemble yaws-lesions which can confuse clinical diagnosis (WHO, 2023).

Similarly, most of the genera that were identified under the phylum Proteobacteria with the 16S rDNA sequencing (e.g., *Pseudomonas*, *Klebsiella*, *E. coli-Shigella*, *Proteous* and *Salmonella*) have been associated with ulcer or wound infection. The *Staphylococcus*, *Streptococcus* and *Corynbacteria* identified in these lesions can also cause ulcers or wound infections (Pastar *et al.*, 2013; Serra *et al.*, 2015; Meinel *et al.*, 2016; Nelson *et al.*, 2016; Talan *et al.*, 2016; Hotterbeekx *et al.*, 2017) though some strains under these genera are normal flora of the skin for natural protection against pathogenic organisms (Meisel *et al.*, 2018; Flowers and Grice, 2020; Chen *et*

al., 2021). However, when opportunity arises, such as compromised immune system or already existing disease (e.g., yaws-like lesions and diabetic foot ulcer), these normal florae can also cause infection (opportunistic infection) (Park *et al.*, 2019; Severn and Horswill, 2022). Some species of these genera, such as *Staphylococcus* (methicillin resistant *Staphylococcus aureus* (MRSA)) and *Pseudomonas* (*Pseudomonas aeruginosa*), are known to be multidrug resistant (MDR) (Raman *et al.*, 2018; Horcajada *et al.*, 2019; Zehra *et al.*, 2019; Tan *et al.*, 2020; Silva *et al.*, 2021). MDR bacteria are resistant to several groups of antibiotics including azithromycin which is used to treat yaws and GUD.

The goal of yaws eradication and elimination of syphilis is to have a total healing of these ulcers to improve the wellbeing of children and adults. The use of single dose of azithromycin might not be effective to treat these MDR bacteria and other organisms which may be present in these yaws-like lesions. Abdulai *et al.*, (2018), reported that yaws-like lesions caused by *H. ducreyi,* or other unknown pathogens, may continue to persist after a single-round of mass treatment. Following the WHO guidelines (WHO, 2012), participants of this study who tested positive on the DPP test were given azithromycin with the aim of seeing total resolution of the lesions. Unfortunately, the COVID-19 pandemic limited the timelines and there were consequently no follow-ups of these participants to determine if the lesions had resolved completely. When lesions do not resolve after treatment, a wrong impression may be created among the populace and health authorities that yaws has not been eradicated from a previously endemic community. This can have a negative perception of yaws eradication programme. All these aspects argue for the need for investment in molecular diagnoses (PCR and sequencing) in yaws endemic countries for diagnosing accurately and treating appropriately these lesions in order to achieve total resolution of these lesions.

5.5 Microbial diversity of yaws-like and syphilis like lesions

Since the etiological agents of yaws (*T. pallidum pertenue*) and syphilis (*T. pallidum pallidum*) are genetically and morphologically identical, and their clinical symptoms resemble each other, it was investigated if the microbiota of yaws-like lesions and syphilis-like lesions are also similar. The alpha diversity (intra sample variation) as measured with the Shannon and Simpson metrics

indicated that the syphilis-like lesions were more diverse than the yaws like lesions, but the beta diversity (inter sample variation) as measured with the Jaccard and Bray Curtis distances metrics suggested no significant dissimilarity between the microbial populations of yaw-like and syphilis-like lesions (Figure 18) (PERMONOVA, F=3.644, p-value=0.061 for Jaccard and PERMONOVA, F=3.595, p-value=0.062 for Bray Curtis). This means that even though both lesions (yaws-like and syphilis-like) share similar microbiota, the abundances of specific genera may differ. This was evident by the finding of all 23 bacteria phyla that were identified in this study in both yaws-like and syphilis-like lesions (Figures 4.10). Additionally, when the phyla that represent at least 1% of reads in terms of abundance in at least one sample were considered, nine of them (*Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, *Fusobacteria*, *Patescibacteria*, *Proteobacteria* and *Tenericutes*) out of 14 were identified in both yaws-like and syphilis-like lesion (Figures 4.11 and 4.12). Also, the top three phyla in syphilis-like lesions in terms of relative abundance were *Proteobacteria* followed by *Actinobacteria* and *Firmicutes* (Figures 4.14). Similarly, the top three phyla in yaws-like lesions were *Firmicutes* followed by *Proteobacteria* and *Actinobacteria* (Figures 4.14).

Chapter 6

6 Conclusion

Syphilis and yaws can both lead to severe physical deformities, affecting the healthy development of children and families. When diagnosed early, these diseases can be cured with antibiotics. They however persist in countries where health care is not accessible to all. It has been shown that potential socio-economic status affects child health and child health relates to future educational and labour market development (Currie, 2009). However, diseases negatively impact on children education (Glewwe and Miguel, 2007; Rai *et al.*, 2009). Consequently, studies seeking to improve children's health status, such as the one presented here, will positively affect not only their own education outcome, but also the economic development of their countries (Glewwe and Miguel, 2007).

This study was conducted in Ghana, one of the countries where yaws is endemic. The prevalence of the treponematoses syphilis and yaws were assessed and the co-occurrence of pathogens causing similar skin lesions determined**.** Additionally, the microbial diversity of yaws-like and syphilis-lesions were evaluated and compared.

A seroprevalence of 17.2% and 10.8% of *T. pallidum* in individuals with yaws-like and syphilis-like lesions, respectively, and the identification of *T. pallidum* with multiplex PCR and 16S rDNA sequencing in this study show the co-occurence of treponeme strains causing yaws and syphilis within the same geographical region, the Ashanti region of Ghana.

Regarding the commonly known organisms that cause lesions similar to those of yaws and syphilis, *H. ducreyi* was more prevalent in yaws-like lesions, while HSV-2 was more prevalent in syphilis-like lesions. Additionally, *Mycobacterium*, also known to cause similar skin-lesions, was found in 3.6% of children with yaws-like lesions.

Although the intra-sample variation (alpha diversity) of the microbiota of syphilis-like lesions were significantly more diverse than yaws-like lesions, these two types of lesions did not show any significant dissimilarity (beta diversity). Other medically important bacteria that can infect the skin or genital area, such as *Pseudomonas*, *Gardnerella*, *Sneathia*, *Escherichia- Shigella*,

Salmonella, *Proteous* and *Acinetobacter* were identified in both yaws-like and syphilis-like lesions.

The presence of other organisms apart from *T. pallidum* in yaws-like and syphilis-like lesions in this study can prevent the total healing of these lesions and full recovery of the patients. This may negatively affect the goal of yaws eradication by 2030 (WHO, 2021a) and elimination of congenital syphilis. This study contributes to a better understanding of the microbiota of yaws-like and syphilis-like lesions from children and adults in Ghana, which should guide the diagnosis, treatment, elimination, and eradication policies of these treponematoses.

Any programs that contribute to improve child education positively affects “the development of respect for human rights and fundamental freedoms”, as stated in Article 29 of the United Nations Convention on the Rights of the Child. Long-term benefits of studies such as the research presented here address the Human Rights, Good Governance and Social Justice global challenge area (GCRF, 2021). In addition, such research contributes to providing an Equitable Access to Sustainable health and wellbeing in Ghana, thereby directly addressing another of the global challenges (GCRF, 2021). These aspects highlight the importance of investing into conducting research such as the one presented here.

Appendices

1 Data used in the review in chapter 2

1.1 The prevalence of yaws and syphilis in published records

Table A1 The 28 Published record data on the prevalence of yaws

Country	location	year	Diagnosis	Sample size	Positive cases	References
Benin	Toff o, Zé, Allada	2012	RPR	900	22	(Mitjà *et al.*, 2015)
Benin	Ze	2018	DPP	1837	4	(Ayelo *et al.*, 2019)
Central Africa Republic	Lobaye	1990	VDRL, TPHA	213	54	(Herve *et al.*, 1992)
Congo	Bétou, Ebyellé	2012	RDT	6215	183	(Coldiron *et al.*, 2013)
Congo DR.	Wasolo	2005	RPR, TPHA	1176	56	(Gerstl *et al.*, 2009)
Cote d'Ivoire	Adzopé	2004	RPR	2182	11	(Konan *et al.*, 2007)
Ecuador	Santiago basin	1993	VDRL, FTA-Abs	1118	69	(Anselmi *et al.*, 1995)
Ecuador	Santiago basin	1998	VDRL, FTA-Abs	1926	68	(Anselmi *et al.*, 2003)

Country	location	year	Diagnosis	Sample size	Positive cases	References
Ghana	Eastern region	2013-2014	DPP	943	103	(Abdulai *et al.*, 2018)
Ghana	Eastern region	2008	Clinical	208,413	4,006	(Agana-Nsiire *et al.*, 2014)
Ghana	Four regions	2020	DPP	625	401	(Basing *et al.*, 2020)
Guyana	Bartica	2000	MHA-TP	1020	52	(Scolnik *et al.*, 2003)
Indonesia	Sumatra	1988	VDRL, TPHA, FTA-Abs, TmpA EIA,	37000	114	(Noordhoek *et al.*, 1991)
Indonesia	Jayapura City	2016	TPHA	322	111	(Sitanggang, 2017)
Nigeria	Garkida	1998	Clinical	1523	64	(Akogun, 1999)
Papau New Guinea	Karkar Island	1988	VDRL, FTS-Abs, and TPHA	632	26	(Backhouse *et al.*, 1998)
Papau New Guinea	Port Moresby-NCD	2001	VDRL, TPHA	227	33	(Manning and Ogle, 2002)

Country	location	year	Diagnosis	Sample size	Positive cases	References
Papau New Guinea	Lihir villages	2015-2016	TPHA, RPR	95353	532	(Mooring, Mitjà and Murray, 2018)
Papua New Guinea	Lihir Island villages	2013	TPHA, RPR	703	287	(Ayove *et al.*, 2014)
Philippines	Mindanao	2017	TPPA, DPP	96	4	(Dofitas *et al.*, 2020)
Solomon Islands	Western and Choiseul Provinces	2013	TPHA, RPR	1497	285	(Marks *et al.*, 2015)
Solomon Islands	Western and Choiseul Provinces	2013	TPHA, RPR, DPP	415	54	(Marks *et al.*, 2015)
Timor-Leste	Oecusse, Bobonaro, Cova Lima, Atauro Island	2007	Clinical	1535	6	(dos Santos *et al.*, 2010)
Vanuatu	Tanna Island	1989	VDRL	20200	464	(Harris *et al.*, 1991)
Vanuatu	Santo Island	2001	VDRL	273	57	(Capuano and Abel, 2003)

Country	location	year	Diagnosis	Sample size	Positive cases	References
Vanuatu	Tanna Island	2008	VDRL, TPHA	306	95	(Fegan *et al.*, 2010)
Vanuatu	Northernmost Provinces	2014	RDT	821	2	(Taleo *et al.*, 2017)
Wallis and Futuna	Wallis and Futuna	2010	RPR, TPHA	264	27	(Guerrier *et al.*, 2011)

This table was generated by an extensive search using the following search engines: University of Westminster Library Search, Science Direct, PubMed, MEDLINE, Google Scholar, and Web of Science for ("yaws" OR "treponematosis" AND "prevalence OR "incidence" OR "diagnosis") OR ("yaws" AND [each current yaws-endemic country]). Studies on the prevalence of yaws can vary greatly in the way they have been conducted. Therefore, articles that provided information on (i) the country where the study was conducted, (ii) the number of positive cases and the studied sample size (iii) The diagnostic method(s) used (either clinical or at least one serological test) and (iv) dates when studies were conducted, started and/or ended (if different) were specifically selected. Abbreviations used in Table 1 are defined as follow: VDRL= Venereal Disease Research Laboratory test, TPHA = *Treponema pallidum* haemagglutination test, RPR= Rapid Plasma Reagin test, DPP= Duah Path Platform syphilis screen and confirmation test, FTA-Abs=Fluorescent Treponemal Antibody Absorbed test, EIA= Enzyme-linked Immunosorbent Assay, RDT=Rapid Diagnostic Test, MHA-TP= Microhemagglutination assay for *Treponema.*

Table A2. The 61 Published record data on the prevalence of syphilis

Country	Location of study	Year of study	Diagnostic method used	Identifiable group	Sample size of study	Number of positive cases of syphilis	References
Saudi Arabia	Riyadh	2005-2015	IgM Elisa	BD	239330	60	(Elyamany *et al.*, 2016)
China	Xi an region	2006-2010	ELISA	BD	159902	575	(Chen *et al.*, 2014)
Italy	Liguria	2009-2013	VDRL, TPHA	BD	419740	132	(Drago *et al.*, 2014)
Togo	Eight cities	2017	DPP	DRUG USERS	477	5	(Ekouevi *et al.*, 2019)
Togo	Lome Sa vana	2011	TPHA, RDT	FSW	1836	41	(Halatoko *et al.*, 2017)
China	Jinan	2008	RPR, TPHA	FSW	363	11	(Liao *et al.*, 2012)
China	Eastern part	2009	ELISA, TRUST	FSW	7118	356	(Chen *et al.*, 2012)
China	Jinan	2009	RPR, TPPA	FSW	432	11	(Liao *et al.*, 2012)
China	Nan-chang	2011-2012	RDT	FSW	361	157	(Tao *et al.*, 2014)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Brazil	Teresina Piaui	2016	NA	FSW	358	27	(Borges *et al.*, 2017)
Morro-cow	Agadir Rabat Fes Tangier	2012	VDRL, TPHA	FSW	1447	256	(Oukouchoud *et al.*, 2017)
Togo	Eight cities	2017	DPP	FSW	1003	8	(Ekouevi *et al.*, 2019)
Brazil	12 cities	2016	RPR	FSW	4245	361	(Ferreira-Júnior *et al.*, 2018)
Brazil	Rio de Janeiro	2015	RPR MHA TP	Inmates	284	28	(Cunha *et al.*, 2015)
Brazil	Sao Paulo State	2003	VDRL, FTA -Abs	Inmates	333	10	(Coelho and Passos, 2011)
Pakistan	Karachi	2007-2008	TPHA	Inmates	349	31	(Kazi *et al.*, 2010)
Brazil	Espirito Santo state	1997	VDRL, FTA -ABS	Inmates	121	19	(Miranda *et al.*, 2000)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Kyrgyz-stan	Prisons in Kyrgyzsta n	2014	RPR, MHA-TP	Inmates	368	71	(Azbel *et al.*, 2016)
Ghana	Nation-wide	2004-2005	TPHA	Inmates	7652	226	(Adjei *et al.*, 2006)
China	NA	2010	VDRL, TPHA	MSM	28739	3276	(Jun-Jie *et al.*, 2011)
USA	Nation-wide	2000	NA	MSM	5873	441	(Heffelfinger *et al.*, 2007)
USA	Nation-wide	2001	NA	MSM	6100	1653	(Heffel-finger *et al.*, 2007)
USA	Nation-wide	2002	NA	MSM	6862	3257	(Heffel-finger *et al.*, 2007)
USA	Nation-wide	2003	NA	MSM	7177	4424	(Heffel-finger *et al.*, 2007)
Ecuador	North-west	2016	VDRL, TPPA	MSM	291	19	(Hernandez *et al.*, 2017)
Moro-ccow	Agadir	2010-2011	VDRL, TPPA	MSM	323	27	(Johnston *et al.*, 2013)
Moro-ccow	Marr-akech	2010-2012	VDRL, TPPA	MSM	346	37	(Johnston *et al.*, 2013)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Brazil	Nation-wide	2012-2014	NA	Inmates	241	21	(Domingues *et al.*, 2017)
Tan-zania	7 regions in Tanzania	2013	RPR, TPHA	FSW	1914	152	(Vu and Misra, 2018)
Rwanda	Nation-wide	2015	RDT, RPR	FSW	1010	156	(Mutagoma *et al.*, 2017)
China	Guangxi Province	2010-2012	ELISA, RPR	FSW	51790	536	(Chen *et al.*, 2015)
China	12 cities in 3 Province	2012-2013	RPR, TPPA	FSW	781	117	(C. Zhou *et al.*, 2014)
Peru	30 Peruvian cities	2002-2003	RPR, TPPA	FSW	4314	56	(Cárcamo *et al.*, 2012)
Indo-nesia	10 cities in Indo-nesia	2007	TPHA	FSW	4324	480	(Majid *et al.*, 2010)
Nepal	Nation-wide	2004-2015	RPR, TPHA	FSW	5958	230	(Kakchapati *et al.*, 2017)

Country	**Location of study**	**Year of study**	**Diagnos-tic method used**	**Identi fiable group**	**Sample size of study**	**Number of positive cases of syphilis**	**References**
Colum-bia	Santa Fe de Bogota	2001-2002	VDRL, MHATP	FSW	514	53	(Mejia *et al.*, 2009)
Argen-tina	six cities	2000-2002	ELISA	FSW	598	273	(Pando *et al.*, 2006)
Burkina Faso	five cities	2013-2014	RDT, RPR	FSW	1045	15	(Ouedraogo *et al.*, 2018)
China	Shanghai	2004-2005	TRUST, TPPA	MSM	475	64	(Choi *et al.*, 2007)
China	61 cities	2008-2009	NA	MSM	47231	5552	(Wu *et al.*, 2013)
China	Guangxi Province	2008	ELISA, RPR	MSM	1146	59	(Wang *et al.*, 2014)
China	Guangxi Province	2009	ELISA, RPR	MSM	1351	90	(Wang *et al.*, 2014)
China	Guangxi Province	2010	ELISA, RPR	MSM	1257	99	(Wang *et al.*, 2014)
China	Guangxi Province	2011	ELISA, RPR	MSM	1314	81	(Wang *et al.*, 2014)
China	Guangxi Province	2012	ELISA, RPR	MSM	1301	109	(Wang *et al.*, 2014)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Burkina Faso	Ouagadougou Bobo Diou-lasso	2013	RDT, RPR	MSM	657	40	(Ouedraogo *et al.*, 2019)
Panama	David	2011	RPR, TPHA	MSM	204	35	(Hakre *et al.*, 2014)
Panama	Panama	2012	RPR, TPHA	MSM	306	77	(Hakre *et al.*, 2014)
Panama	Colon	2011	RPR, TPHA	MSM	93	29	(Hakre *et al.*, 2014)
Italy	Verona	2013-2015	TPHA, RPR	MSM	289	16	(Zorzi *et al.*, 2017)
Nepal	Terai	2016	RPR, TPPA	MSM	167	7	(Storm *et al.*, 2020)
Togo	Eight cities	2017	RDT	MSM	678	4	(Ekouevi *et al.*, 2019)
Brail	Sao Paulo State	2011	NA	MSM	227	44	(Prado *et al.*, 2017)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Brail	Sao Paulo State	2016	NA	MSM	338	103	(Prado *et al.*, 2017)
Indo-nesia	Jakarta	2002	RPR, TPPA	MSM	279	3	(Pisani *et al.*, 2004)
Ghana	Accra	2003	TPPA, VDRL	BD	536	40	(Adjei *et al.*, 2003)
Tanzania	Dar es Salaam	2004-2005	VDRL, TPHA	BD	1597	75	(Matee, Magesa and Lyamuya, 2006)
Burkina Faso	Koudougou	2009	RPR	BD	4520	179	(Nagalo *et al.*, 2011)
Mali	Nation-wide	2007	VDRL	BD	25543	84	(Diarra *et al.*, 2009)
Nepal	Kath-mandu	2008	ELISA	BD	21716	106	(Shrestha *et al.*, 2009)
Nigeria	North Central	2007-2010	NA	BD	9500	85	(Damulak *et al.*, 2013)
Nigeria	Osogbo	2007-2008	RDT, TPHA	BD	1410	16	(Buseri, Seiyaboh and

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
							Jeremiah, 2010)
India	Andra Pradesh	2004-2009	RPR	BD	8067	6	(Bhawani, Rao and Sudhakar, 2010)
Sudan	West sudan	2008	ICT	BD	620	2	(Elfaki, Eldour and Elsheikh, 2008)
Ethiopia	Somali region state	2010-2014	RPR	BD	627	50	(Abate and Wolde, 2016)
Nigeria	Yola	2007-2008	RPR, TPHA	BD	595	7	(Olokoba *et al.*, 2009)
Ghana	Nation-wide	2012	NA	BD	91386	3371	(Sarkodie *et al.*, 2016)
USA	Mary-land	2002	RPR, FTA-Abs	Inmates	3914	23	(Solomon *et al.*, 2004)
Brazil	Sao Paulo State	2007	VDRL, TPHA	Inmates	546	29	(El Maerrawi and Carvalho, 2015)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Pakistan	5 cities	2015-2016	RPR, RDT	Inmates	104	33	(Waheed *et al.*, 2017)
Pakistan	5 cities	2015-2016	RPR, RDT	FSW	101	32	(Waheed *et al.*, 2017)
Pakistan	5 cities	2015-2016	RPR, RDT	DRUG USERS	48	2(4.1)	(Waheed *et al.*, 2017)
USA	Texas	1999-2001	RPR	Inmates	336668	3890	(Baillargeon *et al.*, 2004)
Brazil	Minas Gerais state	1994	VDRL, FTA- Abs	Inmates	63	5	(Catalan-Soares, Almeida and Carneiro-Proietti, 2000)
Brazil	Mato Grosso do Sul	2013	ELISA, VDRL	Inmates	3363	356	(Correa *et al.*, 2017)
Russia	Moscow	2003	RDT	DRUG USERS	455	32	(Rhodes *et al.*, 2006)
Russia	Volgo-grad	2003	RDT	DRUG USERS	514	93	(Rhodes *et al.*, 2006)

Country	Location of study	Year of study	Diagnos-tic method used	Identi fiable group	Sample size of study	Number of positive cases of syphilis	References
Russia	Barnual	2003	RDT	DRUG USERS	504	32	(Rhodes *et al*., 2006)
Ger-many	North Rhine West-phalia	2005	TPHA, FTA-Abs	DRUG USERS	1181	7	(Scher-baum *et al*., 2005)

This table was generated by an extensive search using the following search engines: University of Westminster Library Search, Science Direct, PubMed, MEDLINE, Google Scholar, and Web of Science for “syphilis” OR “treponematosis” AND “prevalence” OR “incidence” OR “diagnoses”. Studies on the prevalence of syphilis can vary greatly in the way they have been conducted. Therefore, articles that provided information on (i) the country where the study was conducted, (ii) the number of positive cases and the studied sample size iii. The specific identifiable group investigated (i.e., blood donors (BD), men who have sex with men (MSM), female sex workers (FSW), drug users (DU) and inmates (prisoners), (iv) the diagnostic method(s) used (either clinical or at least one serological test) and (v) dates when studies were conducted, started and/or ended (if different) were specifically selected. Abbreviations used in this Table are defined as follow: VDRL= Venereal Disease Research Laboratory test, TPHA = *Treponema pallidum* Haemagglutination test, RPR= Rapid Plasma Reagin test, DPP= Duah Path Platform syphilis screen and confirmation test, FTA-Abs=Fluorescent Treponemal Antibody Absorbed test, ELISA= Enzyme-linked Immunosorbent Assay, RDT=Rapid Diagnostic Test, MHA-TP= Microhemagglutination assay for *Treponema,* EIA= Enzyme immunoassay, TRUST for Toluidine Red Unheated Serum Test, ICT= Immunochromatographic Test.

1.2 WHO data on the number of yaws cases reported from yaws endemic countries from 2008-2020 and the national prevalence of syphilis among pregnant women

Table A3: WHO repository data on the number of cases of yaws (2008-2020) (WHO, 2021)

Year Country	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
BEN	NA	NA	45	NA	11	NA	NA	NA	NA	NA	4	11	9
CMR	NA	NA	802	133	59	97	530	463	890	1237	2591	1713	NA
CAF	243	NA	NA	NA	230	NA	NA	NA	NA	NA	NA	NA	NA
COG	NA	646	NA	NA	197	NA	3	10	8	NA	NA	NA	NA
COD	383	NA	NA	NA	NA	NA	NA	NA	429	NA	27	40	45
CIV	NA	NA	3740	3343	3092	2256	2650	1940	1581	1749	3240	1749	564
GHA	20525	35248	18157	9674	8980	18702	2055	1636	1497	1917	1833	1483	4695
IDN	6083	7751	6178	6631	4360	2043	1521	3379	2762	1218	353	673	81
LBR	NA	NA	NA	NA	NA	NA	NA	2	NA	0	24	NA	NA

PNG	28989	25822	29061	28989	17560	19710	29886	28584	33953	25444	54082	69723	81369
PHL	NA	NA	NA	NA	NA	NA	NA	NA	NA	150	NA	NA	NA
SLB	NA	NA	20635	NA	12372	14909	13312	9736	18082	13415	13047	13694	NA
TLS	NA	NA	NA	NA	NA	NA	NA	NA	NA	3098	194	NA	NA
TGO	NA	NA	15	NA	NA	NA	13	7	11	NA	NA	NA	291
VUT	NA	2432	2017	2334	2508	1461	615	317	663	504	728	374	823

BEN= Benin, CRM=Cameroon, CAF= Central Africa Republic, COG= Congo, COD= Democratic Republic of Congo, CIV= Cote de Ivoire, GHA= Ghana, IDN= Indonesia, LBR=Liberia, PNG=Papua New Guinea, PHL=Philippines, SLB= Solomon Islands, TLS= Timor Leste, TGO= Togo, VUT= Vanuatu

Table A4 WHO repository data on national antenatal prevalence of syphilis from the 39 countries in the published records (WHO, 2020)

Country	NPSPW	WHO region	Yaws endemic country
Benin	0.4	Africa	Yes
Central African Republic	4.2	Africa	Yes
Congo	7.1	Africa	Yes
DR. Congo	4.8	Africa	Yes
Cote d Ivoire	3.9	Africa	Yes
Ecuador	0.34	Americas	No
Ghana	2.7	Africa	Yes

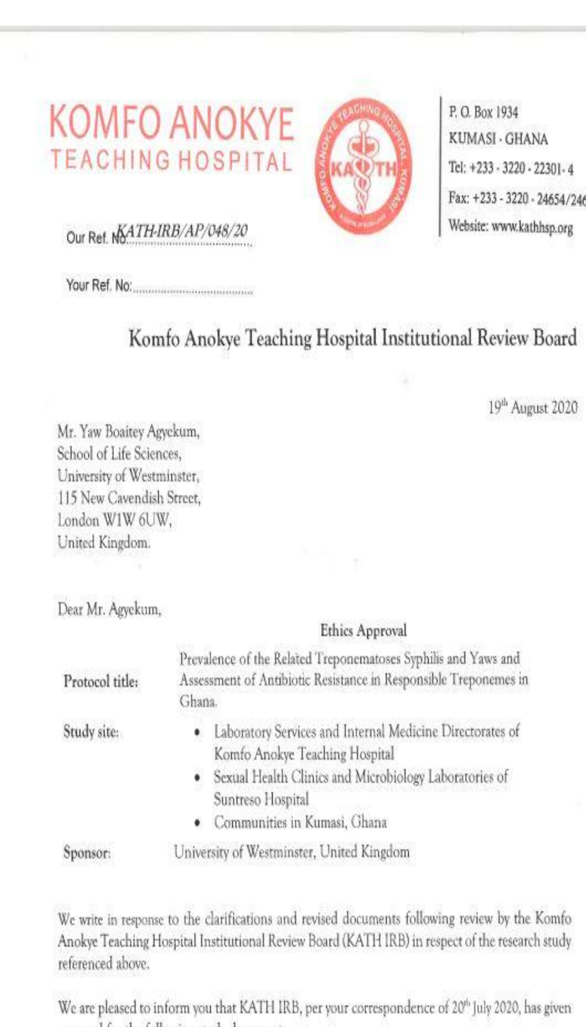
Country	NPSPW	WHO region	Yaws endemic country
Guyana	0.1	Americas	No
Indonesia	1.2	Southeast Asia	Yes
Nigeria	0.4	Africa	No
Papua New Guinea	6.1	West Pacific	Yes
Philippines	1.41	West Pacific	Yes
Solomon Islands	100	West Pacific	Yes
Timor Leste	0.52	South East Asia	Yes
Vanuatu	7.88	West Pacific	Yes
Wallis and Futuna	NA	West Pacific	No
Saudi Arabia	0	East Mediterranea	No
China	0.3	West Pacific	No
Italy	NA	Europe	No
Togo	1.5	Africa	Yes
Brazil	0.8	Americas	No
Pakistan	NA	East Mediterranea	No
Kyrgyzstan	0	Europe	No
USA	NA	Americas	No
Morocco	0.3	East Mediterranean	No
Rwanda	1.4	Africa	No
Peru	0.36	Americas	No
Nepal	NA	Southeast Asia	No
Columbia	1.1	Americas	No
Argentina	4	Americas	No
Burkina Faso	0.7	Africa	No

Country	NPSPW	WHO region	Yaws endemic country
Panama	1.7	Americas	No
Tanzania	NA	Africa	No
Mali	4.4	Africa	No
India	0.1	Southeast Asia	No
Sudan	2.3	Africa	No
Ethiopia	1.5	Africa	No
Russia	NA	Europe	No
Germany	0.1	Europe	No

NPSPW= National prevalence of syphilis among pregnant women

2. Ethics Approvals

2.1 Komfo Anokye Teaching Hospital ethics approval



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Our Ref. No: KATH-IRB/AP/048/20

Your Ref. No:..................................

Komfo Anokye Teaching Hospital Institutional Review Board

19th August 2020

Mr. Yaw Boaitey Agyekum,
School of Life Sciences,
University of Westminster,
115 New Cavendish Street,
London W1W 6UW,
United Kingdom.

Dear Mr. Agyekum,

Ethics Approval

Protocol title:	Prevalence of the Related Treponematoses Syphilis and Yaws and Assessment of Antibiotic Resistance in Responsible Treponemes in Ghana.
Study site:	• Laboratory Services and Internal Medicine Directorates of Komfo Anokye Teaching Hospital • Sexual Health Clinics and Microbiology Laboratories of Suntreso Hospital • Communities in Kumasi, Ghana
Sponsor:	University of Westminster, United Kingdom

We write in response to the clarifications and revised documents following review by the Komfo Anokye Teaching Hospital Institutional Review Board (KATH IRB) in respect of the research study referenced above.

We are pleased to inform you that KATH IRB, per your correspondence of 20th July 2020, has given approval for the following study documents:

- *Protocol version 1.3 last updated 20th July 2020*
- *Informed Consent Form version 1.2 last updated 20th March 2020*
- *Material Transfer Agreement, dated 14 July 2020*

Page 1 of 2

A Centre of Excellence

Approval for the study is in effect until 18th **August** 2021 and it is the responsibility of the Principal Investigator to maintain the study in good standing at the Komfo Anokye Teaching Hospital. The Board anticipates to be notified of the actual start date of your project.

Prior to the expiration of the study approval, you must submit to the KATH IRB an "Application for Continuing Review" along with provision of "Annual Report" when the study is ongoing, or a "Termination Report" if the research has been completed.

You must hastily report to the KATH IRB should a modification to the research be proposed, and without delay if an unanticipated development occurs before the next required review. Regulations do not permit you to modify conduct of the study in its present form prior to ethics approval; except where urgent action is required to eliminate an apparent immediate hazard to a study subject or other person. It is of utmost importance data generated from this study must be used for the intended purposes only.

Thank you.

Sincerely,

Prof. Kwabena Antwi Danso, BSc, MB ChB, FWACS, FGCS, FACOG
Chairman, KATH-IRB

Page 2 of 2

2.2 The University of Westminster Ethics approval

Project title: Prevalence of the related treponematoses syphilis and yaws and assessment of antibiotic resistance in the treponemes causing these diseases in Ghana.

Application ID: ETH2021-0285

Date: 19 Mar 2021

Dear Yaw

Thank you for providing your response to the Conditions set by the Committee.

Your response to Conditions has been considered by the Committee and your proposal is approved.

If your protocol changes significantly in the meantime, please contact me immediately, in case of further ethical requirements.

Yours,

Mandy Walton

LAS Research Ethics Committee

I am advised by the Committee to remind you of the following points:

Your responsibility to notify the Research Ethics Committee immediately of any information received by you, or of which you become aware, which would cast doubt upon, or alter, any information contained in the original application, or a later amendment, submitted to the Research Ethics Committee and/or which would raise questions about the safety and/or continued conduct of the research.

The need to comply with the Data Protection Act 2018 and General Data Protection Regulation (GDPR) 2018.

The need to comply, throughout the conduct of the study, with good research practice standards.

The need to refer proposed amendments to the protocol to the Research Ethics Committee for further review and to obtain Research Ethics Committee approval thereto prior to implementation (except only in cases of emergency when the welfare of the subject is paramount).

The desirability of including full details of the consent form in an appendix to your research, and of addressing specifically ethical issues in your methodological discussion.

The requirement to furnish the Research Ethics Committee with details of the conclusion and outcome of the project, and to inform the Research Ethics Committee should the research be discontinued. The Committee would prefer a concise summary of the conclusion and outcome of the project, which would fit no more than one side of A4 paper, please.

3 PARTICIPANT INFORMATION SHEET

3.1 PARTICIPANT INFORMATION SHEET FOR ADULT

Title of study: Prevalence of the related treponematoses syphilis and yaws and assessment of antibiotic resistance in responsible treponemes in Ghana. (This was the initial PhD topic, but it evolved with time).

Researcher(s): Yaw Agyekum Boaitey (University of Westminster - UoW)

Supervisors: Dr Alex Owusu-Ofori (Kwame Nkrumah University of Science and Technology-KNUST) Dr Saki Raheem (UoW)

You are being invited to take part in a research project on treponematoses syphilis and yaws. Syphilis and yaws are caused by bacteria called treponemes. Both diseases severely damage skin, bone, and cartilage. They are both transmitted by contact with infectious lesions, but syphilis is transmitted sexually or from mother to foetus, while yaws is not. The current distribution of these diseases in Ghana is not known accurately. This study therefore aims at identifying and reporting their occurrence to the Ministry of Health in Ghana and to the World Health Organization so that they can be managed and eradicated. The study aims to establish the burden of treponematoses in Ghana and to provide data on the microbiology of the infections. Data from the study will be used to inform health policies on the management of treponematoses in Ghana; provide education and serve as a foundation for future research on treponematoses in and outside Ghana.
This research is being undertaken as part of the researcher's studies for PhD programme at the UoW and is being carried out in collaboration with Dr Alex Owusu-Ofori at KNUST.
The study will involve:

1) Answering questions about you, including your year of birth, sexual preferences, ethnicity, previous record of treponemal infection and history of antibiotic treatment. This will take about 3 minutes.
2) Collecting finger-prick of blood to perform a point-of-care test that will tell us if you have had or are currently infected by treponemes. The discomfort generated here should not be more than a mild sting. If the test is positive for a current treponeme infection, we will offer you treatment for free with an appropriate antibiotic (penicillin for syphilis or azithromycin for yaws). This will take between 15 and 30 minutes.
3) Collecting swabs/aspirates from skin lesions if you have any. This is done by gently wiping your lesion using a sterile swab by experienced researcher. This will take about 2 minutes. This swab will be stored until further laboratory analyses are completed to diagnose the source of your infection. This information will be reported to inform health policies, but your sample will be destroyed after completion.

Please note:

- Your participation in this research is entirely voluntary.
- You have the right to withdraw at any time without giving a reason.

- Wherever practicable, withdrawal from the research will not affect any treatment and/or services that you receive.
- You have the right to ask for your data to be withdrawn as long as this is practical, and for personal information to be destroyed.
- You do not have to answer particular questions if you do not wish to do so.
- Your responses will be made anonymous, and will be kept confidential unless you provide explicit consent to do otherwise, for example, if your test is positive and further clinical examination need to be done.
- No individuals will be identifiable from any collated data, written report of the research, or any publications arising from it.

All computer data files will be encrypted and password protected. The researcher will keep files in a secure place and will comply with the requirements of the European Data Protection Act.
All hard copy documents, e.g. consent forms, questions answered, will be kept securely and in a locked cupboard, wherever possible. Documents may be scanned and stored electronically. This may be done to enable secure transmission of data to the University's secure computer systems.
As detailed above, this research includes the collection of small amounts of your human tissue (blood and/or lesion samples): If you give your consent for its inclusion in this study, the researchers will ensure it will be destroyed at the end of the study.
Please notify the researchers immediately if any adverse symptoms arise during or after the research.
If you wish, you can receive information on the results of the research. Please indicate on the consent form if you would like to receive this information.
Would you have any questions about this research, the researcher can be contacted during and after participation by email (w1724399@my.westminster.ac.uk) or by telephone (0244 130 557), and either of the project supervisors Dr Alex Owusu-Ofori (owusu_ofori@hotmail.com or 0209 149 370) and Dr Saki Raheem (S.Raheem@my.westminster.ac.uk) can also be contacted.
If you have a complaint about this research project, you can contact the Head of the School of Life Sciences at the University of Westminster Prof. Brendon Noble by email (B.Noble@westminster.ac.uk).

3.2 PARTICIPANT INFORMATION SHEET FOR CHILDREN

Title of study: Prevalence of the related treponematoses syphilis and yaws and assessment of antibiotic resistance in the treponemes causing these diseases in Ghana

Researcher(s): Yaw Agyekum Boaitey (University of Westminster)

Supervisors: Dr Saki Raheem (University of Westminster) and

Dr. Alex Owuso-Ofori (Kwame Nkrumah University of Science and Technology-KNUST)

1. Yaws is a neglected tropical disease caused by bacterium *Treponema pallidum pertenue.*

2. Yaws causes large painful sore on the skin (mainly on arms and legs).

If yaws is not treated early, the bacteria can infect the bones and cartilages which leads to severe pain and disfigurement of some part of the body like the nose.

3. Most cases of yaws (75%) are found in children between ages 1-15.

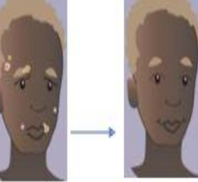
4. Yaws is a highly contagious disease that spread through direct skin contact with the skin of infected individual.

5. Yaws mainly occurs in deprived rural populations in warm, humid and tropical forest areas of Africa, Asia, Latin America and the Pacific.

Map generated in R statistical package using data from WHO, (2018).

6. Yaws can be cured by a single oral dose of the antibiotic azithromycin (WHO, 2012).

7. When an individual has yaws and he or she is given a single dose of azithromycin all the painful sores are healed and the disease is cured.



Drawings of children shown here were taken from yaws.isglobal.org

- Would you have any questions about this research, the researcher can be contacted during and after participation by phone (+233244130557) or email (w1724399@my.westminster.ac.uk), and either of the project supervisors Dr Alex Owusu-Ofori (owusu_ofori@hotmail.com or 0209 149 370) and Dr Saki Raheem (S.Raheem@westminster.ac.uk) can also be contacted.

- If you have any complaints about this research project you can contact the Head of the School of Life Sciences at the University of Westminster Prof. Brendon Noble by email (B.Noble@westminster.ac.uk).

4 CONSENT FORM

Title of Study: Prevalence of the related treponematoses syphilis and yaws and assessment of antibiotic resistance in responsible treponemes in Ghana.

Lead researchers: Yaw Agyekum Boaitey, Dr Alex Owusu-Ofori, Dr Pascale Gerbault

I have read the Participation Information Sheet and I understand its content.

I am willing to act as a participant in the above research study.

Participant's Name: ______________________________

Signature or Thumbprint: __________________

Date: ________________

This consent form will be stored separately from any data you provide so that your responses remain anonymous.

____

I have provided an appropriate explanation of the study to the participant.

Researcher's Name: ______________________________

Signature: ______________________________ **Date:** ________________

5 DATA CAPTURING SHEET

RECORDED INFORMATION	**NOTES**
Participant Number:	Participants will be anonymised using random numbers
Location:	Hospital and community details
Year of birth:	
Sex:	Female (1) / Male (0)
Sexual preferences:	For adults only
Ethnicity:	
Previous record of treponemal infections:	Yes (1) / No (0)
History of antibiotic treatment:	Penicillin (0) / Azithromycin (1) / Unknown (3) / None (4)
Skin lesion present:	None (0) / Genital (1) / Oral or buccal (2) / Arms or legs (3) / Other (4)
Point of care test result:	Negative (-1) / Past (0) / Current (1) infection
Antibiotic offered:	None (0) / Penicillin (1) / Azithromycin (2)
Antibiotic taken:	Yes (1) / No (0)
Lesion sampled:	Yes (1) / No (0)
DNA concentration:	Measured in nanograms
Molecular diagnosis:	*Treponema pallidum pallidum* (TPA) / *Treponema pallidum pertenue* (TPE) / Unclear (0)

6. Microorganisms identified across all samples

Table A5 The results of DPP test, multiplex PCR and 16S rRNA gene sequencing across all samples (110 yaws-like and 46 syphilis-like lesions) for microorganisms that cause yaws-like and syphilis-like lesions in this study

Sample ID	DPP TEST	Multiplex PCR				16S rRNA gene sequencing		
		T. pallidum	*H. ducreyi*	*HSV-1*	*HSV-2*	*Treponema*	*Mycobacterium*	*Haemophilus*
Y001	-	-	-	-	-	-	-	-
Y002	-	-	-	-	-	-	-	-
Y003	+	-	-	-	-	-	-	-
Y004	-	-	-	-	-	-	-	-
Y005	-	-	-	-	-	-	-	-
Y006	-	-	-	-	-	-	-	-
Y007	-	-	-	-	-	-	-	-
Y008	-	-	-	-	-	-	+	+
Y009	-	-	-	+	-	-	-	-
Y010	-	-	-	-	-	-	-	-
Y011	-	-	-	-	-	-	-	-
Y012	-	-	-	-	-	-	-	-
Y013	-	-	-	-	-	-	-	-
Y014	+	-	-	-	-	-	-	-
Y015	-	-	-	-	-	-	-	-
Y016	+	-	-	-	-	-	-	-
Y017	-	-	-	-	-	-	-	-
Y018	-	-	-	-	-	-	-	-
Y019	+	-	-	-	-	-	-	-
Y020	-	-	-	-	-	-	-	-
Y021	+	-	-	-	-	-	-	-
Y022	-	-	-	-	-	-	-	+
Y023	-	-	-	-	-	-	-	-
Y024	-	-	-	-	-	-	-	-
Y025	-	-	-	-	-	-	-	-
Y026	-	-	-	-	-	-	-	-
Y027	-	-	-	-	-	-	-	-
Y028	-	-	-	-	-	-	-	-
Y029	+	-	-	-	-	+	-	-
Y030	-	-	-	-	-	-	-	-
Y031	-	-	-	-	-	-	-	-
Y032	+	-	-	-	-	-	-	-
Y033	-	-	-	-	-	-	-	-
Y034	-	-	-	-	-	-	-	-
Y035	-	-	-	-	-	-	-	-
Y036	-	-	-	-	-	-	-	-
Y037	-	-	-	-	-	-	-	-
Y038	-	-	-	-	-	-	-	-

Sample ID	DPP TEST	Multiplex PCR				16S rRNA gene sequencing		
		T. pallidum	*H. ducreyi*	*HSV-1*	*HSV-2*	*Treponema*	*Mycobacterium*	*Haemophilus*
Y039	-	-	-	-	-	-	-	-
Y040	-	-	-	-	-	-	-	-
Y041	-	-	-	-	-	-	-	+
Y042	-	-	-	-	-	-	-	-
Y043	-	+	-	-	-	-	-	-
Y044	-	-	-	-	-	-	-	-
Y045	-	-	-	-	-	-	-	+
Y046	+	-	-	-	-	-	-	-
Y047	-	-	-	-	-	-	-	-
Y048	-	-	-	-	-	+	-	-
Y049	-	-	-	-	-	-	-	-
Y050	-	-	-	-	-	-	-	-
Y051	+	-	-	-	-	-	-	-
Y052	+	-	-	-	-	-	-	-
Y053	+	-	-	-	-	-	-	-
Y054	-	-	-	-	-	-	-	-
Y055	-	-	-	-	-	-	-	-
Y056	-	-	-	-	-	-	+	-
Y057	-	-	-	-	-	-	-	-
Y058	+	-	-	-	-	-	-	-
Y059	-	-	+	-	-	-	-	-
Y060	-	-	-	-	-	-	-	-
Y061	-	-	-	-	-	-	-	-
Y062	-	-	-	-	-	-	-	-
Y063	-	-	-	-	-	-	-	-
Y064	-	-	+	-	-	-	+	-
Y065	-	-	-	-	-	-	-	-
Y066	+	-	+	-	-	-	-	-
Y067	-	-	-	-	-	-	-	-
Y068	-	-	-	-	-	-	-	-
Y069	-	-	-	-	-	-	-	-
Y070	-	-	-	-	-	-	-	-
Y071	-	-	-	-	-	-	-	+
Y072	-	-	-	-	-	-	-	-
Y073	-	-	-	-	-	-	-	-
Y074	-	-	-	-	-	-	-	-
Y075	-	-	+	-	-	-	-	-
Y076	-	-	-	-	-	-	-	-
Y077	-	-	-	-	-	-	-	-
Y078	-	-	-	-	-	-	-	-
Y079	+	-	-	-	-	-	-	-
Y080	-	-	-	+	-	-	-	-
Y081	+	-	-	-	-	-	-	-

Sample ID	DPP TEST	Multiplex PCR				16S rRNA gene sequencing		
		T. pallidum	*H. ducreyi*	*HSV-1*	*HSV-2*	*Treponema*	*Mycobacterium*	*Haemophilus*
Y082	-	-	-	-	-	-	-	+
Y083	+	-	-	-	-	-	-	-
Y084	-	-	-	-	-	-	-	-
Y085	-	-	-	-	-	-	+	-
Y086	-	-	-	-	-	-	-	-
Y087	-	-	-	-	-	-	-	-
Y088	-	-	-	-	-	-	-	-
Y089	-	-	-	-	-	-	-	-
Y090	-	-	-	-	-	-	-	-
Y091	-	-	+	-	-	-	-	-
Y092	+	-	-	-	-	-	-	-
Y093	-	-	-	-	-	-	-	-
Y094	-	-	-	-	-	-	-	-
Y095	-	-	-	-	-	-	-	-
Y096	-	-	+	-	-	-	-	-
Y097	-	-	-	-	-	-	-	-
Y098	-	-	-	-	-	-	-	-
Y099	-	-	+	-	-	-	-	-
Y100	-	-	+	-	-	-	-	-
Y101	-	-	-	-	-	-	-	-
Y102	-	-	-	-	-	-	-	-
Y103	+	-	-	-	-	-	-	-
Y104	-	-	-	-	-	-	-	-
Y105	-	-	-	-	-	-	-	-
Y106	-	-	-	-	-	-	-	-
Y107	-	-	-	-	-	-	-	-
Y108	-	-	-	-	-	-	-	-
Y109	+	-	+	-	-	-	-	-
Y110	-	-	+	-	-	-	-	+
S001	-	-	-	-	-	-	-	-
S002	-	-	-	-	-	-	-	-
S003	+	-	-	-	-	-	-	-
S004	-	-	-	-	-	-	-	-
S005	+	-	-	-	-	-	-	-
S006	-	-	-	-	-	-	-	-
S007	-	-	-	-	-	-	-	-
S008	-	-	-	-	+	-	-	-
S009	-	-	-	-	+	-	-	-
S010	-	-	-	-	-	-	-	-
S011	-	-	-	-	-	-	-	-
S012	-	-	-	-	-	-	-	-
S013	-	-	-	-	-	-	-	-
S014	+	-	-	-	-	-	-	-

Sample ID	DPP TEST	Multiplex PCR				16S rRNA gene sequencing		
		T. pallidum	*H. ducreyi*	*HSV-1*	*HSV-2*	*Treponema*	*Mycobacterium*	*Haemophilus*
S015	-	-	-	-	-	-	-	-
S016	-	-	-	-	-	-	-	-
S017	-	-	-	-	-	-	-	-
S018	-	-	-	-	+	+	-	-
S019	-	-	-	-		-	-	-
S020	+	-	-	-	-	-	-	+
S021	-	-	-	-	-	-	-	-
S022	-	-	-	-	+	-	-	+
S023	-	-	-	-	+	-	-	+
S024	-	-	-	-	+	-	-	-
S025	-	-	-	-	-	-	-	+
S026	-	-	-	-	+	-	-	-
S027	-	-	-	-	-	-	-	+
S028	-	-	-	-	-	-	-	-
S029	-	-	-	-	+	-	-	+
S030	-	-	-	-	+	-	-	-
S031	-	-	-	-	-	-	-	-
S032	-	-	-	-	-	-	-	-
S033	-	-	-	-	+	-	-	+
S034	-	-	-	-	+	-	-	-
S035	+	-	-	-	-	-	-	-
S036	-	-	-	-	+	-	-	-
S037	-	-	-	-	-	-	-	-
S038	-	-	-	-	-	-	-	-
S039	-	-	-	-	+	-	-	-
S040	-	-	-	-	-	-	-	-
S041	-	-	-	-	-	-	-	-
S042	-	-	-	-	-	-	-	+
S043	-	-	-	-	-	-	-	-
S044	-	-	-	-	-	-	-	-
S045	-	-	-	-	-	-	-	-
S046	-	-	-	-	-	-	-	-

7 Positive control used in the 16S rRNA gene library preparation and sequencing

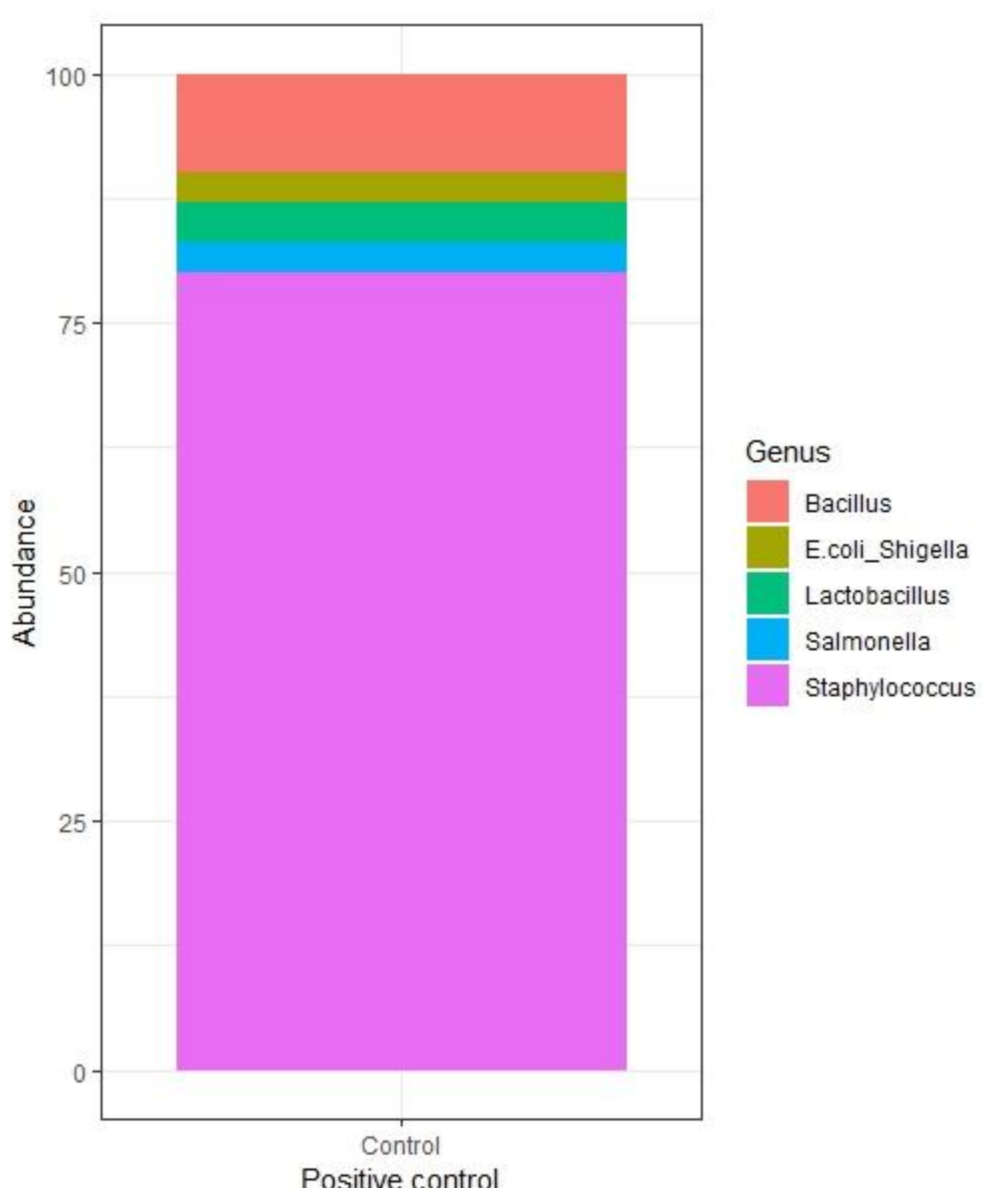


Figure A1. The different taxa (genus level) identified in the positive control of the 16S rDNA sequencing. This indicates the reagents of the PCR and the illumina sequencer used in this study were good.

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