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Serological Specificity in the Detection of Syphilis among Pregnant Women Attending Antenatal in Some Hospitals in Mubi Metropolis, Adamawa State, Nigeria

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ABSTRACT

Syphilis is a sexually transmitted infection (STI) caused by the Treponema pallidum (spirochetes). Syphilis remains a major cause of reproductive morbidity and poor pregnant outcomes in developing countries. This Research work seeks to determine the specificity of serological VDRL test for syphilis against the use of High Vaginal Swab in molecular detection of syphilis among pregnant women attending antenatal in some selected hospitals and clinics in Mubi North and South L.G.A. Adamawa State, Nigeria to subvert the challenges, hence finding lasting solution to the Reproductive and mental health challenges posed by secondary Syphilis. A total of 120 blood samples were collected from 120 consented pregnant women in batches from General hospital, Mubi, Sabon layi clinic, Lokuwa PHC, Lamurde maternity, Kwaja PHC and Alheri Nursing hospital all in Mubi North and South Local Government, during their Antenatal days. In all, 5(4.16%) of the samples were seropositive, with the highest prevalence recorded as 2(10.00%) of the 20 samples collected from Kwajah PHC in Mubi South L.G.A. The HVS of pregnant with positive serological test results were subjected to molecular nPCR test to amplify the tpp47 gene of Treponema pallidum. The only amplified fragment which arose from the positive as the only positive amplification products was analyzed for specificity in an agarose gel developed 0.01 % ethidium bromide for ease of visualization of the band of fragment weight 260 bp tpp47 gene of Treponema pallidum under U.V since all HVS samples showed no bands of amplification, no further sequencing was technically necessary.

Keywords- Syphilis, Specifity, Seropositive and Amplification

I. INTRODUCTION

Syphilis is a sexually transmitted infection (STI) caused by the *Treponema pallidum* (spirochetes). *Treponema pallidum* is a spirochaete bacterium with subspecies that cause treponemal diseases such as syphilis, bejel, pinta, and yaws. It is a helically coiled microorganism usually 6-15µm long and 0.1-0.2 µm wide [1]. The treponemes have a cytoplasmic and an outer membrane. Using light microscopy, treponemes are visible only by using dark field illumination. They are Gram negative, but some regard them as too tiny to be Gram stained [2].

The route of transmission of syphilis is almost always by sexual contact, although there may be congenital syphilis via transmission from mother to child in uterus and it can also be transferred through the transfusion of unscreened blood. If not treated, syphilis can cause serious effects such as damage to the aorta, brain, eyes, and bones. In some cases, this effect may fatal [3]. Syphilis, a chronic systemic infection caused by *Treponema pallidum* subspecies pallidum, is a gram-negative, very mobile bacterium sexually transmitted with untreated cases in resulting in trans-placental transmission [4][5].

Syphilis in pregnant women can result in adverse outcomes of pregnancy in up to 80 % of cases, such as stillbirth and spontaneous abortion 40 %, perinatal death 20 %, and serious neonatal infections and low-birth weight babies 20 % [6].

STIs are widespread in the developing countries and constitute a major public health problem in sub-saharan Africa. More recently, there has been a resurgence of syphilis [7] Testing for syphilis in pregnancy and labor is medically indicated because of the potential risk for congenital infection and fetal loss [8]. Syphilis has also acquired a new potential for morbidity and mortality through association with increased risk for HIV infection [7].

Pregnant women should have serologic test for syphilis at the time of the first prenatal visit. In women suspected of being at increased risk for syphilis or for populations in which there is a high prevalence of syphilis, additional tests should be performed during the third trimester at twenty-eight weeks and again at delivery women should be considered infected and should be treated unless prior treatment with fall in antibody titre is medically documented information regarding syphilis infection in pregnancy in Nigeria shows a wide geographical variation in sero-prevalence. Several models have been proposed to estimate adverse pregnancy outcomes in women infected with syphilis, with resulting estimates ranging from 50 % to 80 % [9]. Transmission occurs more commonly in the last two trimesters, but the spirochete can cross the placenta at any time during pregnancy [10].

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Syphilis is a sexual transmitted infection (STI) caused by a type of bacteria known as *Treponema pallidum.* In 2016, more than 88,000 cases of syphilis were reported in the United States, according to the center for disease control and prevention. The rate of women with syphilis has been declining in the United States, but the rate among men, particularly men who have sex with men, has been rising [11]. *Treponema pallidum* is the causative agent of syphilis. *T. Pallidum* is a thin, elongated (0.10 to 0.18µm) bacterium that cannot be readily visualized by light microscope instead requiring visualization by darkfield microscopy, which uses obliquely applied light.

Scientific Classification of the causative agent of syphilis

Domain	-	Bacteria
Kingdom	-	Eubacteria
Phylum	-	Spirochaetes
Class	-	Spirochaetes
Order	-	Sprochaetale
Family	-	Spirochaetaceae
Genus	-	Treponema
Species	-	*T. pallidum* [2].

Epidemiology of syphilis infection

Globally around 340 million cases of curable new STI occur every year. Of these, syphilis account for an estimated 12 million cases, 2 million of them among pregnant women [12]. In the year 2016 one million pregnant women were at risk for adverse pregnancy outcomes such as stillbirth and neonatal death due to congenital syphilis [13]. Syphilis remains a major cause of reproductive morbidity and poor pregnant outcomes in developing countries, although in sub-Saharan Africa from 2014-2018 only about 4.0 % prevalence of syphilis was recorded among pregnant women [14].

Pathogenesis of syphilis infection

Infection begins when *T. pallidum* penetrates the host usually through intact or abrade mucous membranes. Syphilis remains chronic without treatment and progresses in stages characterized by episodes of active disease interrupted by periods of latent infection. The incubation period is estimated to be between 10 and 90 days (average three weeks) [15]

In early disease, spirochaetes can be found in chancre, the usual first manifestation of syphilis invasion of the bloodstream and lymphatic's by the syphilitic agent which occurs within hours to days of penetration of *T. pallidum* as evidenced by the fact that patients who receive blood transfusion from syphilis donors in the sero-negative incubation period have become infected. All organs of the body can be invaded but the skin lymph nodes and the central nervous system (CNS) are the sites most often invaded. In the skin, *T. pallidum* is found in the dermal epidermal junction zone or throughout the dermis [16].

Mode of infection and transmission of syphilis

In most case syphilis infection is acquired from direct sexual contact with an individual who has an active primary or secondary syphilis lesion. Sharing of needles by I.V drug users, non-genital contact with a mucosal lesion e.g infant contact with a maternal chancre can expose the child to syphilis and transfusion of unscreened blood [11]**.**

Signs and symptoms of syphilis

The first sign of syphilis is a small, painless sore. It can appear on the sexual organs, rectum, or inside the mouth. This sore is called a chancre. People often fail to notice it right away since the chancre appears, disappear and reappear again within days.

Stages of Syphilis Infection

There are four (4) stages of syphilis but it is most infectious in the first two stages, the disease remains active but often with no symptoms. Tertiary syphilis is the most destructive to general mental health leading to neural degeneration and psychosis [17]

Primary syphilis

The primary stage of syphilis occurs about three to four weeks after a person contracts the bacteria. It begins with a small round sore called a chancre. A chancre is painless but it's highly infectious. This sore may appear wherever the bacteria enter the body, such as on or inside the mouth genitals, or rectum [18].

Secondary syphilis

Skin rashes and a sore throat may develop during the second stage of syphilis. The rash won't itch and is usually found on the palms and soles, but it may occur anywhere on the body. Some people don't notice the rash before it goes away. Other symptoms of secondary syphilis may include; headaches, swollen lymph nodes, fatigue, fever, weight loss, hair loss, aching joints [19].

Latent syphilis

The third stage of syphilis is the latent or hidden stage. The primary and secondary symptoms disappear, and there won't be any noticeable symptoms at this stage. However, the bacteria remain in the body. This stage could last for years before progressing to tertiary syphilis [20].

Tertiary syphilis

The last stage of infection is tertiary syphilis. According to the Mayo clinic, approximately 15 to 30 % of people who don't receive treatment for syphilis will enter this stage. Tertiary syphilis can occur years or decades after the initial infection. Tertiary syphilis can be life-threatening.

Risk factors of acquiring syphilis infections

One faces an increased risk of acquiring syphilis if one engages in unprotected sex, having sex with multiple partners, a man who has sex with men are mostly infected with HIV the virus that causes AIDS [11].

Complications of syphilis infection

Without treatment, syphilis can lead to damage throughout your body. Syphilis also increases the risk of HIV infection and for women, can cause problems

during pregnancy. Treatment can help prevent future damage but can't repair or reverse damage that already occurred in the brain and the central nervous system [17]. Blindness, Deafness, Mental illness, Memory loss, Destruction of soft tissue and bone, Neurological disorders, such as stroke, meningitis and heart disease, Pelvic Inflammatory Disease (PID), Neurosyphilis, which is an infection of the brain or spinal cord, ectopic pregnancy are other complications that can accompany syphilis when left undiagnosed and untreated for a long period of time [17]. According to [22] Optic neuropathy from viruses and spirochetes are also complications of major concern.

Pregnancy and childbirth complications

A syphilitic pregnant mother without proper diagnosis and treatment may pass syphilis to her unborn baby known as congenital syphilis. Congenital syphilis greatly increases the risk of miscarriage, still birth or the death of an infant within a few days after birth [23].

The importance of screening syphilis among pregnant women

Screening a pregnant woman for syphilis early in pregnancy is important for their health and that of the fetus. This contributes to monitoring the quality of antenatal care services and services to prevent HIV among pregnant women. It is also a process indicator for assessing the validation of eliminating the mother-to-child transmission of syphilis. Screening of syphilis among pregnant women is very important because, fetal death and morbidity due to congenital syphilis are preventable if the infected mother is identified and treated appropriately screening pregnant women for syphilis is very vital because people can be infected with syphilis and may not know. In light of the often-deadly effects syphilis can have on fetus and unborn children, health officials recommend that all pregnant women be screened for the disease. The screening of syphilis among pregnant women is very important because the USPSTF found convincing observational evidence that the universal screening of pregnant women decreases the proportion of infants with clinical manifestations of syphilis infection [10].

Although [24] reported that there is a declining level of prevalence among women attending antenatal in India.

The Drawbacks of VDRL in the Diagnosis of Syphilis

VDRL test still remain the most common serological methods of diagnosing Syphilis but one major challenge is that abnormal results may be obtained from VDRL test. If the test turns out negative then it is important to confirm the test results using FTA-ABS test or molecular detection of the Virus which is more specific. The VDRL test's the ability to detect syphilis depending on the stage of the diseases. The test is sensitivity to detect syphilis nears 100 % but specificity is less [21].

II. MATERIALS AND METHODS

One percent (1 %) agarose gel containing 0.01 % ethidium bromide, thermal cycler, Isopropanol, Taq polymerases, Phosphate buffer, dNTPs, sterile swab sticks, slides, petri-dishes, 70 % alcohol, cotton wool, ependorff tubes, TE buffer $_{p}$H 8.4, proteinase K, RNase and specific primers;
Tps,5=TTCGATGCAGTTTCTCGCGCCAACC-3=
Tpe,5=CTACTGGGCCACTACCTTCGCACG3=
KO5, 5=-CCCGTTCGCAATCAAAGTCAGCCT-3=
and KO3 B,5=
GACGCGAGCTACACCAATCTGATG-3=

Study Area

The people of Mubi engaged themselves in subsistence farming, cattle or livestock farming and only few are civil servants and businessmen. The climate is tropical with average temperature of about 32.90 ^{o}C in dry season with a relative range of 10-43 % mean annual rainfall i.e about 1050 mm which usually starts in late May and lasted for (6) six months [25].

Statement of the problem and Justification

Serology is the frequently used method in the diagnosis of syphilis but serological test limitations does not allow for diagnosis of active infection in certain organs [21]. The problem of proper diagnosis poses a big challenge in the effective control and treatment of syphilis. The poor habit of most people in developing countries like Nigeria who carelessly do not take routine medical checkup seriously also created room for infections by *Treponema pallidum* linger for a long time unidentified and in the end results in complications such as stillbirth and spontaneous abortion, perinatal death, serious neonatal infections low-birth weight babies and mental debilitation or psychosis. It is therefore important to mitigate these challenges by continuous case finding to prevent spread of the syphilitic agent through routine medical checkup since the bacterial can remain dormant in the body for decades before returning to damage vital organs of the body including the brain.

Aim

To determine the prevalence of syphilis among pregnant women attending antenatal in some selected hospitals and clinics in Mubi North and South L.G.A.

Specific Objectives of the study

i. To diagnose syphilis serologically.
ii. To confirm at Molecular level using nPCR to amplify the conserved *tpp47* gene of *Treponema pallidum* from HVS.

I. Methods

Sample collection

The study was conducted in Mubi North and Mubi South Local Government Area. Six (6) facilities to be selected for the survey included both Government and private hospitals and maternity in Mubi North and South that offer antenatal services. A total number of one hundred and twenty (120) blood samples was collected from the pregnant women visiting the six (6) selected

hospitals and emptied into EDTA bottles carefully labeled with age and educational status was subjected to serological analysis (ELISA).Using sterile swab sticks, HVS (High Vaginal Swab) samples was collected from the pregnant women who test positive serologically in General hospital, Mubi, Sabon layi clinic, Lokuwa PHC, Lamurde maternity, Kwaja PHC and Alheri Nursing hospital all in Mubi North and South Local Government Areas upon consenting and following ethical practices. The HVS swabs was immediately inserted and replaced into the swab holder containing 1ml of sterile phosphate buffer, labeled with age and educational status respectively. The samples were transported in an ice box to the Microbiology laboratory of the Department of Biomedical and Pharmaceutical Technology for further analysis.

Preparation of blood samples for Serology (VDRL)

The blood samples were allowed to stand for clotting to occur i.e sedimentation of the red blood cells. The fresh serum was carefully separated from the sediments of red blood cells into sterile sample bottles for further analysis.

Serological analysis

In VDRL the sealed pouch was carefully opened by tearing along the notch for usage as soon as possible. The test strip was carefully inserted into the blood sample with the arrow side pointing down into the vessel of serum for about 10 seconds, after which it was laid on a clean dry and non-absorbent surface for TP antigen and antibody reaction to take place. After 5-20 minutes the results were read and recorded accordingly for further analysis.

III. MOLECULAR IDENTIFICATION OF THE CONSERVED *TPP47* GENE OF *Treponema pallidum*

The molecular identification was carried out by amplifying the conserved *tpp47* gene using nested Polymerase Chain Reaction was carried out as follows;

DNA extraction from samples of High Vaginal Swab (HVS)

The DNA from High Vaginal Swab (HVS) samples of pregnant women who turns out to be positive for the serological blood test was extracted using the protocol stated by [26] .The DNA was then suspended in buffer solution and the resulting mixture was subjected to centrifugation at 4600 g for 5 min and the resultant pellets was resuspended to make a final volume of 25 µl containing 2 µl of the DNA extract in TE buffer of pH 8.4 and treated with proteinase K (20 mg/ml) followed by treatment with RNases and was incubated for 1 hour at 37 ºC. Further incubation on ice for 5 minutes and was again subjected to centrifugation at 7200 g for 20 min to confirm the serological test Results. The aqueous phase was gently transferred to a new tube and isopropanol (1: 0.5) was added and DNA precipitated and stored at –20 ºC for 16 h until it was subjected to amplification using the nested PCR method.

Nested Polymerase Chain Reaction (nPCR) to amplify tpp47 gene of Treponema pallidum

The Nested Polymerase Chain Reaction (nPCR) cocktail which consist of 0.2 µl of native Taq DNA polymerase (Promega, USA) was made up to µl with sterile distilled water 8 µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 3 minutes followed by a 20 cycles for (PCR1) and 35 cycles for (PCR2) consisting of 94 °C for 1 minute, 68 °C for 30 seconds and 72 °C for 2 minutes 30 seconds; and a final termination at 72 °C for 2 minutes then chilled at 4 ºC [27]. The specific primer sequences used are as follows;

Tps,5=TTCGATGCAGTTTCTCGCGCCAACC-3=
Tpe,5=CTACTGGGCCACTACCTTCGCACG3=
KO5, 5=-CCCGTTCGCAATCAAAGTCAGCCT-3=
and KO3 B,5=
GACGCGAGCTACACCAATCTGATG-3=

The integrity of nPCR product was then analyzed by electrophoresis in a 1.7 % agarose gel. The so formed fragments were visualized by the U.V trans-illumination of ethidium bromide-stained gel with a 800-bp DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular size marker in the presence of a positive and negative control.

Sequencing the amplified fragments

The amplified *tpp47*gene fragments was sequenced using a Genetic Analyzer 3130x*l* sequencer from Applied Biosystems and the sequencing kit BigDye terminator v3.1 cycle sequencing kit was used with matrix standard for fragment sequencing will be followed as described in the manufacturers' manual.

IV. RESULTS

The result for overall prevalence as presented in table 2 stands at 5(4.16 %) of the 120 pregnant women sampled in the two local governments of study is as presented in the table 1.

Table 1: Shows the Distribution of Syphilis Amongst Pregnant Women Attending the Various Antenatal Clinics in Mubi North and South.

Name of hospital/clinic	L.G.A	No. of blood samples collected	No. of Sero- positive blood samples
General hospital, Mubi	Mubi North	20	1(5.00)
Sabon layi clinic	Mubi North	20	1(5.00)

Lokuwa PHC	Mubi North	20	0(0.00)
Lamurde maternity	Mubi south	20	1(5.00)
Kwaja PHC	Mubi south	20	2(10.00)
Alheri Alheri Nursing hospital	Mubi south	20	0(0.00)
Total		120	5(4.16)

Outcome of PCR for Molecular Amplification of tpp47 Gene

Molecular identification of treponemal DNA extract from the HVS comprising quantities of DNA ranging from 0.05 to 250 ng, were used for the nested PCR to detect *tpp47* gene of *Treponema pallidum* in the DNA extracted from the HVS sample of 5(4.16) pregnant women who tested positive for VDRL. Results from the DNA amplification revealed negative results obtained for HVS samples of pregnant women that previously tested positive for treponema antibodies using nested-PCR. Molecular amplification was also negative for all other negative samples but amplification was positive for the positive control from molecularly confirmed syphilitic patient and negative for the negative control (distilled water) to authenticate PCR outcome. This was seen using electrophoretic analysis of the supposed PCR amplified products on a 1 % agarose gel containing 0.01% ethidium bromide as visualised

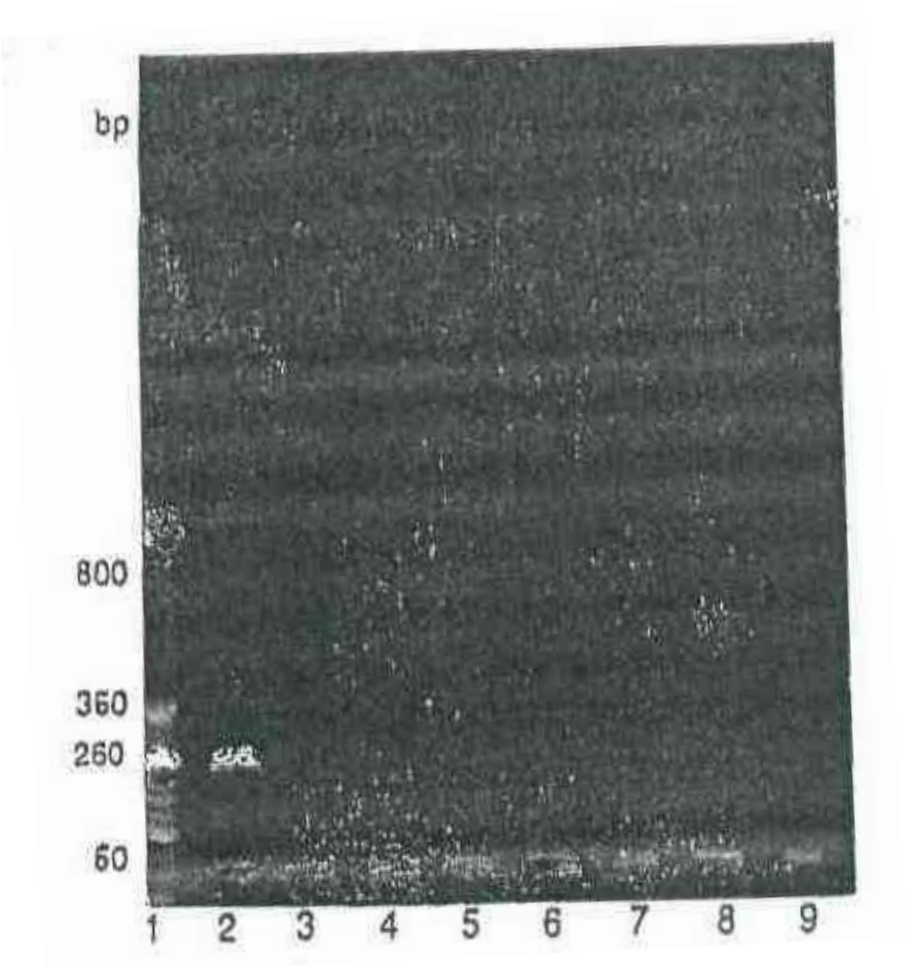

PLATE1: Showing Negative PCR outcome on 1% agarose gel and Viewed Under UV for the Amplification of *tpp47* gene of *Treponema pallidum* in Samples of HVS of pregnant women who tested positive serologically. Lane1: 800-bp Molecular DNA ladder, Lane 2: Positive Control Showing Positive Amplification on 1 % agarose, Lane 3: Distilled Water blank as Negative Control. Lanes 4: HVS from None-reactive subject. Lane 5 and 6; HVS samples from the two (2) Seropositive Pregnant Women from Mubi North, Lane 7-9; HVS samples from the three (3) Seropositive pregnant women from Mubi South.

The negative outcome of the nested PCR carried out on HVS samples of the seropositive pregnant women turned out negative for all samples as presented in table 2.

Table 2: Showing the Negative Outcome of *Treponema pallidum* DNA Amplification by Nested PCR per Serologically Positive Blood Samples

Name of hospital/clinic	L.G.A	No. of blood samples collected	No. of Sero-positive blood samples	% of HVS samples molecularly positive for *tpp47* gene
General hospital, Mubi	Mubi North	20	1(5.00)	Nil
Sabon layi clinic	Mubi North	20	1(5.00)	Nil
Lokuwa PHC	Mubi North	20	0(0.00)	Nil
Lamurde maternity	Mubi south	20	1(5.00)	Nil
Kwaja PHC	Mubi south	20	2(10.00)	Nil
Alheri Nursing hospital	Mubi south	20	0(0.00)	Nil
Total		120	5(4.16)	0

V. DISCUSSION

The molecular aspect of this research work has also shown that there are pregnant women in the two local governments of study who may have entered a latent stage of the syphilis and may congenitally transmit syphilis to their unborn child thereby causing miscarriages low birth weight and even still birth in some cases as no *tpp47 gene* was detected from their HVS PCR test. Therefore, strategies for surveillance by the WHO to eradicate syphilis must intensify campaign for pregnant women to compulsorily get tested for Syphilis using blood samples and skin lesions as suitable samples PCR rather than HVS samples that never confirmed any positive serological results obtained among the pregnant women. The occurrence of positive serological test results which turned out negative for PCR align with the findings of [28] where *PAXgene* of *T. pallidum* spp. *pertenue* could not be detected by PCR in blood samples but was detected in skin lesion of infected persons where they cause yaws. These findings will help address relapses of latent disease thereby promoting transmission to healthy uninfected individuals in Mubi North and South L.G.As which will increase morbidity.

The result for overall prevalence as presented in table 1 stands at 5(4.16 %) of the 120 pregnant women sampled in the two local governments of studies and this shows that they may have entered a latent stage of the syphilis since no practical symptoms were expressed nor morbidity and may congenitally transmit syphilis to their unborn child and may lead miscarriages low birth weight and even still birth in some cases. From the outcome of the molecular aspect in this study as shown in table 2, revealed that nested PCR was unable to detect *tpp47 gene of T. pallidum* in the HVS samples of all pregnant women who were diagnosed through blood test to be seropositive for syphilis, even though Nested PCR has been perceived to be more sensitive than all other PCR types and hence substantiate the findings of [29] where *tpp47* was successfully amplified in blood samples hence confirming blood as a correct sample for molecular screening of syphilis. It is possible that nested PCR might detect *T. pallidum tpp47*gene in more suitable clinical samples but not High Vaginal Swab in line with work of [30]. Since no *tpp47* gene of *Treponema pallidum* was amplified for all the test samples of HVS, no oligonucleotide bands were visualised under UV as well except from the band for the amplified positive syphilis control. Going by this outcome no DNA sequencing was carried out as against what was earlier proposed as there was technically no need for such analysis.

VI. CONCLUSION

In conclusion, 4.16 % of the 120 pregnant women visiting various antenatal in Mubi metropolis (North and South) were serological positive for *Treponema pallidum* antibodies although the specificity of VDRL for diagnosis of syphilis might still be at stake. Interestingly all HVS samples from same women who turned out positive for VDRL serology were confirmed negative for molecular amplification of *tpp47* gene using nested PCR. Therefore it is imperative to state that HVS is not sufficiently the correct sample for molecular Treponema detection despite the drawbacks of VDRL, therefore effective molecular diagnostics that employ the use of suitable samples like skin lesions or blood to specifically targets the syphilitic agents rather than antibodies must be prioritized in our Primary Health Care facilities during antenatal if effective diagnosis of syphilis must be achieved among pregnant women as a giant step towards the eradication of secondary syphilis during pregnancy in a bid to effectively promote and improve Maternal and Child Health in Nigeria.

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