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Original Article

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Molecular characterization of methicillin-resistant *Staphylococcus aureus* isolates from a hospital in Ghana

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Abstract:

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) are a major cause of hospital- and community-acquired infection. They can colonize humans and cause a wide range of infections including pneumonia, endocarditis and bacteraemia. We investigated the molecular mechanism of resistance and virulence of MRSA isolates from a teaching hospital in Ghana.

Methodology: A total of 91 *S. aureus* isolates constituted the initial bacterial sample. Identification of *S. aureus* was confirmed by the VITEK 2 system. The cefoxitin screen test was used to detect MRSA and antibiotic susceptibility was determined using the VITEK 2 system. The resistance (*mecA*, *blaZ*, *aac-aph*, *ermC*, and *tetK*) and virulence (*lukS/F-PV*, *hla*, *hld* and *eta*) genes were amplified by polymerase chain reaction (PCR) and positive samples subjected to DNA sequencing. Pulsed field gel electrophoresis (PFGE) was used to ascertain the relatedness of the isolates.

Results: Fifty-eight of 91 (63.7%) isolates were putatively methicillin resistant by the phenotypic cefoxitin screen test and oxacillin MICs. However, 43 (47%) of the isolates were genotypically confirmed as MRSA based on PCR detection of the *mecA* gene. Furthermore, 37.9% of isolates displayed resistance to tetracycline, 19% to trimethoprim-sulphamethoxazole, 15.5% to clindamycin, 12.1% to gentamicin, 13.8% to ciprofloxacin and erythromycin, 6.9% to moxifloxacin and 7.0% to rifampicin. None of the isolates was positive for inducible clindamycin resistance. The prevalence of resistance (*mecA*, *blaZ*, *aac(6')-aph(2'')*, *tetK*, and *ermC*) and virulence (*hla* and *lukS/F-PV*) genes respectively were 74%, 33%, 22%, 19%, 3%, 5% and 3%, with isolates organized in two highly related clades.

Conclusion: Results indicate a fairly high occurrence of MRSA, which can complicate the effective therapy of *S. aureus* infections, necessitating surveillance and stringent infection control programmes to forestall its spread.

Keywords: MRSA, *mecA*, *blaZ*, *hla*, *lukS/F-PV*

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Caractérisation moléculaire d'isolats de *Staphylococcus aureus* résistants à la méthicilline provenant d'un hôpital du Ghana

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Abstrait:

Contexte: Le *Staphylococcus aureus* résistant à la méthicilline (SARM) est une cause majeure d'infection acquise à l'hôpital et dans la communauté. Ils peuvent coloniser les humains et causer un large éventail d'infections, notamment la pneumonie, l'endocardite et la bactériémie. Nous avons étudié le mécanisme moléculaire de résistance et de virulence des isolats de SARM provenant d'un hôpital universitaire au Ghana.

Méthodologie: Au total, 91 isolats de *S. aureus* constituaient l'échantillon bactérien initial. L'identification de *S. aureus* a été confirmée par le système VITEK 2. Le test de dépistage à la céfoxitine a été utilisé pour détecter le SARM et la sensibilité aux antibiotiques a été déterminée à l'aide du système VITEK 2. Les gènes de résistance (*mecA*, *blaZ*, *aac-aph*, *ermC* et *tetK*) et de virulence (*lukS/F-PV*, *hla*, *hld* et *eta*) ont été amplifiés par une réaction en chaîne de la polymérase (PCR) et des échantillons positifs soumis à un séquençage de l'ADN. Une électrophorèse sur gel en champ pulsé (PFGE) a été utilisée pour déterminer le caractère apparent des isolats.

Résultats: Cinquante-huit des 91 isolats (63,7%) étaient présumés résistants à la méthicilline par le test de dépistage phénotypique à la céfoxitine et par les CMI oxacillines. Cependant, 43 (47%) des isolats ont été confirmés génotypiquement comme SARM sur la base de la détection par PCR du gène *mecA*. En outre, 37,9% des isolats présentaient une résistance à la tétracycline, 19% au triméthoprim-sulfaméthoxazole, 15,5% à la clindamycine, 12,1% à la gentamicine, 13,8% à la ciprofloxacine et à l'érythromycine, 6,9% à la moxifloxacine et 7,0% à la rifampicine. Aucun des isolats n'était positif pour la résistance inductible à la clindamycine. La prévalence des gènes de résistance (*mecA*, *blaZ*, *aac(6')-aph(2'')*, *tetK* et *ermC*) et de virulence (*hla* et *lukS/F-PV*) était respectivement de 74%, 33%, 22%, 19%, 3%, 5% et 3%, avec des isolats organisés en deux clades fortement apparentés.

Conclusion: Les résultats indiquent une présence assez élevée de SARM, ce qui peut compliquer le traitement efficace des infections à *S. aureus*, nécessitant une surveillance et des programmes de contrôle des infections rigoureux pour prévenir sa propagation.

Mots-clés: SARM, *mecA*, *blaZ*, *hla*, *lukS/F-PV*

Introduction:

Staphylococcus aureus cause a variety of infections, including mild skin infections such as boils, stye and furuncles as well as more severe infections such as meningitis, pneumonia, phlebitis, mastitis, urinary tract infections, endocarditis and osteomyelitis (1). Methicillin resistance is problematic for many healthcare facilities worldwide as is the occurrence of community strains of methicillin resistant *Staphylococcus aureus* (MRSA) which also

harbor genes associated with increased virulence (2, 3). MRSA strains are produced by *S. aureus* when the *mecA* gene is acquired by methicillin susceptible *S. aureus* (MSSA). The *mecA* gene is borne on the mobile element referred to as the staphylococcal cassette chromosome (SCC) *mec* (4, 5).

There is considerable variability in the prevalence as well as the epidemiology of MRSA within and between countries (6) with limited data on the antibiotic susceptibility patterns and

molecular epidemiology of MRSA in Africa. Several African countries report a MRSA prevalence ranging from 4.8% to 20.0% (7-14). This study investigated the antibiotic susceptibility and molecular characterization of MRSA isolates from clinical samples in a Ghanaian hospital.

Materials and methods:

Ethical considerations

Ethical clearance was obtained from Biomedical Research Ethics Committee of University of KwaZulu-Natal (BE068/15) and the Committee on Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (CHRPE/AP/220/15).

Collection and identification of bacterial isolates

A total of 91 *S. aureus* isolates from different patients were collected from the Komfo Anokye Teaching Hospital in Ghana between May and September 2015. Presumptive staphylococci were identified by colony morphology (cream to golden yellow colour) on blood agar and the coagulase test (slide method) conducted by the Microbiology Unit of the hospital. Isolates were subjected to further tests including mannitol fermentation and the tube coagulase test. The identity of isolates was also confirmed by the automated VITEK 2 system (BioMérieux, Marcy-L'Etoile, France). Fifty-eight isolates were confirmed as MRSA (the cefoxitin screen test) and included for further study.

Detection of MRSA and antibiotic susceptibility profiles

The cefoxitin test was used to screen for MRSA according to CLSI guidelines (15). The MICs for MRSA isolates were determined using the automated VITEK 2 system for the following antibiotics: oxacillin, gentamicin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, fusidic acid, rifampicin and trimethoprim-sulfamethoxazole, and results interpreted

according to CLSI guidelines (15). *S. aureus* ATCC 25923 was used as the reference strain. Inducible clindamycin resistance was tested by the 'D-zone' (16).

DNA extraction, PCR and sequencing of virulence and resistance genes

Genomic DNA was extracted using the Roche High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and the concentration and purity were determined by Nanodrop™ 1000 spectrophotometer (Thermo Scientific, USA). The resistance genes *mecA*, *blaZ*, *aac-aph*, *ermC*, *tetK* conferring resistance to oxacillin, penicillin, aminoglycosides, macrolide-lincosamide-streptogramin B and tetracyclines respectively and the virulence genes *lukS/F-PV*, *hla*, *hld* and *eta* encoding Panton-Valentine Leucocidin (PVL), alpha haemolysin, delta haemolysin and exfoliative toxin A respectively were amplified. Amplification of genes was done using T100™ Thermal cycler (Bio-Rad, USA), using primers described elsewhere (17-20) (Table 1).

Genes investigated are those that confer resistance to some of the most commonly prescribed antibiotics in Ghana and virulence genes were selected based on their importance in staphylococcal infections. PCR experiments were carried out using the 2x PCR master mix (ThermoScientific, USA), 1.25 µL each of forward and reverse primers, and 50-200 ng of template DNA in a total reaction volume of 25 µL. PCR products were run on 1.5% agarose gel at 65V for 90 min and visualized by UV transillumination using Bio-Rad ChemiDoc™ MP System (Bio-Rad, UK). The PCR products were sequenced (Inqaba Biotech, Pretoria, South Africa) to confirm the identity of the genes. Analysis of the sequences was done using ChromasPro 1.9.9.1 (Technelysium, Queensland Australia), BioEdit and BLAST 2.0 available on the National Center for Biotechnology Information (NCBI) website <http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi>.

Table 1: Oligonucleotide primers and cycling conditions for the detection of genes in this study

Gene	Primer sequence	PCR program	Amplicon size (bp)	Reference
<i>mecA</i>	F-AACAGGTGAATTATTAGCACTTGTAAG R-ATTGCTGTTAATATTTTTGAGTTGAA	3min 95°C, 30s 55°C, 1min 72°C	174	Ref 17
<i>blaZ</i>	F-ACTTCAACACCTGCTGCTTTC R-TGACCACTTTTATCAGCAACC	3min 95°C, 30s 55°C, 1min 72°C	173	Ref 17
<i>tetK</i>	F-TCGATAGGAACAGCAGTA R-CAGCAGATCCTACTCCTT	3min 95°C, 30s 51°C, 1min 72°C	169	Ref 18
<i>aac (6')-aph (2'')</i>	F-TAATCCAAGAGCAATAAGGGC R-GCCACACTATCATAACCACTA	3min 95°C, 30s 54°C, 1min 72°C	227	Ref 19
<i>ermC</i>	F-CTTGTTGATCAGGATAATTTC R-ATCTTTTAGCAAACCCGTATTC	3min 95°C, 30s 52°C, 1min 72°C	190	Ref 17
<i>eta</i>	F-GCAGGTGTTGATTTAGCATT R-AGATGTCCCTATTTTGCTG	3min 95°C, 30s 51°C, 1min 72°C	93	Ref 20
<i>lukS/F-PV</i>	F-ATCATTAGGTAATGTCTGGACATGATCCA R-GCATCAAGTGTATTGGATAGCAAAAGC	3min 95°C, 30s 58°C, 1min 72°C	443	Ref 20
<i>hla</i>	F-CTGATTACTATCCAAGAAATTCGATTG R-CTTCCAGCCTACTTTTTATCAGT	3min 95°C, 30s 55°C, 1min 72°C	209	Ref 20
<i>hld</i>	F-AAGAATTTTATCTTAATTAAGGAAGGAGTG R-TTAGTGAATTTGTTCACTGTGTCGA	3min 95°C, 30s 55°C, 1min 72°C	111	Ref 20

PFGE typing

Strain typing was conducted using pulsed field gel electrophoresis (PFGE) as described by Tenover *et al.*, (21) using contour-clamped homogeneous electric field apparatus (CHEF DR-III; BioRad, Hercules, CA). Restriction was done with the *Sma*I restriction enzyme for *S. aureus*. Analysis of results was done using BioNumerics software version 6.6 (Applied Maths NV, Belgium) using the Dice coefficient and represented by unweighted pair group method with arithmetic mean (UPGMA) with optimization settings and position tolerance set at 0.5% and 1% respectively was used to analyze the electrophoretic patterns. Clusters were defined as described by Tenover *et al.*, (21).

Results:

Overall, fifty-eight (63.7%) isolates were putatively methicillin resistant as detected by the phenotypic cefoxitin screen test and oxacillin MICs. The

detailed phenotypic and genotypic profiles of MRSA isolates are available in the supplementary material. Thirty-five (60.3%) isolates were from blood, 5 (8.6%) from urethral swabs, 4 (6.9%) from urine and 14 (24.1%) from unknown sources.

Table 2 delineates the percentage resistance to different antibiotics tested. Generally, 37.9% of MRSA isolates displayed resistance to tetracycline, 19% to trimethoprim-sulphamethoxazole, 15.5% to clindamycin, 12.1% to gentamicin, 13.8% to ciprofloxacin and erythromycin, 6.9% to moxifloxacin and 7.0% to rifampicin. None of the MRSA isolates tested positive for inducible clindamycin resistance. All isolates were fully susceptible to linezolid, teicoplanin, vancomycin, tigecycline and fusidic acid.

Table 3 shows the distribution of resistance and virulence genes screened. The prevalence of resistance (*mecA*, *blaZ*, *aac(6')-aph(2'')*, *tetK* and *ermC*) and virulence (*hla* and *lukS/F-PV*) genes respectively were 74%, 33%, 22%, 19%,

3%, 5% and 3%, with isolates organized into two highly related clades. None of the

isolates expressed the *hld* and *eta* virulence genes (Fig 1).

Table 2: Antibiotic susceptibility profile of MRSA isolates (n=58)

Antibiotic	No sensitive (%)	No intermediate (%)	No resistant (%)
Oxacillin	0 (0.0)	0 (0.0)	58 (100.0)
Gentamicin	49 (84.5)	2 (3.4)	7 (12.1)
Ciprofloxacin	49 (84.5)	1 (1.7)	8 (13.8)
Moxifloxacin	54 (93.1)	0 (0.0)	4 (6.9)
Erythromycin	49 (84.5)	1 (1.7)	8 (13.8)
Clindamycin	18 (31.0)	31 (53.4)	9 (15.5)
Linezolid	58 (100.0)	0 (0.0)	0 (0.0)
Teicoplanin	58 (100.0)	0 (0.0)	0 (0.0)
Vancomycin	58 (100.0)	0 (0.0)	0 (0.0)
Tetracycline	36 (62.1)	0 (0.0)	22 (37.9)
Tigecycline	58 (100.0)	0 (0.0)	0 (0.0)
Fusidic acid*	15 (26.3)	42 (73.7)	0 (0.0)
Rifampicin*	37 (65.0)	16 (28.0)	4 (7.0)
Trimethoprim/sulphamethoxazole	46 (79.3)	1 (1.7)	11 (19.0)

*n=57 as MICs could not be determined for 1 isolate each

Table 3: Distribution of virulence and resistance genes (%) in MRSA strains

Gene	%
<i>MecA</i>	74
<i>BlaZ</i>	33
<i>ErmC</i>	3
<i>aac(6')-aph(2'')</i>	22
<i>TetK</i>	19
<i>Hla</i>	5
<i>Hld</i>	0
<i>Eta</i>	0
<i>lukS/F-PV</i>	3

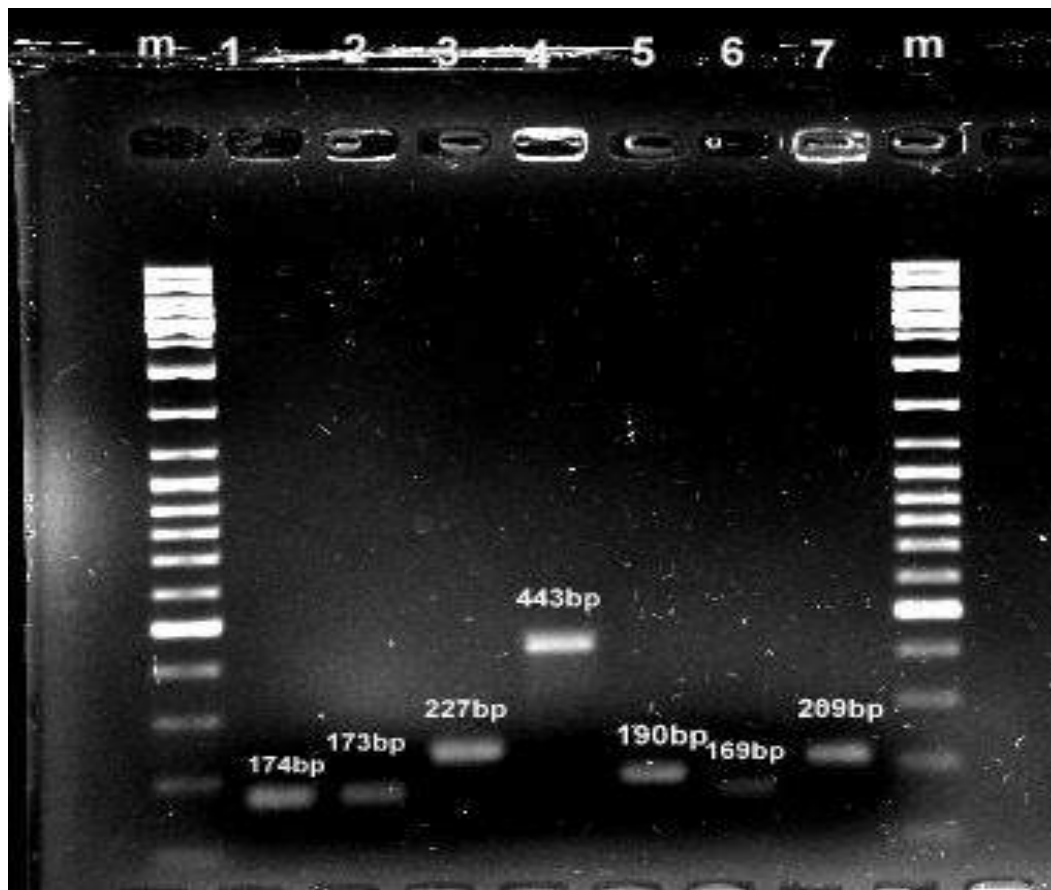


Fig 1: PCR mapping

Lanes M: DNA molecular marker, Lane 1: *mecA*, Lane 2: *blaZ*, Lane 3: *aac(6')-aph(2'')*, Lane 4: *lukS/F-PV*, Lane 5: *ermC*, Lane 6: *tetK*, Lane 7: *hla*

Three single nucleotide polymorphisms (SNPs) were observed for isolate S15, in the *hla* gene (C→T [12], T→A [15] and T→C [204]) as well as an insertion of G [8] and an SNP (G→C [429]) in the *lukS/F-PV* gene (KY056259). A missense mutation of N41S was observed in the *hla* protein (compared with the *hla* protein of *S. aureus* gb|AIG51324.1). Two SNPs (G→A [10] and A→C [111]) were also observed in the *blaZ* gene for isolate S31. A missense mutation of N98T was present in the *blaZ* protein (compared with the *blaZ* protein of *S. aureus* gb|ACP40660.1). Two putatively

novel nucleotide sequences for *hla* and *blaZ* genes were submitted to GenBank and the following accession numbers were assigned; KY056259 (*hla*) and KY056260 (*blaZ*). Detailed report of sequence analysis can be found in supplementary material at www.afrcem.org

PFGE analysis showed two major clades (A and B) of highly related isolates. The most resistant isolates against the most commonly prescribed antibiotics (i.e. gentamicin, ciprofloxacin, erythromycin, clindamycin, tetracycline and trimethoprim-sulfamethoxazole) belonged to clade B type (Fig 2).

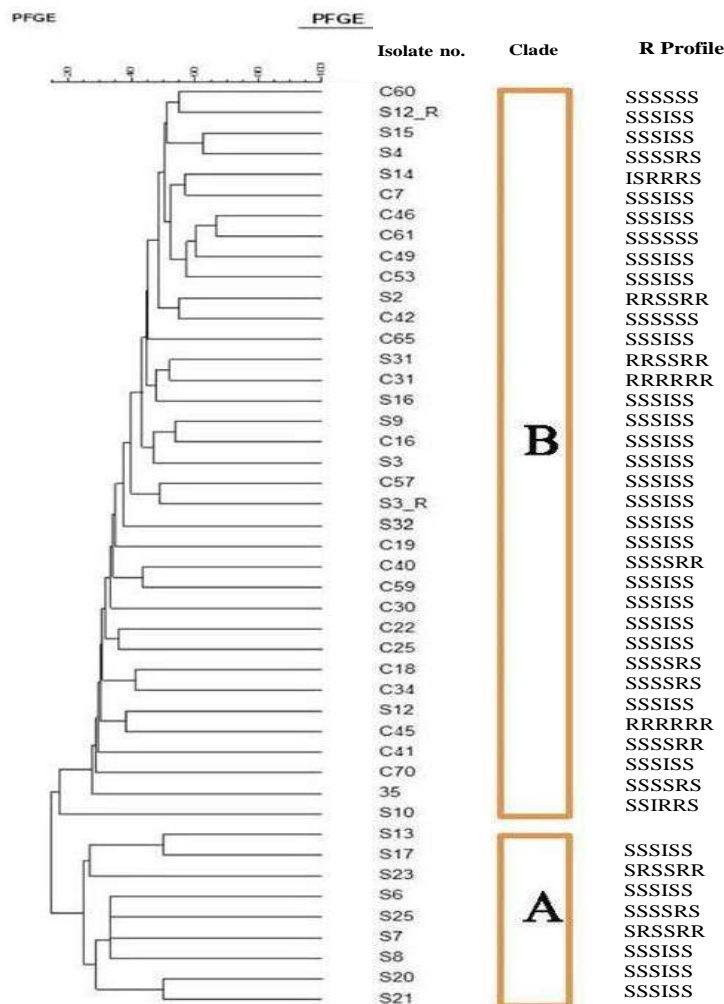


Fig 2: PFGE *SmaI* genotypic types generated from clinical MRSA isolates

The S, I and R (susceptible, intermediate and resistant) indicate resistance or susceptibility to gentamicin, ciprofloxacin, erythromycin, clindamycin, tetracycline and trimethoprim/sulfamethoxazole respectively

Discussion:

This study reports on the prevalence of MRSA from a Ghanaian hospital, with 58 (63%) of the 91 *S. aureus* isolates phenotypically methicillin or oxacillin resistant, and 43 (74%) were genotypically confirmed as MRSA. This figure is relatively high compared to the prevalence of 5.7% (11) in a Ghana study. However studies in Ethiopia, Egypt and Algeria showed an MRSA prevalence of 55.0%, 52.0% and 45.0% respectively in clinical isolates (22).

The use of tetracycline is common in Ghanaian communities, based on

anecdotal evidence and may be obtained over-the-counter, which may partly explain the high resistance to this antibiotic. The resistance level to trimethoprim-sulphamethoxazole was lower in this study compared to resistance levels found in studies in Nigeria (90.9%) (9), Tanzania (50.0%) (23), and Ghana (58.3%) (24). However Egyir *et al* in 2015 reported a low level of resistance (7.0%) to trimethoprim-sulphamethoxazole in Ghana (25). Hence, MRSA from Ghana are likely to be susceptible to trimethoprim-sulphamethoxazole than those from these other countries. None of the isolates was resistant to all antibiotics tested and the

susceptibility of all isolates to linezolid, teicoplanin, tigecycline, fusidic acid and vancomycin shows that these drugs remain tenable alternatives in the treatment of MRSA infections in Ghana. However, the high costs and limited availability of these drugs in Ghana means treatment options for MRSA infections are still limited in Ghana.

Furthermore, the relatively high susceptibility levels of MRSA isolates to the quinolones, ciprofloxacin and moxifloxacin, erythromycin, gentamicin and trimethoprim-sulphamethoxazole indicates that these agents may be relied upon when the first line anti-MRSA antibiotics are not immediately available.

Of the 58 isolates identified as MRSA by phenotypic tests (disc diffusion and MIC), 43 (74.0%) carried the *mecA* gene. Methicillin resistance in staphylococcal strains that do not carry the *mecA* gene may be linked with another mechanism of methicillin resistance such as changes in affinity of penicillin-binding proteins for oxacillin (15). In addition, there are other chromosomally determined components aside *mecA* that are implicated in methicillin resistance. For example, *FemA* and *FemB*, which encode proteins involved in the formation of the pentaglycine side chain of peptidoglycan, influence the methicillin resistance expression level in *S. aureus* (26). Again, methicillin resistance in the *mecA*-negative isolates could be mediated by the *mecC* or *mecB* genes (27).

The frequency of *tetK* gene found in this study (19.0%) is close to that reported in another study in Ghana (16.3%) (28). Tetracycline resistance determinants are common and are usually found in multidrug resistant bacteria (19). *TetK* mediates resistance to tetracycline but not to minocycline, and is a more widespread tetracycline resistance gene than *tetL* and *tetO* which are uncommon (29). The *tetK* and *tetM* genes code for ribosomal target site alteration and/or efflux mechanism, limiting the effectiveness of the drug. The observation that some isolates were resistant and yet

showed no *tetK* genes upon genotypic screening could be due to fact that their resistance to tetracycline may be mediated by other tetracycline resistant genes like *tetM*, which was not investigated in this study. Resistance to tetracycline is frequently due to the acquisition of new genes related to either conjugative plasmids or transposons (19). Some isolates were susceptible to tetracycline by phenotypic testing although they possessed the *tetK* gene, which suggests that the gene may be present but silent or minimally expressed in the absence of selection pressure. This assertion appears more likely given that the use of tetracycline for *S. aureus* infections in hospitals is on the decline in Ghanaian hospitals.

Of the eight isolates resistant to erythromycin and clindamycin (by phenotypic screening), only two possessed *ermC* gene upon molecular analysis. Resistance to erythromycin and clindamycin in the isolates that did not have *ermC* may be alternatively mediated by the *msrA* and *linA* genes respectively. *MsrA* encodes an efflux pump that actively pumps macrolides from the bacterial cell before they can bind their ribosomal target site. This mechanism however does not create resistance to lincosamides (16). *MsrA* and *linA* genes were not investigated in this study.

Clindamycin may be useful even in HA-MRSA when susceptibility testing shows it has activity, given the limited options for MRSA infections in Ghana. However, one major concern regarding the use of clindamycin in MRSA infections is the possible development of resistance in the presence of macrolide inducers such as erythromycin (30). This has resulted in some clinicians avoiding or abandoning the use of clindamycin in staphylococcal infections whenever there is resistance to erythromycin. The 'D-zone' test therefore distinguishes strains with intrinsic genetic possibility of resistance from those that are fully susceptible to clindamycin. None of the isolates tested was positive for inducible clindamycin resistance. Those that did not possess the clindamycin

resistance genes and were susceptible to clindamycin by susceptibility testing can therefore be safely reported as susceptible to clindamycin.

The prevalence of the *aac(6')-aph(2'')* gene found in this study was 22.0%. Report of a previous study in Ghana showed a prevalence of 17.0% for this gene (25). The presence of the *aac(6')-aph(2'')* gene in some isolates that showed no resistance to gentamicin implies the gene is not sufficiently expressed in these isolates. Again, some isolates carrying the *aac(6')-aph(2'')* gene expressed intermediate susceptibility towards gentamicin and the borderline susceptibility in these isolates may be ascribed to the expression of this gene.

LukS/F-PV gene, detected in two (3.0%) of the isolates is commonly associated with community-acquired MRSA (31). Genes encoding PVL, which is a cytotoxin that causes destruction of leucocytes and tissue necrosis, were not frequently encountered until recently, being produced by less than 5% of *S. aureus* strains worldwide. The frequency of PVL-positive *S. aureus* strains associated with clinical infections in England and Wales was reported to be 1.6% by Holmes *et al* (32), while a study in South Africa found it to be 0.3% (12). It is not therefore surprising that just two isolates carried the gene for PVL considering they are clinical isolates. Studies in Ghana however showed PVL prevalence rates of 17.0% (5/30) and 63.8% among hospital *S. aureus* isolates (25, 28). Although PVL genes are mostly associated with community strains of MRSA, data from West and Central Africa showed that no less than 40.0% of clinical methicillin susceptible *S. aureus* (MSSA) isolates in these regions are PVL-positive (7, 13). Thus, the acquisition of *mecA* gene by PVL-positive MSSA and the potential spread of PVL-positive CA-MRSA could pose a challenge in the control of infections in countries with inadequate resources in Africa. There was an insertion of G (8) and an SNP (G→C [429]) in the *lukS/F-PV* gene.

Alpha haemolysin is encoded by *hla*

was found in 5% of the isolates and plays an important role in staphylococcal infections (20). This is in contrast to reports from Uganda (14), United States (33) and Iran (20) where the frequency of *hla* recorded were 100.0%, 100.0% and 93.15% respectively, which suggests that the geographical distribution of this gene may be lower in Ghana. Alpha haemolysin, when secreted by *S. aureus*, integrates into host cell membranes and causes lysis of eukaryotic cells, with erythrocytes being particularly susceptible (34).

None of the isolates harbored the gene for the exfoliative toxin, *eta*. This is consistent with data from another study in Ghana (25). Three SNPs were observed for isolate S15, viz, in the *hla* gene (C→T [12], T→A [15] and T→C [204]). A missense mutation of N41S was observed in the *hla* protein (compared with the *hla* protein of *S. aureus* gb|AIG51324.1). The effects of SNPs at different positions of the codon may affect the expression and functioning of this gene. Alpha haemolysin hyperproduction in *S. aureus* is a multifactorial process affected at both the genomic and transcriptional levels (35). A study conducted by Liang *et al.*, in 2011 identified SNPs in the *hla* gene at positions 2376, 2483 and 2484 from the start codon, associated with alpha toxin hyperproduction (35).

The most resistant isolates against the most commonly prescribed antibiotics such as gentamicin, ciprofloxacin, erythromycin, clindamycin, tetracycline and trimethoprim-sulfamethoxazole belonged to clade B type. Detailed patient demographic data was unavailable, making the discussion of clonal relatedness as well as hospital-associated and community-acquired comparisons challenging.

Conclusion:

In conclusion, this study provides information on the occurrence of MRSA from a major referral hospital in Ghana over a 5-month period. Results indicated a fairly high occurrence of MRSA. Some

isolates carried the *mecA*, *blaZ*, *tetK*, *aac(6')-aph(2'')*, *ermC*, *lukS/F-PV* and *hla* genes. The high occurrence of MRSA found in this study is worrying, considering that there are limited treatment options for antibiotic-resistant *S. aureus* in Ghana. There is an urgent need to institute effective surveillance mechanisms to monitor MRSA and implement stringent infection prevention and control programmes to forestall its spread.

Conflict of interest:

Professor Essack is a member of the Global Respiratory Infection Partnership (GRIP) and the Global Analgesic Steering Committee sponsored by unrestricted educational grants from Reckitt and Benckiser.

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Anti-Salmonella activity of metabolites from African soldier termites, *Macrotermes bellicosus*

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Abstract:

Background: The global emergence and rapid dissemination of multidrug resistant *Salmonella* strains necessitate research to find new antimicrobials that will effectively be used against these pathogens. In the present study, anti-*Salmonella* activity of metabolites from African Soldier Termites, *Macrotermes bellicosus* was demonstrated and subsequently compared with a potent antibiotic, ciprofloxacin.

Materials and Methods: N-hexane, ethylacetate, methanol and aqueous extracts of metabolites from the *M. bellicosus* were assayed for anti-*Salmonella* activity using the agar dilution method in the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The inhibitory activities of the extracts were compared to ciprofloxacin (256µg/ml). Also, the bioactive components of the extracts were determined using standard techniques.

Results: At 4000 µg/ml, N-hexane extract inhibited the growth of *Salmonella* Typhi, *S. Paratyphi* A, B and C while ethylacetate extract was able to inhibit *S. Paratyphi* A and C. Methanolic and aqueous extracts at the same concentration were unable to inhibit these strains of *Salmonella*. Furthermore, our findings revealed that the MIC of ethylacetate extract was 2000µg/ml for *S. Paratyphi* A and B, 250µg/ml for *S. Typhi*, and 125µg/ml for *S. Paratyphi* C. Also, the MIC of hexane extract was 4000µg/ml for *S. Paratyphi* B, 2000 µg/ml for *S. Paratyphi* C, 500µg/ml for *S. Typhi* and 250µg/ml for *S. Paratyphi* A respectively. The screening of bioactive components revealed the presence of cardiac glycosides and alkaloids.

Conclusion: Our results provide evidence of anti-*Salmonella* action of metabolites from African Soldier Termites, *M. bellicosus*. N-hexane and ethylacetate extracts of *M. bellicosus* may be explored as novel antimicrobials for the treatment of typhoid and paratyphoid fevers thereby reducing the pressure exerted on available antibiotics.

Keywords: *Salmonella*, antimicrobials, insects, extracts

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Activité anti-*Salmonella* des métabolites de termites soldats africains, *Macrotermes bellicosus*

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Abstrait:

Contexte: L'émergence et la dissémination rapide de souches de *Salmonella* multirésistantes nécessitent des recherches pour trouver de nouveaux antimicrobiens qui seront utilisés efficacement contre ces agents pathogènes. Dans la présente étude, l'activité anti-*Salmonella* de métabolites de *Macrotermes bellicosus*, African Soldier Termites, a été démontrée et comparée par la suite à un antibiotique puissant, la ciprofloxacine.

Matériels et méthodes: L'activité anti-*Salmonella* a été dosée avec du N-hexane, de l'acétate d'éthyle, du méthanol et des extraits aqueux de métabolites provenant de *M. bellicosus* en utilisant la méthode de dilution en gélose dans la détermination de la concentration minimale inhibitrice (CMI) et de la concentration bactéricide (MBC). Les activités inhibitrices des extraits ont été comparées à la ciprofloxacine (256µg/ml). En outre, les composants bioactifs des extraits ont été déterminés à l'aide de techniques classiques.

Résultats: À 4000µg/ml, l'extrait de N-hexane inhibe la croissance de *Salmonella* Typhi, *S. Paratyphi* A, B et C alors que l'extrait d'acétate d'éthyle est capable d'inhiber *S. Paratyphi* A et C. Les extraits méthanoliques et aqueux à la même concentration ne peuvent inhiber ces souches de *Salmonella*. En outre, nos résultats ont révélé que la CMI de l'extrait d'acétate d'éthyle était de 2000µg/ml pour *S. Paratyphi* A et B, de 250 µg/ml pour *S. Typhi* et de 125µg/ml pour *S. Paratyphi* C. De plus, la CMI de l'extrait d'hexane était de 4000µg/ml pour *S. Paratyphi* B, 2000µg/ml pour *S. Paratyphi* C, 500µg/ml pour *S. Typhi* et 250µg/ml pour *S. Paratyphi* A respectivement. Le dépistage des composants bioactifs a révélé la présence de glucosides et d'alcaloïdes cardiaques.

Conclusion: Nos résultats fournissent des preuves de l'action anti-*Salmonella* des métabolites de termites de soldat africains, *M. bellicosus*. Des extraits d'N-hexane et d'acétate d'éthyle de *M. bellicosus* peuvent être explorés comme nouveaux antimicrobiens pour le traitement des fièvres typhoïde et paratyphoïde, réduisant ainsi la pression exercée sur les antibiotiques disponibles

Mots-clés: *Salmonella*, antimicrobiens, insectes, extraits

Introduction:

Typhoid and paratyphoid fevers are a severe, life-threatening disease caused primarily by serovars Typhi and Paratyphi A, B, or C of *Salmonella enterica* subspecies enterica. Typhoid accounts for approximately 90% of enteric fevers in Africa, Asia, and Latin America, mostly regarded as endemic, with high morbidities and mortalities (1). It is transmitted by the faecal-oral route via contaminated food and water and is therefore common where sanitary conditions are inadequate and access to clean water is limited (2).

Although, antibiotics have been used for the treatment and control of *Salmonella* infections in human and animals, recent studies show increase resistance to conventional drugs, including fluoroquinolones and third generation

cephalosporins which are the drugs of choice for infections caused by *Salmonella* and other members of the Enterobacteriaceae family (3, 4). The emergence of *Salmonella* strains with multidrug-resistant (MDR) genes has constituted a serious public health problem, resulting in higher treatment cost, longer stay in the hospital, and increase morbidity and mortality especially in developing countries (5). Therefore, an urgent search for novel anti-*Salmonella* agents with high potency which will be used as alternatives to conventional drugs is imperative.

Insects have been recognized recently to possess highly potent immune defenses that synthesize constitutive and inducible antimicrobial compounds capable of combating a wide spectrum of pathogens (6). Consequently, insects are now the major target as the abundant

potential source of different antimicrobial compounds (7, 8). Although insects make up 90% of the total number of animals on earth, insect-derived antimicrobial peptides (AMPs) only account for approximately 10% of more than 2,830 AMPs listed in the Antimicrobial Peptide Database; thus there are more AMPs with activity just waiting to be discovered (9).

African Soldier Termites, *Macrotermes bellicosus* are well distributed in Nigeria and other African countries. These termites can survive and propagate in diverse ecological environments due to their ability to develop resistance mechanisms against different diseases (10). This study, therefore, investigated the *In vitro* anti-*Salmonella* activity of different extracts from African soldier termite, *M. bellicosus* against strains of *Salmonella*.

Materials and Methods:

Zoological Method

Termites were sampled from the mound using a hoe in Minna, North-Central Nigeria by methods previously described (11). The samples were put in plastic bags with mound soil and immediately transported to the laboratory. The termites were further picked using forceps and transferred to a bottle containing methanol, and were identified as African soldier termites, *M. bellicosus*, by the Entomology section of the Department of Biological Sciences, Federal University of Technology, Minna, Nigeria.

Preparation and Screening of Extracts for Bioactive Components

The head of the termite which is distinct from the body was carefully removed using a sterile scalpel, which was air-dried for two weeks and pounded using clean mortar and pestle. Successive reflux extraction of the powdered termites' heads (80g) was carried out using solvents ranging from non-polar to polar to obtain various soluble portions. The resulting mixtures were filtered using Whatman filter paper No. 1 while the soluble portion was evaporated to dryness

using a steam bath. At the end of the extraction protocol, four extracts of N-hexane, ethylacetate, methanol, and aqueous extracts were obtained and stored in sterile universal bottles at 4°C until further processing. The extracts obtained were screened for the presence of bioactive components as previously described (12). Different bioactive components including saponins, tannins, phlobatannin, cardiac glycosides, alkaloids and reducing sugars were sought for.

Bacterial Strains

Pure clinical strains of *Salmonella* Typhi and *S. Paratyphi* A, B and C were collected from the Vaccine Laboratory, Department of Microbiology, Federal University of Technology Minna, Nigeria. The strains were further confirmed by biotyping using the Microbact™ 12A (12 E) kit.

Anti-Salmonella Activity

The extracts were reconstituted in DMSO and distilled water as 0.4g of the different extract was dissolved in 1ml of DMSO plus 4ml of distilled water. The anti-*Salmonella* activities of all the extracts were determined using agar dilution method (13). Under aseptic conditions, 1 ml of each reconstituted extract was dispensed into sterile Petri plates, and 19 ml of sterilized nutrient agar was added to make a final concentration of 2000 µg/ml, after which the plates were swirled for homogeneity. The plates were prepared in duplicates and control plates consisted of organism viability control (OVC), extract sterility control (ESC), medium sterility control and DMSO control plate was also prepared. The activities of extracts were compared with that of ciprofloxacin (256µg/ml). Ciprofloxacin, which is a fluoroquinolone known to be potent against *Salmonella*, was obtained from General Hospital, Minna, Nigeria and dissolved in sterile water before use.

Minimum Inhibitory Concentration Determination

The broth dilution method of Cheesbrough (13) was employed for MIC

determination. The hexane and ethylacetate extracts were diluted with nutrient broth to obtain 4000µg/ml, 2000 µg/ml, 500µg/ml, 250µg/ml and 150µg/ml respectively. A loopful of each *Salmonella* strain was added to the diluents and incubated at 37°C for 24hr. The tubes with the least concentration of the extracts that showed no turbidity were recorded as the MIC.

Minimum Bactericidal Concentration Determination

The MBC of the extracts were determined as earlier described (13). Briefly, the MIC tubes were sub-cultured on sterile nutrient agar and incubated at 37°C for 24hr. The plates with the least concentration of the extracts that showed no growth after sub-culturing was recorded as the MBC.

Results:

The anti-*Salmonella* activity of crude extracts of *M. bellicosus* are

presented in Table 1. The N-hexane extracts showed activity against the four isolates, *S. Typhi* and *S. Paratyphi* A, B and C screened while activity was only recorded against *S. Paratyphi* A and C from the Ethylacetate extract respectively. There were no anti-*Salmonella* activities recorded for both methanol and aqueous extracts.

The minimum inhibitory concentration (MIC) of N-hexane extract showed activity ranged between 4000 to 250µg/ml only for *S. Typhi* and *S. Paratyphi* A, B and C while that of Ethylacetate extract ranged between 2000 to 125µg/ml only for *S. Typhi* and *S. Paratyphi* A, B and C (Tables 2 and 3). The presence of bioactive components as obtained from the N-hexane and Ethylacetate extracts indicated the presence of cardiac glycosides and alkaloids (Table 4).

Table 1: Anti-*Salmonella* activity of crude extracts of *Macrotermes bellicosus*

Test organisms	Crude Extract (4000 µg/ml)			
	ST ^{SH}	ST ^{SE}	ST SM	ST ^{SW}
<i>S. Typhi</i>	+	-	-	-
<i>S. Paratyphi</i> A	+	+	-	-
<i>S. Paratyphi</i> B	+	-	-	-
<i>S. Paratyphi</i> C	+	+	-	-

+ = activity; - = no activity; ST^{SH} = N-hexane extract; ST^{SE} = Ethylacetate extract; STSM = Methanol extract; ST^{SW} = Aqueous extract

Table 2: Minimum Inhibitory Concentration (MIC) of N-Hexane Extract Crude Extract of *Macrotermes bellicosus*

Test organisms	N-hexane (µg/ml)					
	4000	2000	1000	500	250	125
<i>S. Typhi</i>	-	-	-	+	-	-
<i>S. Paratyphi</i> A	-	-	-	-	+	-
<i>S. Paratyphi</i> B	+	-	-	-	-	-
<i>S. Paratyphi</i> C	-	+	-	-	-	-

Activity = +; - = No activity

Table 3: Minimum Inhibitory Concentration (MIC) of Ethylacetate Extract Crude Extract of *Macrotermes bellicosus*

Test organisms	Ethylacetate (µg/ml)					
	4000	2000	1000	500	250	125
<i>S. Typhi</i>	-	-	-	-	+	-
<i>S. Paratyphi A</i>	-	+	-	-	-	-
<i>S. Paratyphi B</i>	-	+	-	-	-	-
<i>S. Paratyphi C</i>	-	-	-	-	-	+

+ = activity; - = no activity

Table 4: Bioactive components of the N-hexane and Ethylacetate extracts

Bioactive components	Extract	
	N-hexane	Ethylacetate
Tannin	-	-
Phlobatannin	-	-
Reducing sugar	-	-
Alkaloids	-	+
Steroids	-	-
Saponin	-	-
Cardiac glycosides	+	+

+ = Present; - = Absent

Discussion:

The study demonstrates that metabolites from the head of African soldier termites have antibacterial activities that are effective against some human pathogens. The pathogens tested were found to show different degree of susceptibility to various extracts obtained from the head of African soldier termites. In this study, N-hexane extract inhibited the growth of typhoid and paratyphoid bacilli while the Ethylacetate extract inhibited *S. Paratyphi A* and *C* only. Termites have developed the ability to deal with a rich microbial community inhabiting their nests and feeding sites. One of the ways which they do this is by the synthesis of antimicrobial peptides, among other defense mechanisms (14). These antimicrobial peptides may explain

the reason for the anti-*Salmonella* activity observed in this study. This claim was supported by the observation of Zeng (15), who reported the antibacterial activity of some peptides, spinigerin and termicin obtained from termites against pathogenic organisms. Furthermore, spinigerin and termicin peptides that are rich in cysteine and contained α -helical properties have high ability to permeate microbial cytoplasmic membranes (16). This was further corroborated by Lee (17), who studied the antimicrobial properties of spinigerin.

Although extracts used in this study were not obtained from plants, it has been suggested that for plants to be used for medicinal purposes, their extracts should possess antimicrobial activity with MIC < 1000 µg/mL (18). With reference to

their report, results of the MIC obtained from this study indicated that N-hexane extracts possess strong antimicrobial activity for *S. Typhi* and *S. Paratyphi A* while Ethylacetate extracts possess antimicrobial activity against *S. Typhi* and *S. Paratyphi C* since their MICs were less than 1000µg/mL. The evaluation of the MBC to ascertain the bactericidal effect of the N-hexane and Ethylacetate extracts on the *Salmonella* strains examined at various concentrations did not showed any bactericidal activity (results not presented). This further shows that a higher concentration above the MIC is required for bactericidal activity.

Phytochemicals are known to have complementary and overlapping mechanisms of action in the body including modulation of hormone metabolism and enzyme detoxification, antioxidant effects, stimulation of the immune system and antibacterial activity (19, 20). The qualitative screening of bioactive components from the head of African soldier termites shows the presence of some plant secondary metabolites; alkaloids and cardiac glycosides. The presence of these components in termites could undoubtedly arise from their host plants. This is because this species of termites is mainly xylophage. They are herbivorous whose diets consist primarily of wood. As such, Phyto-compounds present in the plants consumed may still be in circulation within the insects in relatively high amounts (21, 22). The presence of these bioactive components may also be the reason for the antimicrobial activity recorded in this study.

It was observed that alkaloids and cardiac glycosides also significantly decreased the growth and proliferation of pathogenic *Klebsiella pneumoniae* and *Staphylococcus aureus* (23). Similarly, Fernandez-Melendez (24) observed that the alkaloids in fire ant, *Solenopsis invicta* inhibited the growth of Gram-positive and Gram-negative bacteria acting as a broad-spectrum antimicrobial agent. Their findings are in consonance with the results obtained from this current study.

The extracts of *M. bellicosus* at 4000µg/ml displayed similar bacteriostatic effects on the test organisms when compared with ciprofloxacin (265µg/ml). It is noteworthy to further state that such results as obtained from this study can be attributed to the crude state of the extract used. Furthermore, upon purification and quantification of the extracts used in this study, their activities may be more potent and biocidal even at lower concentrations.

Conclusion:

The findings of the current study show that metabolites from the head of soldier termites can inhibit the growth and survival of some pathogenic bacteria. However, further studies are needed to standardize potential use of *M. bellicosus* as complementary agent for eliminating pathogenic bacteria. Also, synergistic effect of the use of metabolites of *M. bellicosus* and conventional antibiotics can also be examined.

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Original Article

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Prevalence of hepatitis B virus infections among blood donors in Federal Capital Territory, Abuja, Nigeria

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Abstract

Background: Transfusion of hepatitis B virus (HBV) infected blood represents a major but avoidable means of HBV transmission, which unfortunately still account for millions of global HBV infections annually.

Method: This study determined the prevalence of HBV infection among 550 blood donors aged 18 to 60 years from selected hospitals and blood transfusion centres within the Federal Capital Territory, Nigeria, using hepatitis B surface antigen (HBsAg) rapid diagnostic test (RDT) kit and Enzyme linked Immuno-sorbent Assay (ELISA). Representative positive and negative samples for RDT and ELISA were tested by both conventional and real-time polymerase chain reaction (PCR) assay

Results: Forty nine (8.9%) and 14 (2.8%) out of the 550 blood donors tested positive for HBsAg with the RDT and ELISA respectively. Replacement donors had higher prevalence rate of the HBV infection than voluntary donors. The highest prevalence of HBV infection was recorded among the 30–39 year age group. The difference in the rate of infection between the males and the females was not statistically significant ($p>0.05$). A perfect agreement between RDT and PCR and fair agreement between ELISA and PCR were observed.

Conclusion: This study report a high prevalence of hepatitis B virus infections among blood donors in Abuja, Nigeria which underscores the need for proper screening of blood for transfusion to completely eliminate the incidence of transfusion transmitted HBV infections.

Key words: Blood, Malaria, Hepatitis, Rapid, Immuno-sorbent, Polymerase

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Prévalence de l'infection par le virus de l'hépatite B chez les donneurs de sang dans le Territoire de la capitale fédérale, Abuja, Nigéria

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Abstrait

Contexte: La transfusion de sang infecté par le virus de l'hépatite B (VHB) représente un moyen important mais évitable de transmission du VHB, qui représente malheureusement toujours des millions d'infections mondiales au VHB chaque année.

Méthode: Cette étude a déterminé la prévalence de l'infection à VHB chez 550 donneurs de sang âgés de 18 à 60 ans de certains hôpitaux et centres de transfusion sanguine situés dans le Territoire de la capitale fédérale, au Nigéria, à l'aide du kit de test de diagnostic rapide (TDR) de l'antigène de l'hépatite B (HBsAg) et Essai immuno-sorbant lié à une enzyme (ELISA). Des échantillons positifs et négatifs représentatifs pour les TDR et ELISA ont été testés à la fois par le test de réaction en chaîne par polymérase (PCR) classique et en temps réel

Résultats: Quarante-neuf (8,9%) et 14 (2,8%) des 550 donneurs de sang ont été testés positifs pour HBsAg avec le TDR et le test ELISA, respectivement. Le taux de prévalence de l'infection à VHB était plus élevé chez les donneurs de remplacement que chez les donneurs volontaires. La prévalence la plus élevée d'infection par le VHB a été enregistrée dans le groupe d'âge des 30 à 39 ans. La différence de taux d'infection entre les hommes et les femmes n'était pas statistiquement significative ($p > 0,05$). Un accord parfait entre TDR et PCR et un accord juste entre ELISA et PCR ont

Conclusion: Cette étude fait état d'une prévalence élevée d'infections par le virus de l'hépatite B chez les donneurs de sang à Abuja, au Nigéria, ce qui souligne la nécessité d'un dépistage approprié du sang par transfusion afin d'éliminer

Mots-clés: sang, paludisme, hépatite, rapide, immuno-sorbant, polymérase

Introduction:

About 2 billion people worldwide have been infected with Hepatitis B Virus (HBV). An estimated 360 million people remain chronically infected with 2 million deaths annually (1). Hepatitis B viral infections are caused by the HBV; an envelope virus containing a partially double stranded circular DNA genome and classified within the family hepadnaviridae (2). HBV infects the liver, thereby impairing liver functions as the virus replicates within the hepatocytes during acute and chronic phases of HBV infections (3). According to the Centre for Disease Control and Prevention (CDC), HBV is present in the blood, blood products and body fluids such as vaginal secretions, semen and in low concentrations in the saliva of the carriers (4).

Among the various routes of transmission of HBV infection, blood transfusion transmission represents a deliberate process of transmission that can be avoided even with the slightest vigilance (5). In 2001, WHO estimated that transfusion of unsafe blood alone accounted for 8-16 million hepatitis B virus infections annually. Although, the

incidence of transfusion transmitted hepatitis B has been gradually controlled within the intervening period, the available records show that HBV remains yet the viral infection with the greatest risk of transmission through blood transfusion especially in developing countries. An obviously high variability in the prevalence of HBV is seen across the countries, with higher incidence and prevalence observed in developing nations such as 1.6%–7.7% in Brazil, 19.6% in Egypt and 2-10% across India (6). In Nigeria, the pooled prevalence of HBV is reported to be about 13.6% (7).

Even though there are routine tests for demonstrating the presence or otherwise of this infection, the greatest risk are blood donations made in the infectious window period, which is the time between development of infectious viraemia and/or reactivity to routine serological or nucleic acid technology (NAT) based screening tests (8). The safety of blood can be guaranteed by application of assay technologies with high specificity and sensitivity so as to be able to identify all true positive blood units and true negative blood units (9). Screening of blood donors is a critical issue as the outcome of the test if flawed for whatever reason can result in serious consequences

for either the blood service or the blood donor. False positive result can lead to a larger number of blood donors being deferred, while a false negative testing may jeopardize blood safety (10).

In Nigeria, the prevalence of HBV infection has been speculated to vary with regard to the screening method used with prevalence rates varying from 12.3% by enzyme linked immunosorbent assay, 17.5% by immuno-chromatography and 13.6% by HBV DNA polymerase chain reaction (11). It is therefore necessary to monitor the safety of blood supply and the effectiveness of the presently employed screening procedures since pregnant women and children are the major recipient. The aim of this study was to determine the prevalence of Hepatitis B Virus among blood donors and to compare the sensitivity of HBV detection methods employed in the study area.

Materials and methods:

Study area

The study areas are blood bank units of Asokoro, Maitama, Wuse District Hospital; National Blood Transfusion Services; Federal Medical Centre Jabi, Karishi and Nyanya General Hospital, all in the Federal Capital Territory (FCT), Abuja, Nigeria.

Study design

The study is a descriptive cross sectional survey of blood donors which include voluntary, family/replacement donors, and commercial/paid donors which are not recorded.

Ethical clearance

The study was approved by the Health Research Ethics Committee of the Federal Capital Territory, Abuja and other ethics body of the chosen blood bank centres where applicable.

Study population

There were a total of 550 subjects comprising 521 (94.7%) males and 29 (5.3%) females who came to the blood bank units of the hospitals to donate

blood. Sixty-three (11.5%) out of the 550 were voluntary donors while 487 (88.5%) were family replacement donors. No commercial/paid donors participated in the study. Participation in the study was based on informed consent while demographic data about subjects were collected through a structured questionnaire.

Inclusion criteria

All the subjects satisfied the qualifying criteria of age between 18 - 60 years, body weight of above 50 kg depending on the body mass index (BMI), haemoglobin requirement of more than 12.5 gm/dl and absence of significant medical or surgical history. All donors were offered pre and post donation counseling.

Exclusion criteria

Donors who did not meet the conditions spelt out in the inclusion criteria as well as those who did not consent to participate in the study, lactating mothers, pregnant or menstruating women, donors who did not meet the haemoglobin requirements, those who have donated more than the prescribed 3 times in the last 12 months and other requirements were excluded from participation in the study.

Samples collection and analysis

Five milli-liters of whole blood were obtained via venipuncture from the donors using 5 ml syringe (12). To ensure confidentiality of the donors, numbers were used. Two milli-liters of these were placed in ethylene diamine tetra acetic acid (EDTA) bottle for haematological screening of HBsAg. The remaining 3mls were dispensed into a red top vacutainer tube, then allowed to clot naturally at room temperature, centrifuged at 3000rpm for 5minutes to obtain the serum and was pipetted out of the vacutainer tube which was used for immunological analysis of HBsAg.

Rapid Diagnostic Test for HBsAg detection

Twenty five micro liters of serum

was added to the specimen well of the RDTs with the aid of pipette. The serum reacted with the particle coated with monoclonal anti-HBsAg. The mixture then migrates upward along the membrane by capillary action and reacts with polyclonal anti-HBsAg, which are pre-coated on the test region. After 15 minutes, invisibility of the control line indicated invalid result. The presence of two distinct lines in both the control and test regions indicated a positive result while one red line in the control region is a negative result. Strips used were from ACON® on the instructions of the manufacturers.

Enzyme Linked Immuno Sorbent Assay (ELISA) for HBV antibody detection

ELISA assay (ADALTIS, S. r. l, Milano, Italy) was used to reconfirm blood samples that yielded negative results from RDTs screening tests to quantify the absence of anti-bodies to HBsAg. The procedure was carried out according to the manufacturer's instructions. First, 20 µL of assay diluent was added into each well except the blank, and 100 µL of positive control, negative control and specimen were added into their respective wells except the blank. The plates were covered and incubated for 60 minutes at 37°C. After incubation, plate cover were discarded and 50 µL conjugate was added into each well (except the blank) and mix by tapping the plate gently. Each plate was covered and incubated for 30 minutes at 37°C. Following incubation, plate cover was discarded and each well was soaked for 30-60 seconds before washing for 5 times with diluted washing buffer. After the final cycle of washing, the plate was turned down on a blotting paper and gently tapped to remove any remainders.

To each well of the plate, including the blank, 50 µL of substrate solution "A" and 50 µL of substrate solution "B", were added. The plate was incubated at 37°C for 30 minutes without exposure to light. The enzymatic reactions that occurred between the substrate solutions and the conjugate produced blue colour in positive control and HBsAg positive sample wells. 50 µL of stop solution was added into each

well and mixed gently. Intensive yellow colour developed in positive control and HBsAg positive sample wells. Plate reader was calibrated with the blank well and the absorbance read at 450 nm. The cut of value was calculated and result evaluated by reading the absorbance within 10 minutes after stopping the reaction.

Polymerase chain reaction (PCR) assays for HBV DNA

Three RDT positive HBsAg samples, and two positive and seven negative ELISA samples were subjected to both conventional PCR (BIONEER, USA) and real time PCR (DAAN Gene, Biotechnology, China) assays, at the DNA LABS Limited, Q5 Danja, off Katuru Road, Kaduna State, Nigeria, according to the manufacturer's instructions.

DNA extraction

DNA was extracted from 100 µL serum sample using DAAN Gene DNA kit (cat.# DA-451, China) as follows; 50 µL of Proteinase K was pipetted into the bottom of a 1.5 mL centrifuge tube. 200 µL of fluid sample was added to the tube, and 200 µL of virus lysing solution was then added and mixed by pulse-vortexing for 15 seconds. The resulting mixture was incubated at 72°C for 10 min followed by a short spin down. 250 µL of ethanol (96-100 %) was added to the sample, mixed by vortexing and briefly centrifuged to remove drops from the inside of the tube lid. The mixture was applied carefully to the spin column (in a 2mL collection tube) without wetting the rim. The cap was closed and column in the collection tube was centrifuged at 12,000 rpm for 1 min.

The Mini spin column was placed in a clean 2 mL collection tube, and the tube containing the filtrate discarded. 500 µL of inhibitor removal and 500 µL of desalting solution (twice) were added to spin column without wetting the rim and centrifuged at 12,000 rpm for 1 min at room temperature and the spin column was replaced in a clean 2 mL collection tube. Filtrate was discarded in each step. Finally, the spin column was placed at room temperature in a clean centrifuge tube, 50 µL elution buffers was added to

the column and centrifuged at 14000 rpm for 3 minutes. The filtrate in this step resembles the isolated DNA.

Conventional PCR assay

Conventional PCR was performed using Bioneer AccuPower PCR PreMix a ready-to-use PCR reagent, which consist of lyophilized mix of; thermostable enzyme, dNTPs, reaction buffer, stabilizer, tracking dye and PCR product purification. A water/primer mix was prepared in a 0.5 mL Eppendorf tube on ice consisting of 2 µL of each primer (forward and reverse) and 280 µL of ultra-pure water. One strip of 6 Bioneer Premix tubes was labeled 1 to 6 including the positive and negative control and 16 µL of the water/primer mix was added to each of the 6 tubes with 2 µL of sample. These were placed in thin walled tubes inserted in the programmed PCR thermal cycler (Biometra's T Gradient).

The amplification reaction started with 3 min at 95°C for 1 cycle, followed by 30 seconds at 94°C, 45 seconds at 45°C, 1 minute at 72°C for 35 cycles, followed by final extension at 72°C for 7 minutes, and then soaked for 1 minute at 20°C. The primer pair, HepBP1: 5'-TCA CCA TAT TCT TGG GAA CAA GA-3' and HepBS1-2: 5'-CGA ACC ACT GAA CAA ATG GC-3', were used. The PCR products were electrophoresed on 3% agarose gels and DNA was visualized by ultraviolet trans-illuminator. Bands were compared by size with the presence of 350 bp DNA ladder fragment indicating a positive result.

Real Time PCR assay

Real Time PCR was performed using ABI Prism 7500 (Applied Biosystem, USA). All positive controls, negative controls and samples were tested in duplicates. 27 µL HBV-PCR and 3 µL Taq enzyme for each sample were properly mixed. A filter tip was used to add 20 µL supernatant of extracted DNA, HBV negative quality control product, HBV strong positive quality control product, HBV critical positive quality control product, and positive quantitative reference, into the HBV reaction tube. The tube was centrifuged at 8,000 rpm for several seconds and samples were

transferred to PCR amplification detection region using the cyclic conditions, temperatures and channels as follows; In stage 1 with ABI Prism 7500 instrument setup, detection channels used are FAM, VIC and ROX at 93°C for 2 minutes of 1 cycle. In stage 2 with Light cycler 4800 setup, detection channels used are FAM and VIC at 93°C for 45 minutes and 55°C for 60 seconds of 10 cycles. In stage 3 with DA7600 instrument setup, detection channels used are FAM and HEX at 93°C for 30 seconds and 55°C for 45 seconds, of 30 cycles

Statistical analysis:

Data generated were analyzed using the Statistical Package for the Social Sciences (SPSS) version 20.0 and Chi-square (X^2) test was used to measure significant relationship between different sociodemographic characteristics among blood donors at 95% confidence level.

Results:

Prevalence of hepatitis B virus infections

Out of the 550 blood samples obtained from the blood donors, 49 (8.9%) were positive for HBsAg with the RDTs while 14 (2.8%) were positive with ELISA. The age range of the donor is 18 to 60 years, with the majority of the donors (335) aged between 30 and 39 years, which also has the highest prevalence of HBV infections (Table 1). Both males and female donors tested positive for HBV (Table 2), with higher rates observed among the male subjects, 47 (9.0%) with RDT and 14 (2.9%) with ELISA compared to female subjects, 2 (6.8%) with RDT and none (0%) with ELISA. From the result of the RDT, replacement donors had a significantly higher rate of infection than voluntary donors ($p = 0.008$) (Table 3). DNA analyses of 12 samples (3 positive with RDT, 2 positive with ELISA and 7 negative with ELISA) were carried out by PCR (both real time and conventional PCR) as shown in Figures 1, 2 and 3. The samples were randomly selected upon screening them for HBsAg with RDT and confirmation with ELISA.

Table 1: Age group distribution of donors with HBV infections

Age group (years)	Number Examined	Number infected (RDT) (%)	Number infected (ELISA) (%)
< 20	1	0 (0.0)	0 (0.0)
20 – 29	83	8 (9.6)	1 (1.3)
30 – 39	335	31 (9.3)	11 (3.6)
≥ 40	131	10 (7.6)	2 (1.6)
Total	550	49	14
χ^2		0.464	2.017
<i>p</i> value		0.927	0.569

Table 2: Gender distribution of hepatitis B virus infection among blood donors

Gender	RDT		χ^2	<i>p</i> value	ELISA		χ^2	<i>p</i> value
	No examined	No positive (%)			Number examined	No positive (%)		
Male	521	47 (9)	0.003138	0.9553	473	14 (2.9)	0.5180	0.4717
Female	29	2 (6.8)			17	0		
Total	550	49 (8.9)			490	14 (2.8)		

Table 3: Prevalence of hepatitis B virus infection among blood donor types

Types of Blood Donors	RDTs (%)		ELISA (%)	
	Number examined	Number infected	Number examined	Number infected
Voluntary	63	0 (0.0)	63	2 (3.2)
Family/Replacement	487	60 (13.6)	427	12 (2.7)
Commercial	0	0 (0.0)	0	0 (0.0)
χ^2		6.959		0.043
<i>p</i> value		0.008**		0.835

The identification codes for the 12 samples are stated besides the Figures. Three (100%) positive samples from RDTs were positive with PCR. Also, 2 (22.2%) positive and 7 (77.8%) negative samples with ELISA were negative with real time PCR. In Figure 1, the curves in the graph represent positive samples with different viral load values of HBV DNA in the samples upon confirmation with real time

PCR, while absence of values and curves in Figure 2 showed negative samples. In Figure 3, conventional PCR also confirmed the 2 positive and 1 negative ELISA samples as negative with marker at 350 bp DNA ladder. Tables 4, 5 and 6 show the sensitivity, specificity and accuracy of RDTs, ELISA and real time PCR methods of HBV detection used in this study.

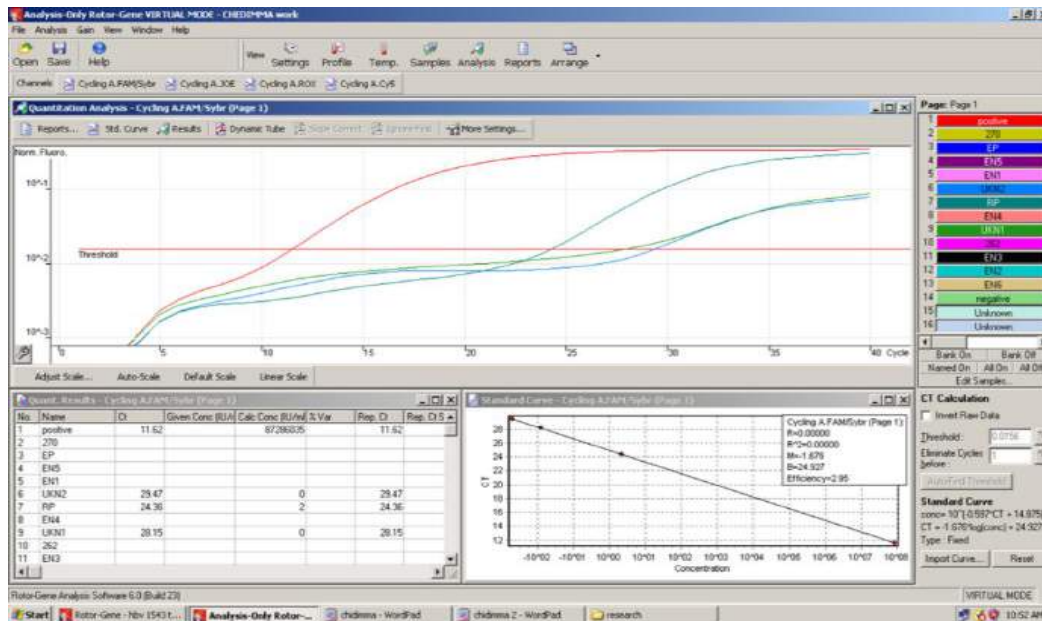


Fig 1: Amplification of extracted DNA from 3 positive RDTs, 2 positive ELISA and 7 negative ELISA samples using rt PCR. The curves on the graphs with the values on the table signify positive HBV samples from positive control and positive RDTs samples

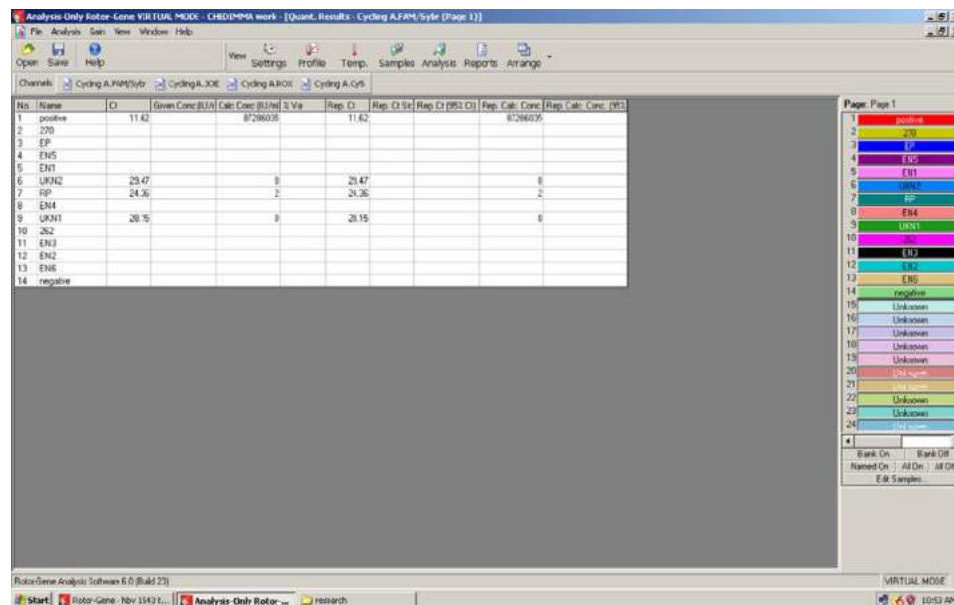


Fig 2: Amplification of extracted DNA from 3 positive RDTs, 2 positive ELISA and 7 negative ELISA samples using real-time PCR. The absence of curves on the amplified graphs with absence of values on some of the columns signifies that the 2 positive and 7 negative samples from ELISA tested negative after using real- time PCR

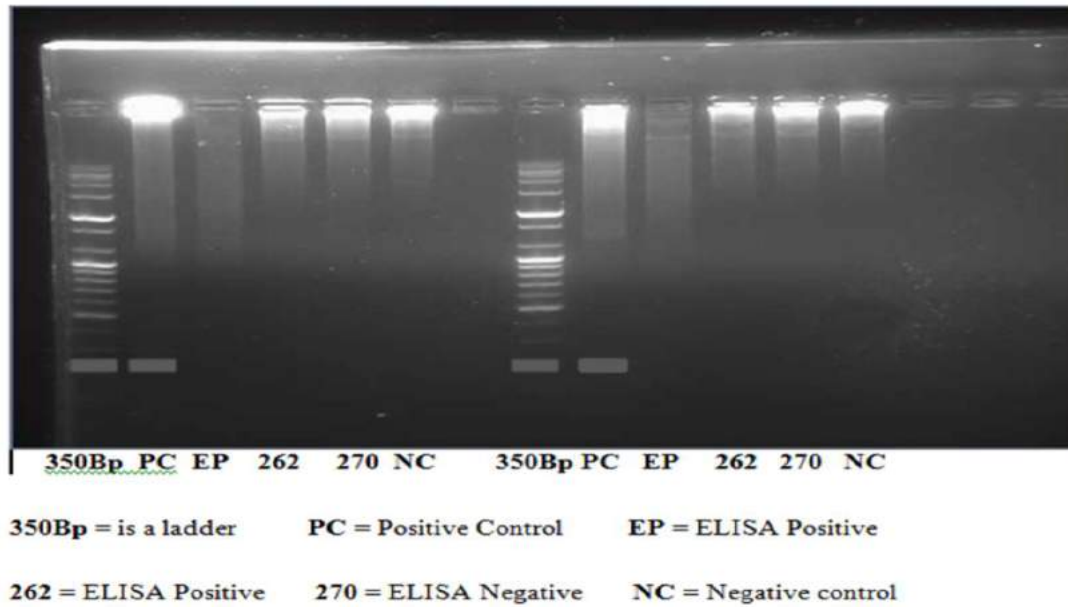


Fig 3: Agarose electrophoresis use to reconfirm 2 positive and 1 negative ELISA samples that were found to be negative using rt PCR

Table 4: Detection rate of HBV by RDT and ELISA tests

		ELISA		Total (%)	Kappa
		No reactive (%)	No non-reactive (%)		
RDT	Reactive	3 (100.0)	0 (0.0)	3 (100.0)	0.304*
	Non-reactive	2 (22.2)	7 (77.8)	9 (100.0)	
Total		5 (41.7)	7 (58.3)	12 (100.0)	

* = there's agreement between the two test methods. The two test methods will agree on 304 out of 550 samples

Table 5: Detection rate of HBV using ELISA and RT PCR

		ELISA		Total (%)	Kappa
		No reactive (%)	No non-reactive (%)		
RT PCR	Reactive	3 (100.0)	0 (0.0)	3 (100.0)	0.304
	Non-reactive	2 (22.2)	7 (77.8)	9 (100.0)	
Total		5 (41.7)	7 (58.3)	12 (100.0)	

* = there's agreement between the two test methods. The two test methods will agree on 304 out of 550 samples

Table 6: Detection rate of HBV using RDT and PCR

		PCR		Total (%)	Kappa
		No reactive (%)	No non-reactive (%)		
RDT	Reactive	3 (100.0)	0 (0.0)	3 (100.0)	1.000*
	Non-reactive	0 (0.0)	9 (100.0)	9 (100.0)	
	Total	3 (25.0)	9 (75.0)	12 (100.0)	

HBV infections and sociodemographic characteristics of blood donors

The prevalence of HBV infection estimated by RDTs and ELISA methods in relation to marital status was higher with 16.7% rate in divorced/separated donors (with RDTs), and 5.0% in singles (with ELISA) but this relationship is not statistically significant ($p>0.05$). In relation to occupational status, farmers had a higher prevalence of HBV infection with 17.0% but this was also not statistically significant ($p>0.05$). For educational status, the infection rate was higher in those with primary level education with 100% rate.

Analysis by risk factors in Table 7 shows association of HBV infections with factors such as tattooing, cauterization, blood transfusion, scarification, lack of vaccination, unprotected sex, multiple sex partners, anal sex, intravenous drug use, and injuries from sharp objects, and others.

Discussion:

According to the WHO criteria, the prevalence rate of 8.9% for HBsAg among blood donors in the Federal capital Territory, Abuja, Nigeria reported in our study is high (13). This rate is higher than 1.1% reported in Port-Harcourt (14), 2.4% in Yola (15), 4.2% in Zaria (16) and 5.9% in Ibadan (17). But our rate is lower than 20% reported in Benue State (18)

and 17.5% in Abuja by Agbesor et al. (19). In Burkina Faso, a high prevalence rate of 17.3% was reported (20), 11.2% in Cameroon (21) but low rate of 2.48% was reported in Northern Karnataka, India (22). Although it is difficult to explain the differences in the rate reported in our study and those of others, the variations observed may be related to the differential knowledge about hepatitis risk factors, mode of transmission, and information or lack of it on how to make a diagnosis of hepatitis B virus infections in health centres.

The majority of donors (88.5%) tested in this study were family replacement donors rather than voluntary non-remunerated donors (11.5%). The number of voluntary blood donors has continued to decline over the years in Nigeria and the net result is that commercial and family replacement donors persist. Family replacement donors accounted for a significant number of HBV infection compared to voluntary non-remunerated donors. This observation is consistent with the WHO suggestion (23) that the safest source of blood is voluntary non-remunerated donors. This is because replacement donors have long been regarded as higher risk based upon the assumption that friends or relatives are more likely to deny or ignore risk factors that invite further inquiry, removing the protection afforded by risk-screening questionnaires in favor or

coercion to donate. Furthermore, where there is urgent need to procure donors, family members may pay donors for their services thereby compounding transfusion risk.

The associated risky behaviours of the blood donors positively correlated with the rate of HBV infection among the blood donors. This was obvious in the case of people who admitted to having incomplete or no HBV immunization history. The prevalence rate of HBV was significantly high among the donors who are divorcee (30%). This may be attributed to exhibition of risky sexual behaviours that

put them at risk of infection. The age group 20 – 29 years, which is the group with highest infection rate (by RDT) in this study, is the sexually active and mostly independent adult group which incidentally constitutes the work force of the population. Appropriate agencies of the Federal Government of Nigeria and Non-governmental Organizations (NGO) need to redress by re-launching infection control programmes to curtail the spread of HBV infections. This should be aimed at changing high-risk behaviours, which are usually denied, among the youths.

Table 7: Infection rate of HBV on social demographic characteristics of donors

Social demographic characteristics		No examined	RDTs reactive	ELISA reactive	
Marital Status	Single	99	8 (4.8)	8 (5.0)	—
	Married	183	35 (11.1)	5 (1.8)	—
	Divorced	7	3 (30.0)	0 (0.0)	—
	Undisclosed	26	3 (5.3)	1 (1.9)	—
χ^2			11.734	4.476	—
p value			0.008	0.214	—
Occupation	Civil Servant	130	20 (8.2)	7 (3.1)	—
	Business	78	14 (10.3)	2 (1.6)	—
	Student	27	4 (8.5)	1 (2.3)	—
	Farmer	36	8 (17.0)	3 (7.3)	—
	Others	44	3 (3.9)	1 (1.4)	—
χ^2			6.664	4.431	—
p value			0.155	0.351	—
Level of Education	Primary	1	1 (100.0)	0 (0.0)	—
	Secondary	95	16	2	—
	Tertiary	196	30	12	—
	Others	23	2	0	—
χ^2			10.749	3.465	—
p value			0.013	0.325	—
Immunization against HBV	Yes	188	0 (0.0)	11 (2.8)	—
	No	127	49 (8.9)	3 (2.6)	—
χ^2			122.578	0.24	—
P -value			0.000	8.76	—
Exposed to any risk	Surgery	37	14 (23.7)	0 (0.0)	—
	Blood Transfusion	82	10 (7.1)	0 (0.0)	—
	Cauterization	45	3 (4.8)	4 (6.6)	—
	Scarification	37	16 (35.6)	0 (0.0)	—
	Tattooing	101	5 (2.5)	8 (4.1)	—
	None	13	1 (2.4)	2 (5.0)	—
χ^2			69.576	11.156	—
p value			0.000	0.048	—
Risky Sexual Behavior					
Unprotected sex		41	5 (7.9)	1 (1.7)	—
Multiple sexual partners		12	2 (15.4)	1 (9.1)	—
Anal sex		16	4 (14.8)	1 (4.3)	—
Sexually transmitted diseases		19	4 (12.9)	1 (3.7)	—
None		227	34 (8.2)	10 (2.6)	—
χ^2			2.793	2.212	—
p value			0.593	0.697	—

In this study, PCR test achieved 100% sensitivity, and this extremely high sensitivity score was not at the expense of specificity, which also had a perfect performance. The sensitivity of a test is the probability that the test will produce a true positive result when used in an infected population while specificity is the probability that a test will produce a true negative result when used in a non-infected population. Based on the predictive values, RDTs test achieved 100% Positive Predictive Value (PPV), while Negative Predictive Value (NPV) dropped to 81%. PPV refer to the probability that a person is infected with HBV when a positive test result is observed while NPV is the probability that a person is not infected when the test result is negative. Generally, in this study, ELISA test failed to diagnose NPV of PCR negative samples.

The best detection method that will be suitable in screening blood donors for the purpose of eradicating HBV transfusion infections was statistically calculated using kappa (as shown in Tables 4, 5 and 6). Kappa is a measure of agreement that tells the extent to which two methods or raters will agree with each other beyond what might be expected by chance alone (Cohen, 1960). Landis and Koch (1977) went further in interpreting the results of kappa values from 0-1 as follows; poor agreement = 0.20 or less, fair = 0.20 to 0.40, moderate = 0.40 to 0.60, good = 0.60 to 0.80 and perfect = 0.80 to 1.00. The kappa results reported in this study indicated a significant fair agreement, showing that both RDTs and ELISA, and ELISA and PCR methods will agree on 304/550 ($k=0.304$). Also RDTs and PCR agree on 550/550 ($k=1.000$), indicating a perfect agreement between the two test methods as reported in Table 6.

Similarly, Kappa can be related to diagnostic likelihood ratios which are not yet commonly reported in peer-reviewed literature, but they can be a valuable tool for comparing the accuracy of several tests to the gold standard. The positive diagnostic likelihood ratio (PDLR)

represents the odds ratio that a positive test result will be observed in an infected population compared to the odds that the same result will be observed among a non-infected population. The negative diagnostic likelihood ratio (NDLR) which represents the odds ratio that a negative test result will be observed in non-infected population compared to the odds that the same result will be observed among an infected population.

Although there are false negative samples observed in this study, the false positive rate was minimal. Providing a safe blood for the recipients still remains a major priority, especially in identifying donors at risk of spreading transmissible transfusion infections (TTIs) due to window period. New techniques of testing for HBV will result to zero risk and unnecessary deferral of donors with the introduction of nucleic acid amplification techniques (NAAT), in combination with other methods despite its cost.

Conclusively, this study recorded a higher prevalence rate of hepatitis B infection among blood donors in the Federal Capital Territory, Abuja, Nigeria. No voluntary donor was predisposed to hepatitis B infection with a higher significant difference at $p = 0.008$. In comparing the sensitivity of HBV detection methods namely, RDTs, ELISA and PCR, a fair agreement was observed between RDTs and ELISA and also between ELISA and PCR. A perfect agreement was observed between RDT and PCR.

It is recommended that upgrade of blood bank centres with modern advanced technology such as NAAT, in combination with other methods, should be employed. Encouraging 100% voluntary blood donation will reduce TTIs. Compulsory universal vaccination against HBV should be introduced in Nigeria (especially vaccination of neonates at birth in case of positive mothers) and the appropriate agencies of the Federal Government of Nigeria in conjunction with Non-Governmental Organizations (NGO) need to redress by re-launching infection control programmes to curtail the spread of HBV infections, with inclusion of HBV

testing and treatment in the list of laboratory tests covered by the National Health Insurance Scheme (NHIS) in the country.

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Original Article

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The risk of transfusion transmitted malaria and the need for malaria screening of blood donors in Abuja, Nigeria

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Abstract:

Background: Blood transfusion saves life but it is also a major risk factor in the transmission of certain infections such as malaria, which remains a public health problem in tropical and sub-Saharan Africa.

Methodology: This study investigated the prevalence of malaria among 550 blood donors aged 18 to 60 years from blood bank units of some selected hospitals in Federal Capital Territory (FCT), Abuja, using gold standard microscopy for malaria parasite detection.

Results: Two hundred and fifty two (45.8%) donors were positive for malaria parasites. Replacement donors had higher prevalence rate of malaria compared to voluntary donors. The distribution of infection on the basis of age revealed the highest prevalence rate of malaria among the 20- 29yrs age group. The rate of infection among the males and the females was not significantly different ($p > 0.05$). No association was observed between the blood group types and the rate of malaria infection ($p > 0.05$).

Conclusion: A high prevalence of malaria parasitaemia was observed among blood donors in FCT, Abuja, Nigeria in this study. The introduction of malaria screening as part of routine screening for blood donation and the provision of modern blood screening equipment within healthcare facilities are highly advocated.

Keywords: Blood, Malaria, Microscopy, ABO Blood group

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Risque de paludisme transmis par transfusion et nécessité d'un dépistage du paludisme chez les donneurs de sang à Abuja, Nigéria

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Abstrait:

Contexte: La transfusion sanguine sauve des vies, mais elle constitue également un facteur de risque majeur dans la transmission de certaines infections, telles que le paludisme, qui reste un problème de santé publique en Afrique tropicale et en Afrique subsaharienne.

Méthodologie: Cette étude a examiné la prévalence du paludisme chez 550 donneurs de sang âgés de 18 à 60 ans appartenant aux banques de sang de certains hôpitaux sélectionnés du Territoire de la capitale fédérale (FCT), à Abuja, en utilisant la microscopie de référence pour la détection des parasites du paludisme.

Résultats: Deux cent cinquante deux (45,8%) donneurs étaient positifs pour les parasites du paludisme. Le taux de prévalence du paludisme était plus élevé chez les donneurs de remplacement que chez les donneurs volontaires. La répartition de l'infection sur la base de l'âge a révélé le taux de prévalence du paludisme le plus élevé parmi le groupe d'âge des 20-29 ans. Le taux d'infection chez les hommes et les femmes n'était pas significativement différent ($p > 0,05$). Aucune association n'a été observée entre les types de groupes sanguins et le taux d'infection palustre ($p > 0,05$).

Conclusion: Une prévalence élevée de parasitémie paludéenne a été observée chez les donneurs de sang à FCT, à Abuja, au Nigeria, dans cette étude. L'introduction du dépistage du paludisme dans le cadre du dépistage systématique des dons de sang et la fourniture d'équipements modernes de dépistage du sang dans les

Mots-clés: Sang, Paludisme, Microscopie, Groupe sanguin ABO

Introduction:

Blood is a unique type of organic liquid that is produced within a living body which is indispensable for normal bodily function and even survival. In spite of the advances made in medicine, man has not been able to produce a functional substitute for human blood as a result of which blood transfusion will remain a clinically important medical procedure for a long time to come (1). Every second someone somewhere in the world needs blood as trauma, surgery, anaemia and complications of pregnancy in every countries of the world makes the need inevitable (2). Blood transfusion is an age-long important life-saving clinical intervention which unfortunately has also played a role in the transmission of infectious diseases when proper screening of donated blood is not carried out (World Health Organization (3, 4).

Blood donation occurs when a healthy person referred to as a blood donor voluntarily has his or her blood drawn for the purpose of saving another person's life. A blood donor may fall into any of three categories namely; voluntary (or allogeneic) donor, family (or replacement or directed) donor and

commercial (or paid) donors (5, 6, 7). Blood transfusion is safer today than it has ever been. However, any blood borne pathogen has the potential to be transmitted by blood transfusion if adequate vigilance is not maintained (8). It has been suggested that the safest source of blood supply is from voluntary non-remunerated donors usually seen as a population at low risk for transfusion-transmissible infections (3). Since blood safety is a major concern all over the world, certain factors such as the prevalence of asymptomatic carriers in the case of transmission transfusion malaria (TTM) should not be neglected.

Malaria is a protozoan parasitic infection caused by *Plasmodium* species; *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi* (9). The species differ widely in morphology, geographical distribution, characteristics and clinical presentation (10). In addition to being transmitted through the bite of an infected female Anopheles mosquito, malaria can also be transmitted by transfusion of blood from infected donors (11).

Transfusion Transmitted Malaria (TTM) was first reported in 1911 (12). But

due to high interest in human immunodeficiency virus (HIV), hepatitis B virus (HBV) and syphilis safety in blood transfusion, TTM has been a neglected topic until the year 2010 (13). Even with the interest in TTM leading to its inclusion by the World Health Organization among the diseases for which blood must be screened before transfusion, blood transfusion is still done without regard for TTM screening in Nigeria. This is despite the consequences for a substantial segment of the Nigerian populace among who are sickle cell disease patients for whom malaria is very dangerous. It is against this background that this study was designed to assess the danger associated with the non-inclusion of malaria screening for blood donors in the Federal Capital Territory, Abuja, Nigeria.

Materials and method:

Study area

Blood samples were collected at the blood bank units of Asokoro, Maitama, Wuse District hospitals, National Blood Transfusion Services, Federal Medical Center at Jabi and Karishi, and General Hospital, Nyanya. The choice of the hospitals reflects the level of patronage by most residents of Abuja especially in emergency cases. The donors were individuals without symptoms of malaria.

Ethical clearance

The study was approved by the Health Research Ethics Committee of the Federal Capital Territory, Abuja.

Study population

A total of 550 apparently healthy blood donors who came to the blood bank units of the hospitals to donate blood participated in this study based on informed consent. All the donors who satisfied the qualifying criteria of age (18-60 years), body weight (above 50 kg depending on the BMI), haemoglobin requirement (more than 12.5 gm/dl) and who had no significant medical or surgical history were qualified for the donation

process. All donors were offered pre and post donation counseling. Lactating mothers, pregnant, or menstruating women, and those who have donated more than the prescribed 3 times in the last 12 months were excluded from the study. The study was also designed to include different types of blood donors namely; voluntary, family/replacement donors and commercial/paid donors which were absent. The data obtained were analyzed to determine significant relationship between different socio-demographic characteristics of the blood donors.

Sample collection

Five milli-liters of whole blood were obtained via venipuncture from the donors using 5 ml syringe (14). To ensure confidentiality of the donors, numbers were used. Two milli-liters of these were placed in ethylene diamine tetra acetic acid (EDTA) for parasitological (malaria) screening.

Screening tests for malaria parasites

Thick and thin films were prepared following the procedure described by Cheesbrough (14) within 1-2 hours of collection. A drop of each blood sample was placed in grease-free clean slide and smeared with micro-pipette and cover slip to give a thick and thin film respectively. Slides were air-dried and thin films were fixed with methanol. All the films were stained with 1% Giemsa stain diluted with 9 ml of distilled water for 15 minutes and rinsed. The slides were dried at room temperature. Prepared slides were viewed under 100x objective lens of microscope with the aid of immersion oil and were reconfirmed by a medical laboratory scientist.

The search for the parasite was done by viewing the edges and the tails of the films, since the parasite cells tend to be found along these sites (14). The process of searching was characterized by continuous adjustment of the stage of the microscope to get different fields of each slide. A positive slide of malaria parasite

showing the different stages of the parasite was used as a guide in the identification of the parasite. Film was considered positive (p+) when any of the erythrocytic stage was observed and negative (p-) when absent (14).

ABO blood grouping

The ABO/Rhesus blood groupings were performed for all subjects using the slide method. Blood from each sample were placed separately on a clean tile. To each drop of blood, one of the antisera; anti A, anti B or anti D was added and then mixed with the aid of a glass rod. The blood groups were determined on the basis of agglutination.

Statistical analysis

Data was analyzed using Statistical

Package for the Social Sciences (SPSS) version 20.0, and Chi square (χ^2) was used to test significant association between malaria prevalence and different socio-demographic characteristics of the blood donors at 95% confidence level.

Results:

Prevalence of malaria

Out of the 550 blood donors sampled and analyzed for ABO Rhesus blood group and malaria, 521 (94.7%) were males while 29 (5.3%) were females. Malaria parasite was detected in 252 (45.8%) blood samples comprising 237 (45.5%) from males and 15 (51.7%) from females (Table 1).

Table 1: Gender Distribution of Malaria among Donors

Gender	Number examined	Number infected (%)
Male	521	237 (45.5)
Female	29	15 (51.7)
Total	550	252 (45.8)
χ^2		0.430
<i>p</i> value		0.512

Table 2: Frequency distribution of malaria with respect to types of blood donors

Types of blood donors	Gender			
	Male		Female	
	Number Examined	Number infected	Number Examined	Number infected (%)
Voluntary	60	19 (31.6)	3	1(0.3)
Family/Replacement	461	218 (47.2)	26	14 (53.8)
Commercial/Paid	0	0 (0.0)	0	0 (0.0)
	χ^2			5.675
	<i>p</i> value			0.017*

Statistical analysis of the result showed that there was no significant relationship between the gender and the prevalence of malaria ($p=0.512$). It was observed that there were 63 (11.5%) voluntary donors, 487 (88.5%) family/replacement donors, with no record of commercial/paid donors. In Table 2, replacement donors 218 (47.2%) had significantly higher rate of malaria than voluntary donors 19 (31.6%) ($p= 0.017$).

Age group and malaria

The age distribution ranges from 18 to 60 years with the majority of the donors 335 (60.9%) aged 30-39 years

(Table 3). The age group with the highest prevalence of malaria was the <20 years (100%). There was no significant difference in the age groups in relation to malaria ($p> 0.05$).

Blood group and malaria

Table 4 shows the distribution of malaria on the basis of blood group which revealed that O- blood group (58.3%) had the highest prevalence of malaria, followed by A- (55.6%), AB+ (53.3%), B- (50.0%), B+ (47.1%), A+ (47.0%), O+ (43.7%), and AB- (25.0%). However, there is no significant association between the prevalence of malaria and ABO blood groups ($p= 0.507$).

Table 3: Frequency distribution of malaria with respect to age

Age (18-60 years)	Number examined	Number infected (%)
< 20	1	1 (100.0)
20 – 29	83	36 (43.4)
30 – 39	335	158 (47.2)
≥40	131	57 (43.5)
χ^2		1.908
p-value		0.592

Table 4: Predisposition to malaria with relation to blood group

Blood Group	Number examined	Number infected (%)
A+	117	55 (47.0)
A-	9	5 (55.6)
B+	85	40 (47.1)
B-	10	5 (50.0)
AB+	15	8 (53.3)
AB-	4	1 (25.0)
O+	286	125 (43.7)
O-	24	14 (58.3)
χ^2		6.286
p value		0.507

Discussion:

This risk associated with malaria is worsened by the fact that absence of symptoms even for a long period does not necessarily mean lack of infectivity and malaria parasites survive well in stored blood. In this study, malaria prevalence rate of 45.8 % was recorded among blood donors in F.C.T, Abuja. This is higher than the 6.0% among blood donors in Maiduguri (15). This rate appears to be lower when compared to 77.4% obtained from blood donors in Owerri (16) and 93.4% obtained in Odoakpu, Onitsha South (17). A study in Sudan reported a figure lower than this study, 13% (18). These differences in the prevalence rate of malaria documented above are suggestive of local variations in the prevalence of plasmodia infection. A high prevalence of the malaria parasite in blood already screened for transfusion should be a cause for concern since majority of blood recipients are usually sickle cell disease patients, pregnant women, children and those with already compromised immune systems.

It was observed that female blood donors (51.7%) had higher infection rate with malaria than their male counterparts, though no significant difference was obtained ($p > 0.05$). The reason for this difference may be due to higher number of male donors than the female donors. Generally, males donate blood more often than females, particularly in developing countries (19). The reason has been attributed to socio-cultural influences and beliefs (20).

Blood group O+ was the dominant blood group type in this study with O- highly predisposed to malaria. There was no significant relationship between the ABO blood groups and malaria infections however. This finding contradicts the earlier reports of Migot-Nabias *et al.* (21) and Pathirana *et al.* (22) who observed low malaria parasitaemia among blood group O individuals. In their reports, they concluded that blood group O seems to confer a certain degree of protection

against severe course of malaria because of the absence of either A or B triglyceride antigen believed to enhance plasmodial cyto-adherence and rosette formation which in turn increases pathogenicity. However the even distribution of malaria observed among blood groups and their rhesus factor showed that any of the ABO blood groups may be equally predisposed to malaria. This observation is consistent with the findings of Fischer and Boone (23), Uneke (24), and Otajevwo (25).

In conclusion, the prevalence rate of 45.8% asymptomatic carriage of malaria among blood donors in F.C.T Abuja poses a serious threat to certain categories of recipients of the blood such as pregnant women and sickle cell disease patients. This finding underscores an urgent need to review the policy on the safety of blood for transfusion in Nigeria towards the inclusion of malaria as part of routine screening for donors. Awareness campaign to educate the public on the benefits of maintaining environmental practices which reduces the breeding of mosquitoes is strongly recommended.

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Sero-prevalence of human T-lymphotrophic virus 1/2 among HIV-1 infected individuals in Ilorin, Nigeria

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Abstract:

Background: HTLV-1 or 2 co-infection in individuals infected with HIV-1 can lead to increased morbidity. The shared routes of transmission of HTLV with HIV-1 may increase the prevalence of HTLV among HIV-1 infected population and subsequently affect patient's management.

Methods: Sera were collected from 144 HIV-1 infected individuals attending the highly active anti-retroviral therapy (HAART) clinic of the University of Ilorin Teaching Hospital between the months of May and August 2016. Sera were tested for anti-HTLV IgM and IgG antibodies to HTLV-1&2 using the sandwich enzyme-linked immunosorbent assay.

Results: Out of the 144 participants tested, 47 (32.6%) and 37 (25.7%) were positive for HTLV IgG and IgM respectively. Twenty-one participants (14.6%) had both IgG and IgM antibodies to HTLV-1&2. Ten individuals were anti-retroviral drug naïve out of which, four and six were positive to anti-HTLV IgG and IgM respectively.

Conclusion: Findings from this study revealed that there is high sero-prevalence of HTLV IgG and IgM antibodies among HIV-1 sero-positive individuals in Ilorin. The high rate of co-infection supports routine screening for HTLV-1/2 co-infection among HIV-1 infected individuals in Ilorin, Nigeria so that the purpose of HAART treatment and monitoring of patients to prevent progression to AIDS will not be aborted.

Keywords: Human T-cell lymphotropic Virus, Human immunodeficiency virus-1, IgG, IgM, CD4+ counts

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Prévalence sérologique du virus T-lymphotrophe humain 1/2 chez les personnes infectées par le VIH-1 à Ilorin, au Nigéria

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Abstrait:

Contexte: La co-infection par le HTLV-1 ou 2 chez les personnes infectées par le VIH-1 peut entraîner une morbidité accrue. Les voies de transmission partagées du VIH-1 par le VIH-1 peuvent augmenter la prévalence du virus dans les populations infectées par le VIH-1 et, par conséquent, affecter la prise en charge du patient.

Méthodes: Des sérums ont été recueillis auprès de 144 personnes infectées par le VIH-1 fréquentant la clinique de traitement antirétroviral hautement actif (HAART) de l'hôpital

universitaire de Ilorin entre mai et août 2016. Les sérums ont été testés pour la détection des IgM et IgG anti-HTLV. anticorps anti-HTLV-1 et 2 en utilisant l'essai sandwich immuno-absorbant lié à une enzyme.

Résultats: Sur les 144 participants testés, 47 (32,6%) et 37 (25,7%) étaient positifs pour les IgG et les IgM du HTLV, respectivement. Vingt et un participants (14,6%) avaient à la fois des anticorps IgG et IgM anti-HTLV-1 et 2. Dix personnes n'avaient jamais reçu d'antirétroviraux, dont quatre et six étaient positives aux IgG et aux IgM anti-HTLV, respectivement.

Conclusion: Les résultats de cette étude ont révélé une prévalence élevée de l'anticorps IgG et IgM du HTLV chez les sujets séropositifs pour le VIH-1 à Ilorin. Le taux élevé de co-infection favorise le dépistage systématique de la co-infection par HTLV – 1/2 chez les personnes infectées par le VIH – 1 à Ilorin, au Nigéria, de sorte que l'objectif du traitement HAART et du suivi des patients pour prévenir la progression du sida ne soit pas avorté.

Mots-clés: Virus lymphotrophe humain à cellules T, Virus de l'immunodéficience humaine-1, IgG, IgM, Compte de CD4+

Introduction:

Human T-lymphotrophic viruses (HTLV) and human immunodeficiency viruses (HIV-1) are two distinct family members of *Retroviridae* causing significant infections worldwide. These viruses have common modes of transmission and share an in vivo tropism for cells of the immune system particularly T lymphocytes, resulting into co-infections of a number of individuals worldwide (1, 2). HTLV is made up of two major types associated with disease in humans which are HTLV-1 and HTLV-2. Two other types have been discovered which are HTLV-3 and HTLV-4 (3). HTLV-1 is endemic in many well-defined geographic areas like Sub-Saharan Africa in which Nigeria is included. However, there is no recent representative data regarding prevalence of HTLV-1 among HIV-1 infected individuals in Nigeria but majority of serological studies carried out were on blood donors (4–8). Research has shown that HIV-1/HTLV-1 and HIV-1/HTLV-2 co-infection probably occur more frequently than physicians are aware of since routine testing for HTLV-1/2 is not usually performed.

HIV-1 infection is sexually transmitted therefore people with HIV-1 disease are at risk of having HTLV infection because both have same routes of transmission and similar cell tropism. Therefore, co-infection of both viruses will likely influence the pattern of progression to AIDS, since both viruses preferentially infect CD4+ T-cells. The common link between HTLV-1/2 in HIV-1 patients is that both have been linked

normal or high CD4+ T cell count hinder proper treatment as a result of delay in introduction of highly active anti-retroviral therapy (HAART) in co-infected patients. It has also been reported that co-infection of HTLV-1/2 and HIV-1, may accelerate progression to AIDS and significantly shorten survival time in such individual (9).

A major issue in developing countries including Nigeria is lack of routine screening practice for HTLV, thus there exist gaps in knowledge and awareness of transmission of HTLV infection from both healthy individuals and those who are co-infected with HIV-1. Little is known about the pattern of sero-prevalence of HTLV-1 in Ilorin; this study is therefore designed to determine the sero-prevalence of HTLV-1 among HIV-1 infected individuals attending the HAART clinic of UITH, Ilorin

Materials and method:

Study design and participants

This descriptive cross-sectional study was conducted at the HAART clinic among HIV-1 positive patients 18 years and above that visited University of Ilorin Teaching Hospital (UITH), Ilorin, Kwara State, Nigeria. At the HAART clinic, diagnoses of new cases of HIV infection are made and over 4 000 HIV-1/AIDS patients on therapy are monitored.

Sample size determination

Sample size was estimated using fisher's formula (10) adopting a prevalence rate of 10.47% (11) as follows; $N = Z^2pq/d^2$ where 'N' is the required sample size, 'Z' is the

confidence interval at 95% (1.96), 'p' is estimated prevalence of HIV-1 Infection, 'q' is $1 - p$, and 'd' is the degree of accuracy set at 0.05. This gives a sample size of 144. Patients were consecutively recruited after giving informed consents. Under aseptic conditions, 5 ml of venous blood was collected from each consenting participant using a hypodermic needle. Aliquots of blood specimen were decanted into tubes without anticoagulant and EDTA anti-coagulated tubes to determine the CD4 count. The blood sample bottles were labeled with sample code L001- L144.

Serum from blood samples in tubes without anticoagulant were separated by allowing the blood to clot at room temperature, and then centrifuged at 2500 rpm for 10 minutes. The serum samples were transferred into cryovials and stored at -20°C until required for analysis. The serum samples were analyzed for IgM and IgG antibodies to HTLV using enzyme-linked immunosorbent assay based on manufacturer's instruction.

Determination of CD4+ T-cell count in peripheral blood of HIV-1 patients

The CD4 T-cell count enumeration was done within 6 hours of blood specimen collection in HAART clinic laboratory in UITH. The blood sample dispensed inside the EDTA anti-coagulated tubes was used to determine the CD4 T-cell count in whole blood using Partec Cytoflow analyser (12).

Determination of anti-HTLV IgM and IgG antibodies in serum of HIV-1 patients

Analysis of IgG and IgM Antibodies to HTLV were detected using human T-lymphotrophic virus sandwich ELISA. The micro-ELISA strip plates were pre-coated with an antigen specific to HTLV IgG and IgM antibodies respectively. Standards test samples were added to appropriate micro-ELISA strip plate wells and combined to specific antigen. Then a horseradish peroxidase (HRP)-conjugated antigen specific to HTLV was added to each micro-ELISA strip plate well and incubated. Free components were washed away. The TetraMethylBenzidine (TMB) contained HTLV substrate solution was added to

each well. Only those wells that contained HTLV-IgG or IgM in different micro-ELISA strip plate respectively and HRP conjugate HTLV antigen appeared blue in colour and then yellow after addition of the stop solution. The optical density (O.D) was measured using a spectrophotometer at a wavelength of 450nm. The presence of anti-HTLV IgG or IgM antibodies in different micro-ELISA strip plate, respectively, was determined by comparing the O.D of sample to CUT OFF value of the plate according to manufacturers' instruction.

Statistical analysis

Data entry and analysis was carried out using the Epi info version 7.14 (2014) software packages (CDC). Results were presented in tables and charts. Chi-square test was used for statistical significance of the difference for different variables respectively. p value of < 0.05 was regarded as significant.

Ethical Considerations

This study was conducted in compliance with the Helsinki Declaration of 1975, as revised in 2008 and was approved by the Health Research and Ethics Committee of the University of Ilorin Teaching Hospital (ERC PAN/2016/04/1532). The participants gave their written informed consent before they were enrolled in the study. All data were analyzed anonymously throughout the study.

Results:

A prevalence of 47 (32.6%) and 37 (25.7%) was reported for IgM and IgG antibodies to HTLV respectively in the study population. The prevalence of anti-HTLV IgG and IgM antibodies among HIV-1 patients in different age groups showed that more individuals within age group of 40-49 years had more anti-HTLV IgG and IgM antibodies as shown in Figure 1.

Both IgG and IgM antibodies to HTLV were detected together in 21 HIV-1 patients among the different age groups. Among those between 30-39 years, six individuals had both IgG and IgM antibodies to HTLV, while among

age group 40–49 years, 10 individuals had both IgG and IgM antibodies to HTLV, and also among age group 50–59 years, three individuals had both IgG and IgM antibodies to HTLV and in the age group greater than 60 years, two persons had both IgG and IgM

antibodies to HTLV. Of the 144 HIV-1 seropositive patients recruited in this study, 40 (27.8%) were males and 104 (72.2%) were females. The females recruited in the study had HTLV IgG and IgM antibody than the males as shown in Table 1.

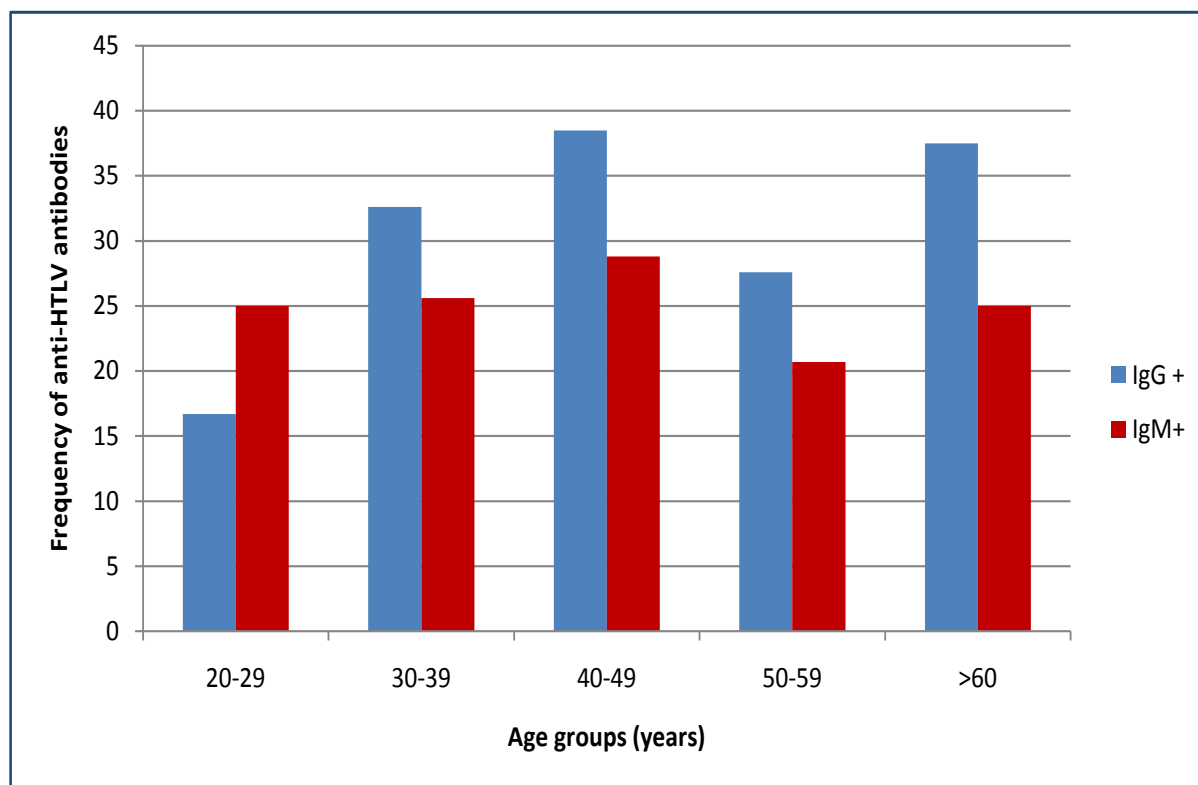


Figure 1: Frequency of anti-HTLV IgG/IgM antibodies among HIV-1 patients by age groups

Table 1: Gender distribution of HTLV IgG/IgM antibodies among HIV-1 seropositive patients at UITH

Gender	Total N (%)	IgG + N (%)	IgM + N (%)	IgG+ IgM+ N (%)
Male	40	12 (30)	9 (22.5)	4
Female	104	35 (33.7)	28 (26.9)	17
Chi-square		0.175	0.296	
<i>p</i> value		0.675	0.586	
Total	144	47 (32.6)	37 (25.7)	21

The distribution of HTLV IgG and IgM Antibodies by CD4 grouping among HIV-1 seropositive patients at UITH revealed that a number of participants with CD4 cell count below 200 cell/mm³ had IgG and IgM antibodies to HTLV but with a low frequency while individuals whose CD4 cell count was above 200cell/mm³, had a high possibility of producing detectable IgG and IgM antibodies to HTLV. The CD4+ T-cell count of individuals that IgM antibodies to HTLV has been detected when compared to individuals not infected with HTLV is not significant as shown in Table 2.

Table 2: Distribution of HTLV IgG and IgM antibodies by CD4 grouping among HIV-1 seropositive patients at UITH

CD4 grouping	Total	%IgG+	%IgM+
≤ 200	40	9 (22.5)	7 (17.5)
>200	104	38 (36.5)	30 (28.8)
Chi-square		2.589	1.948
p-value		0.108	0.163
Total	144	47 (32.6)	37 (25.7)

Table 3 shows sero-prevalence of HTLV IgG and IgM antibodies among HIV-1 sero-positive patients by ART status. Participants not on ART but sero-positive for HTLV IgG are more than those not on ART but sero-positive for HTLV IgM.

Table 3: Sero-prevalence of HTLV infection among HIV-1 seropositive individuals by ART drug status

HAART drugs	Total	IgG+ (%)	IgM+ (%)
Yes	134	41 (30.6)	33 (24.6)
No	10	6 (60)	4 (40)
Chi-square		3.659	1.152
p-value		0.056	0.283
Total	144	47 (32.6)	37 (25.7)

Discussion:

In this study, an overall sero-prevalence of 32.6% and 25.7% was

reported for anti-HTLV IgG and IgM antibodies respectively in HIV-1 infected individuals in UITH, Ilorin. Fourteen percent of the study participants had both IgG and IgM antibodies to HTLV, which is an indication that this group has been recently infected with HTLV and are at the point of seroconversion. Only 16 participants with IgM antibodies to HTLV can be said to be recently infected among the participants recruited. This observation is not conclusive because there was lack of information on time of onset of HIV-1 and HTLV infection but we can suggest that HTLV infection acquired is a marker of high risk behavior that might be associated with exposures to HIV-1.

In our study, we discovered that higher percentage of the participants infected were females, with 33.6% and 26.9% of them having IgG and IgM antibodies to HTLV respectively. Although, the number of females recruited in this study was more than the males, this could be because there is more efficient transmission from men to women during sexual intercourse. The HTLV sero-prevalence among HIV-infected individuals in this study is higher than the prevalence reported in previous studies, and this may be adduced to the reluctance in adapting to behaviours that are less risky especially amongst those with HIV in Ilorin. Also, the high sero-prevalence rate may be as a result of the method employed in this study. Nasir *et al.* (13) reported an overall sero-prevalence of 4.9% for HIV-1/HTLV co-infection among ART naïve patients in Abuja. In Brazil, HTLV-1 prevalence of 1.9% was reported in HIV-1 patients using the Polymerase Chain Reaction assay (14). Rego *et al.* (15) reported the prevalence of HTLV/HIV-1 co-infection to be 1.8% in KwaZulu-Natal, South Africa. In another study conducted in rural Guinea-Bissau among HIV-1 and HIV-2-infected women, overall HTLV-1 prevalence of 5.2% was reported (16). The difference is most likely due to the difference in the design, population and method of detection of HTLV. Molecular methods were used to confirm HTLV proviral DNA in the studies mentioned, whereas antibody detection was solely used in

this study.

In our study, the highest prevalence of HTLV IgG and IgM antibodies was among age group 40–49 years. This was in contrast with Nasir et al. (13) study who reported highest prevalence of HTLV-1 among age group 21–30 years. This could be due to the fact that the population of those mostly recruited falls among this age group in our study. Also, there is a presumption that the accumulation of sexual exposures with age in women of this population might contribute to the results obtained in this study.

Thirty out of 37 patients with IgM antibodies to HTLV had CD4+ lymphocyte count more than 200 cells/mm³ in our study, which is an indication that co-infected individuals may present with a seemingly normal CD4+ cell counts that may not correctly reflect the true immune status of the individual. We cannot interpret in clear terms that the seven individuals with CD4+ lymphocyte count less than 200 cells/mm³ who produced IgM antibodies to HTLV is associated with HIV-1 immune status of the individuals. In this scenario the immune system will be incompetent to produce neutralizing antibodies since HIV-1 infection can also lead to extensive defects in the humoral arm of the immune system (17).

There are reports that CD4+ lymphocyte count cannot always be considered a reliable marker of immunological competence in HIV-1 infected people, especially in patients co-infected with HTLV (3, 18, 19). There was no significant association between CD4+ cell count in HTLV/HIV-1 co-infected patients compared to HIV-1 infected individuals ($p=0.163$). CD4+ cell count is one of the important criteria used to determine eligibility for HAART in HIV-1-infected individuals especially in resource-limited settings nevertheless in the event of HTLV/HIV-1 co-infections, CD4 cell counts may not be reliable.

Conclusion:

Our findings in this study revealed that there is high sero-prevalence of HTLV IgG and IgM among

HIV-1 sero-positive patients in UITH. The high rate of co-infection supports routine screening for HTLV-1/2 co-infection among HIV-1 infected individuals in Ilorin, Nigeria so that the purpose of HAART treatment and monitoring of patients to prevent progression to AIDS will not be aborted.

Treatment modality in individuals co-infected with HIV-1 and HTLV-1/2 is recommended because these patients present normal or unexpectedly high CD4+ T cell counts which does not account for the immunosuppression experienced.

Competing interest:

The authors have no competing interest to declare

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Optimization of culture conditions for antimetabolite production by a rare tea garden actinobacterial isolate, *Amycolatopsis* sp. ST-28

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Abstract:

Background: Microbial metabolites are of great importance to the pharmaceutical industries. There is an urgent need of novel microbial metabolites in the present scenario to combat antimicrobial resistance. Selection and screening of potent microbial strains for production of antimicrobial metabolites as well as optimization of their culture conditions is of utmost importance in drug discovery. Therefore, the study was carried out to evaluate the effect of nutritional and cultural conditions on the production of bioactive metabolites by a rare tea garden actinobacterial strain *Amycolatopsis* sp. ST-28.

Materials and methods: Submerged fermentation of the actinobacterial isolate was carried out on different culture media and different culture conditions such as carbon and nitrogen sources, inoculum volume, pH, fermentation period and agitation speed. The culture filtrate was assayed against *Staphylococcus aureus*. Agar well diffusion method was employed to determine the maximum diameter of zone of inhibition (mm). The dried mycelial weight (mg) in a fixed volume of culture media was used for the determination of the total biomass produced.

Results: Maximum bioactive metabolite and biomass production was observed when submerged fermentation was carried out with mannose and peptone respectively as a sole carbon and nitrogen source. Maintaining other environmental parameters viz. inoculum 11% (v/v), pH of 6.5, temperature of 32°C and incubation period of 11 days at 150 rpm were found optimum for maximum antimicrobial activity.

Conclusion: This study demonstrated optimized cultural conditions for improved production of antimicrobial compound by *Amycolatopsis* sp. ST-28

Keywords: *Amycolatopsis*, antimicrobial, submerged fermentation, optimization.

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Optimisation des conditions de culture pour la production d'antimétabolites par un isolat rare d'actinobactéries de jardin de thé, *Amycolatopsis* sp. ST-28

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Abstrait:

Contexte: Les métabolites microbiens revêtent une grande importance pour les industries pharmaceutiques. Il existe un besoin urgent de nouveaux métabolites microbiens dans le scénario actuel pour lutter contre la résistance aux antimicrobiens. La sélection et le criblage de souches microbiennes puissantes pour la production de métabolites antimicrobiens, ainsi que l'optimisation de leurs conditions de culture, revêtent une importance capitale pour la découverte de médicaments. Par conséquent, l'étude a été réalisée pour évaluer l'effet des conditions nutritionnelles et culturelles sur la production de métabolites bioactifs par une rare souche d'actinobactéries de jardin de thé, *Amycolatopsis* sp. ST-28.

Matériels et méthodes: La fermentation immergée de l'isolat actinobactérien a été réalisée sur différents milieux de culture et différentes conditions de culture tels que les sources de carbone et d'azote, le volume d'inoculum, le pH, la période de fermentation et la vitesse d'agitation. Le filtrat de culture a été testé contre *Staphylococcus aureus*. La méthode de diffusion sur puits d'agar a été utilisée pour déterminer le diamètre maximum de la zone d'inhibition (mm). Le poids du mycélium séché (mg) dans un volume fixe de milieu de culture a été utilisé pour la détermination de la biomasse totale produite.

Résultats: Une production maximale de métabolites bioactifs et de biomasse a été observée lors de la fermentation en immersion avec du mannose et de la peptone, respectivement, comme seule source de carbone et d'azote. Maintenir d'autres paramètres environnementaux à savoir. inoculum 11% (v / v), pH de 6,5, température de 32 ° C et période d'incubation de 11 jours à 150 tr / min ont été jugés optimaux pour une activité antimicrobienne maximale.

Conclusion: Cette étude a démontré des conditions de culture optimisées pour une production améliorée de composé antimicrobien par *Amycolatopsis* sp. ST-28

Mots clés: *Amycolatopsis*, antimicrobien, fermentation en immersion, optimisation

Introduction:

Natural products with industrial applications are produced by the metabolism of living organisms (plants, animals or microorganisms). The most economical natural compounds produced by microorganisms, other than enzymes and recombinant proteins, are the low molecular weight primary and secondary metabolites (1). One microbe usually produces more than one compound, for example, a gentamicin-producing strain of *Micromonospora* produces 50 isolatable secondary metabolites (2).

Microbes isolated from nature usually produce extremely low levels of such metabolites. In order for a natural product to become a commercial reality, overproduction must be achieved initially at the laboratory level. Screening of proper strain and knowledge of microbial physiology is crucial to achieving higher metabolite production. The nutrition, growth and death rates, transport, energy, building blocks, polymer synthesis, regulation of enzyme synthesis, action and degradation, as well as cellular differentiation are some of the

factors influencing natural metabolite production.

Production of antibiotics by microorganisms differs qualitatively and quantitatively depending on the strains and species of microorganisms used as well as on their nutritional and cultural conditions (3). Changes in the culture medium and the sole source of carbon and nitrogen have great influence on the growth and antibiotic production by microorganisms as reported by different researchers (4, 5, 6). Thus, a mastery of the fermentation process for each new strain, sound engineering knowledge of media optimization, and the fine-tuning of process conditions are required to yield integrated and successful processes (7).

Microbial products have so long been exploited for their richness in the medical field. There has been tremendous progress made and success recorded in the field of antibiotic since the discovery of penicillin. However, the war against infectious diseases is yet to be won because of ever increasing threats of antimicrobial resistance of the microorganisms. One of such threats is

Staphylococcus aureus, an important pathogen of public health concern that has evolved into multiple antimicrobial resistant strains now considered a major problem. The organism can cause a wide variety of diseases ranging from superficial infections to severe life-threatening diseases such as pneumonia, endocarditis, septicaemia, and variety of toxin-mediated diseases including staphylococcal scalded-skin syndrome and toxic shock syndrome (8, 9, 10).

The search for new antibiotics should therefore be continued in order to overcome resistance of microorganisms. In this respect, efforts are being made to exploit the chemical diversity of the rare actinobacteria isolated from unexplored habitats, which may increase the chances of discovering novel structures of biotechnological importance (11). One such possible candidate of this rare actinobacterial group is the genus *Amycolatopsis*, proposed by Lechevalier et al., in 1986 on the basis of 16S rRNA gene sequence analysis (12). *Amycolatopsis* belongs to family *Pseudonocardiaceae* (13, 14) which are Gram positive, non acid fast, non motile, catalase positive actinobacteria. There has been intense scientific interest and focus on this genus due to its ability to produce diverse group of antibiotics and secondary metabolites. Some important antibiotics such as balhimycin, dethymycin, rifamycin and vancomycin are produced by *Amycolatopsis* strains. There is therefore the need to conduct more elaborate studies on this prolific group of rare actinobacteria for better understanding of its hidden potential and diversity (15-18).

Northeast India has been identified as the Indo-Burma hotspot due to its rich variation in the flora and fauna (19). However, information about the microbial diversity of this region is sparse in the literature. Owing to its pristine location, it is a treasure house of diverse microorganisms and novel metabolites that is waiting to be discovered for medical use. In our course of investigation for novel microbial metabolites that have both pharmaceutical and agricultural

importance, *Amycolatopsis* sp. ST-28, a tea garden isolate was screened against *Staphylococcus aureus*. In this study, attempt was made to determine the influence of different culture media, various carbon and nitrogen sources, inoculum volume, temperature, pH, aeration, and incubation period on *invitro* optimum growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28.

Materials and methods:

Microbial strains

Amycolatopsis sp. ST-28 was isolated using various selective isolation procedures (20, 21) from tea garden soil in Golaghat district, Assam, India (N 26027.534', E 093055.859'). Identification and characterization of the actinobacterial strain was done on the basis of colony morphology, biochemical and physiological properties (22, 23). The identity of the isolate was confirmed by PCR based 16S rRNA gene sequence analysis and the isolate has been deposited in GenBank with accession number (KY111723) (24). The strain was maintained in ISP-2 medium (yeast and malt extract medium) composed of yeast extract 4.0g, malt extract 10g, dextrose 4g, agar 15g and pH 7.3. The test organism, *Staphylococcus aureus* MTCC-737 was collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The organism was maintained on Mueller Hinton agar (HiMedia, India) and preserved at 4°C for two months and in deep freezer at -70°C in 15% (v/v) glycerol for longer period (25).

Submerged fermentation

Inoculum preparation

Two different media were used for production of inoculum with the following composition (g/L) (26); (i) IM-1 (Organic medium) composed of beef extract 3.0g, tryptone 5.0g, dextrose 1.0g, potato starch 24.0g, CaCo₃ 2.0g, and (ii) IM-2 (Complex medium) composed of soyabean meal 10.0g, corn steep solid 10.0g,

glucose 5.0g and CaCO_3 5.0g. The colonies from the previously grown pure culture of *Amycolatopsis* sp. ST-28 in ISP-2 medium was scrapped with 5ml of distilled water to make a colony suspension (26). This was then transferred to 45ml of each medium contained in 250ml capacity conical flasks which were incubated on a rotary shaking incubator at $28 \pm 1^\circ\text{C}$ and 220 rpm for 48 hours. A 10% (v/v) inoculum was transferred to a production medium (composed of sucrose 2.0g, malt extract 10g, yeast extract 4.0g, dipotassium hydrogen phosphate 5.0g, sodium chloride 2.5g, zinc sulphate 0.04g, calcium carbonate 0.4g, 1.0L distilled water, and pH 7.0). The fermentation was run at 30°C for 5 days on a rotary shaker at 220 rpm. The growth and antimicrobial activity were determined at the end of incubation period, and the best inoculum medium was used for optimization studies (26).

Basal media preparation

Seven fermentation media (HiMedia Laboratories, Mumbai, India) were inoculated with the *Amycolatopsis* sp. ST-28 strain for the production of antimicrobial secondary metabolites containing the following compositions (g/L) at pH of 7; (i) Starch casein medium (SCM) with soluble starch 10.0g, casein 1.0g, $\text{CaCO}_3 \cdot 2\text{H}_2\text{O}$ 10.0g and K_2HPO_4 0.5g (22); (ii) Bennett's medium (BM) with D-glucose 10.0g, beef extract 1.0g, yeast extract 1.0g and N-Z amine type A (casein hydrolysate) 2.0g (27); (iii) Soyabean medium (SM) with Dextrin 15.0g, soyabean 30.0g, $\text{CaCO}_3 \cdot 2\text{H}_2\text{O}$ 10.0g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0g (28); (iv) C medium (CM) with D-glucose 10.0g, soluble starch 35.0g, casein hydrolysates 5.0g, yeast extract 8.0g, meat extract 3.5g, soyabean meal 3.5g and $\text{CaCO}_3 \cdot 2\text{H}_2\text{O}$ 2.0g (29); (v) ISP-2 medium with glucose 4.0g, malt extract 10.0g and yeast extract 4.0g (30); (vi) ISP-4 medium with soluble starch 10.0g, K_2HPO_4 1.0g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0g, NaCl 1.0, $(\text{NH}_4)_2\text{SO}_4$ 2.0g, CaCO_3 2.0g (22); and (vii) Glycerol asparagine medium (GAM) with L-asparagine 1.0g, glycerol 10.0g, K_2HPO_4 1.0g, and trace

salt solution 1.0 ml (22).

The colony suspension of *Amycolatopsis* sp. ST-28 was prepared as described earlier. A 10% (v/v) inoculum was transferred to each of the above seven media and incubated at $30 \pm 1^\circ\text{C}$ for 10 days on a rotary shaking incubator at 220rpm. Growth and antimicrobial activity were determined at the end of incubation period and the best medium was selected as the basal medium which also serves as control for further experiments.

Invitro antibiosis and microbial growth

The culture broth of *Amycolatopsis* sp. ST-28 was centrifuged and filter sterilized (with $0.4 \mu\text{m}$ cellulose acetate). This was then bio-assayed against *Staphylococcus aureus* using agar well diffusion technique (31). Briefly, 0.1 ml of test bacterial suspension containing 3×10^8 cells/ml was spread aseptically on Mueller Hinton agar and 50 μL of culture broth was pipetted onto agar wells prepared by a sterile cork borer (6.00 mm in diameter). The diameter of zone of inhibition was recorded after 24 hours of incubation at $30 \pm 2^\circ\text{C}$. Growth was measured in terms of dry mycelial weight in a fixed volume (50 ml) of culture medium after drying the cell in an oven at 70°C overnight (25).

Optimization studies

Optimization of antimicrobial metabolite production was carried out with the removal and supplementation of nutrients based on single-dimension optimization (32). To determine the ideal condition for growth and maximum bioactive metabolite production, starch casein medium (SCM) with or without carbon and nitrogen sources was selected as the basal medium. A 10% (v/v) inoculum was transferred to the basal medium of 50ml. Flasks were incubated under stationary condition at $30 \pm 1^\circ\text{C}$ for 10 days.

Various parameters influencing the growth and antimicrobial metabolite production viz effect of medium supplements such as carbon and nitrogen sources and effect of the physiological conditions such as pH, temperature,

incubation period, inoculum size and agitation were studied. The experiments were conducted in triplicates.

Carbon and nitrogen supplement

Lactose, glucose, mannose, glycerol, galactose, starch, mannitol, starch, ethanol, succinic acid and sucrose were used as carbon source, while sodium nitrate, potassium nitrate, ammonium sulphate, L-asparagine, peptone, beef extract, ammonium chloride, malt extract, yeast extract and L-tryptophan were used as nitrogen source. Each carbon and nitrogen source was incorporated separately at 1.0% (w/v) level into the basal medium. The carbon sources were ether-sterilized to prevent denaturation (22).

Effect of temperature, pH, agitation, incubation period and inoculum volume

The optimum temperature for antimicrobial metabolite production was determined using different incubation temperatures ranging from 20 to 45±1°C in accordance with the method described by Suetsuna and Osajima in 1990 (33). Different pH values ranging from 5 to 9 were used after adjusting pH level of the basal medium with 1N HCl or NaOH. To determine the effect of aeration on growth and active metabolite production, culture flasks were incubated at 32±1°C in an orbital shaking incubator at 150rpm as well as at stationary phase. The effect of incubation period for maximum growth and antimicrobial metabolite production was observed up to 20 days of incubation. For all the previous experiments, 10% (v/v) fresh culture was used as inoculum but in the present experiment, varying inoculum volumes of 2 to 15% (v/v) were evaluated. Fermentation was carried out and bioactive metabolite produced was assayed (26).

Statistical analysis

The mean and standard errors of mean (±SE) were calculated for each experiment. Differences observed between various tested variables were determined using Analysis of Variance (ANOVA) and

Fisher's LSD was calculated with Statistical Package for the Social Sciences (SPSS) software version 18.0. Significance was considered where calculated *p* value was less than 0.05.

Results:

Choice of target organism

Amycolatopsis sp. ST-28 was assessed for its antimicrobial activity against *Staphylococcus aureus* and it showed prominent antimicrobial activity with zone of inhibition of 25±0.1 mm, hence for the study, it was taken as the choice target organism for culture filtrate assay.

Standardization of inoculum and basal media

Two different media were tested for inoculum production, IM-1 and IM-2. IM-1 (Organic medium) produced growth with dry mycelial weight of 42±0.3 mg/250ml and antimicrobial activity with diameter of inhibition of 9±0.1mm while IM-2 (Complex medium) produced growth with dry mycelial weight of 67±0.02 mg/250 ml and antimicrobial activity with diameter of inhibition of 14 ±0.66 mm. Therefore IM-2, which supported maximum yield in terms of growth and antimicrobial activity, was used for further experiment.

Amycolatopsis sp. ST-28 produced highest biomass (65.33±1.2 mg) as well as bioactive metabolite with maximum zone of inhibition (26.66±0.33 mm) against *Staphylococcus aureus*, when grown on Starch Casein (SC) medium (Fig 1). The other six media also supported the growth of the strain however the bioactivity was less compared with SC medium. Therefore, SC medium with or without carbon and nitrogen sources was used as the basal medium (control) for rest of the experiments.

Effect of carbon and nitrogen source

All of the test carbon sources supported the growth of *Amycolatopsis* sp. ST-28 (Fig 2). Maximum growth and bioactive metabolite production were

obtained in the basal medium supplemented with mannose as sole carbon source followed by basal medium with dextrose and glycerol (Fig 2). The result showed that mannose at a concentration of 1% (w/v) produced maxi-

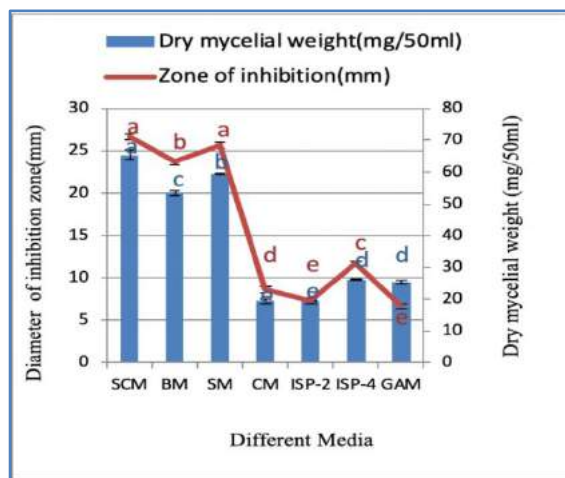


Fig 1: Effect of different culture media on growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p value < 0.05).

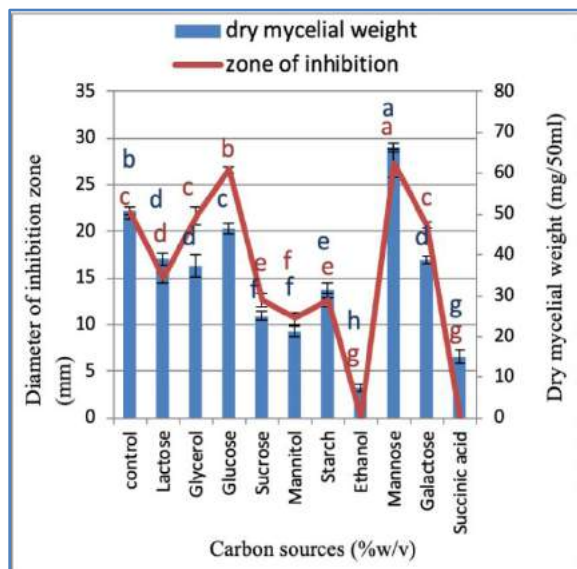


Fig 2: Effect of different carbon sources on growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p value < 0.05). The control used here is basal media.

mum cell mass (66.33 ± 1.2 mg/50ml) and inhibition zone (27.33 ± 1.45 mm) against *S. aureus*. Further optimization of mannose concentration showed that mannose at a concentration of 1.5% (w/v) was optimum for growth and bioactive metabolite production (Fig 3).

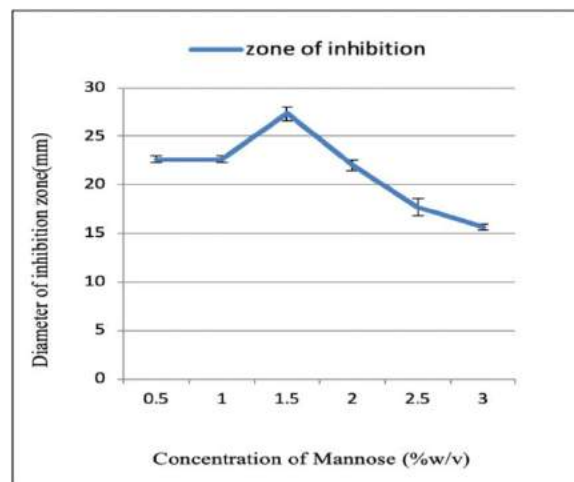


Fig 3: Effect of concentration of Mannose on bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE).

The nitrogen sources demonstrated significant effect on growth and metabolism of *Amycolatopsis* sp. ST-28. Only few nitrogen sources were able to support the growth and bioactive metabolite production by the strain (Fig 4). Peptone as sole nitrogen source in the basal medium produced maximum mycelial growth (66.66 ± 1.20 mg/50ml) and zone of inhibition (27.66 ± 1.20 mm), followed by basal medium with beef extract and yeast extract. Some nitrogen sources such as NaNO_3 , KNO_3 , NH_4NO_3 , NH_4Cl and L-asparagine had inhibitory effect on bioactive metabolite production and growth of the strain. The optimum concentration for better growth and antimicrobial metabolite production was 0.8 % (w/v) peptone (Fig 5).

Effect of temperature and pH

The results showed that the most suitable temperature for growth and antimicrobial metabolite production of *Amycolatopsis* sp. ST-28 was in the range of $30\text{--}32 \pm 1^\circ\text{C}$. Beyond this temperature,

growth and metabolite production of the strain considerably decreased. Maximum mycelial yield (67.33 ± 0.88 mg/50ml) and antimicrobial metabolite production (27.33 ± 0.33 mm) were recorded at $32 \pm 1^\circ\text{C}$ (Fig 6). Thus *Amycolatopsis* sp. ST-28 has very narrow range of temperature for its growth and antimicrobial activity.

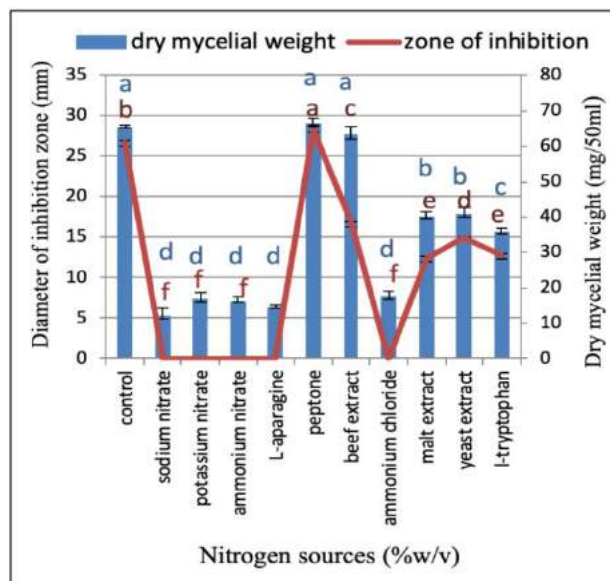


Fig 4: Effect of nitrogen sources on the growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p-value < 0.05).). The control used here is basal media.

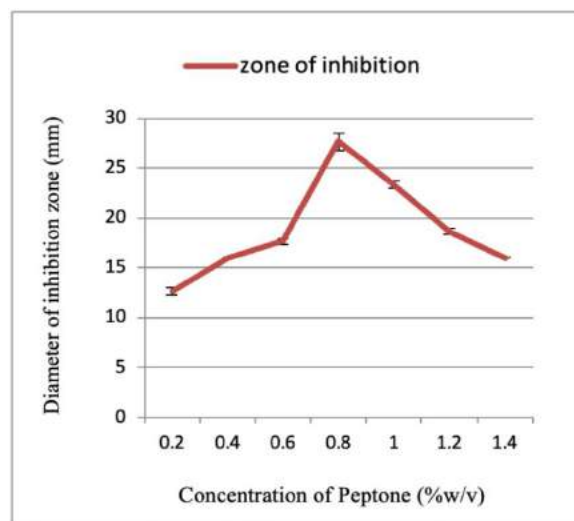


Fig 5: Effect of concentration of Peptone on bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE).

With regards to pH, the experiment revealed that *Amycolatopsis* sp. ST-28 grew when the initial pH of the medium was adjusted to pH 5 to 9, and the biosynthesis of antimicrobial metabolite and growth were maximal at pH 6.5 with 27.00 ± 0.00 mm and 65 ± 0.88 mg/50ml respectively. The growth and antimicrobial metabolite production decreased beyond pH 6.5 (Fig 7).

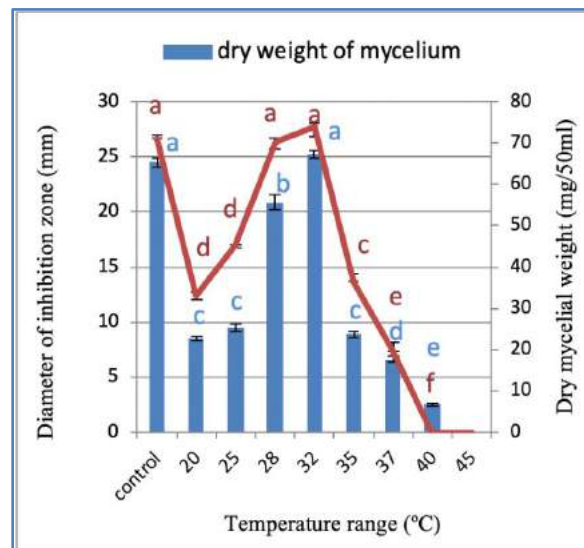


Fig 6: Effect of different temperature ranges on growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p-value < 0.05).). The control used here is basal media.

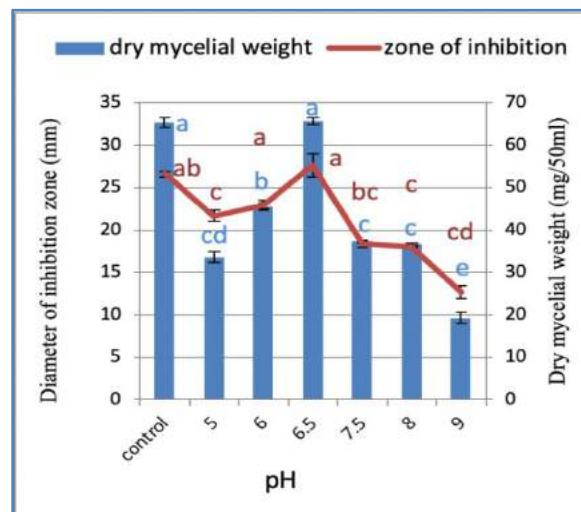


Fig 7: Effect of different pH ranges on growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p-value < 0.05).). The control used here is basal media.

Effect of incubation period, agitation speed and inoculum volume

The results showed that under good aeration, maximum growth and antimicrobial metabolite production were obtained. The optimum incubation time was 11 days under shaking condition. There was gradual decrease of antimicrobial activity and growth with

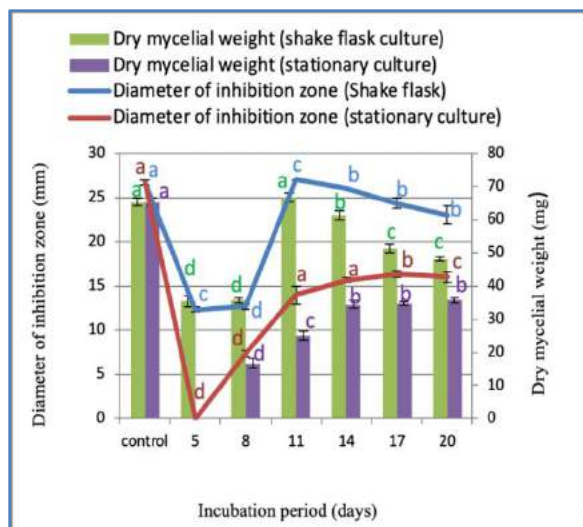


Fig 8: Effect of shaking and stationary condition on the growth and bioactive metabolite production *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p -value < 0.05). The control used here is basal media

further extension of incubation time (Fig 8). Inoculum dose of 11% (v/v) concentration was most effective for maximum growth and yield of antimicrobial metabolite (Fig 9). Table 1 shows the detailed description of optimized culture conditions and nutritional requirements for maximum production of antimicrobial compounds.

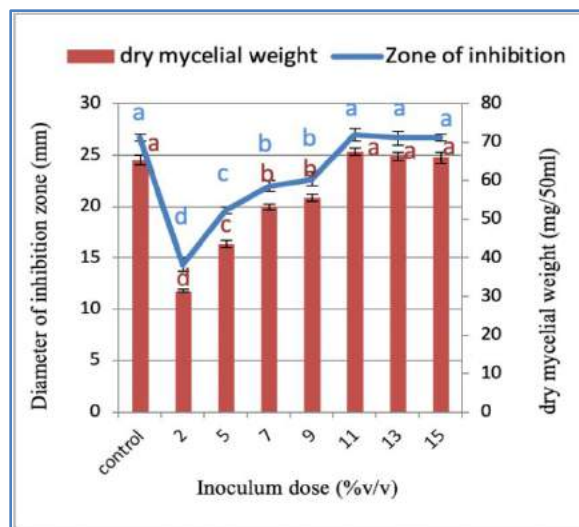


Fig 9: Effect of different levels of inoculums on growth and bioactive metabolite production by *Amycolatopsis* sp. ST-28. Vertical bars represent standard error (\pm SE). Means with different letters within an assay were significantly different (p -value < 0.05). The control used here is basal media

Table 1: Optimized cultural parameters for the growth and antimetabolite production by rare actinobacterial isolate *Amycolatopsis* sp. ST-28

Parameters	Optimum value	Dry mycelial weight (mg/50ml)	Zone of inhibition (mm)
Carbon source	Mannose	66.33 \pm 1.2	27.33 \pm 1.45
Mannose concentration	1.5%	-	27.33 \pm 0.33
Nitrogen source	Peptone	66.66 \pm 1.2	27.66 \pm 1.2
Peptone concentration	0.8%	-	27.66 \pm 0.88
Temperature	32°C	67.33 \pm 0.88	27.33 \pm 0.33
pH	6.5	65.00 \pm 0.88	27.66 \pm 1.3
Inoculum volume	11%v/v	67.66 \pm 0.88	27.00 \pm 0.57
Incubation period	11 days	66.66 \pm 1.4	27.00 \pm 00
Agitation	150 rpm	66.66 \pm 1.4	27.00 \pm 00

Discussion:

New drugs, especially antibiotics, are urgently needed to counter the threats of antibiotic resistant pathogens and to combat life threatening infectious diseases (34). The prevalence of methicillin-resistant *S. aureus* in hospitals has increased from 3% in the early 1980's to as high as 40% in recent times (35) and reports also suggest that coagulase negative *S. aureus* (CoNS) are becoming increasingly important in hospital settings where they cause serious infections (36). The focus, therefore, is on the rarer group of non-Streptomyces actinobacteria, which are yet to be well exploited for their rich chemical diversity. These rare actinobacteria produce diverse and unique, unprecedented, sometimes very complicated compounds with excellent bioactive potency, and usually low toxicity (37, 38).

Attempts are also being made to study the secondary metabolism of these rare actinobacteria for their ability to produce bioactive metabolites such as antibiotics. Nutritional parameters and growth conditions exert strong influence in production of secondary metabolite by microbes (39). The medium constitution together with the metabolic capacity of the producing organisms greatly affects antibiotic biosynthesis (32). Therefore, this present investigation was conducted to determine the optimal cultural conditions such as nutritional, physical and chemical parameters for enhanced production of antimicrobial compounds by *Amycolatopsis* sp. ST-28.

The results of our study show that *Amycolatopsis* sp. ST-28, which is a rare actinobacterium, exhibited prominent antimicrobial activity against *S. aureus*. Previous reports have demonstrated antibiotic activity of *Amycolatopsis balhimycina* and *Amycolatopsis orientalis* against methicillin resistant *S. aureus* strains (15, 40). Therefore during the optimization experiment in our study, *S. aureus* was selected as the target organism for the antimicrobial assay. *Amycolatopsis* sp. ST-28 showed highest

growth and antimicrobial metabolite production in Starch casein broth (SCB) as compared to the other tested media in our study. Similar findings were observed with *Arthrobacter* sp. SAA16 and *Streptomyces afghaniensis* VPTS3-1, when inoculated in different media, with SCB proving to be the best medium for growth and antimicrobial metabolite production in these studies (39, 41). This informed our selection of SCB as the basal medium (with or without C-sources) for the optimization studies.

Carbon sources are utilized by microorganisms for production of cell mass, primary and secondary metabolites and also for energy (42). Of the various carbon sources tested, basal medium supplemented with mannose at a concentration of 1.5% as sole carbon source supported the maximum growth as well antimicrobial metabolite production. Similar study on *Streptomyces* by Kreig and Holt reported that mannose was one of the most fermentable carbon sources by the actinobacteria for antibiotic production (43). However, further increase in the concentration of mannose did not increase the antimicrobial metabolite production in our study. This is probably because higher concentration of carbon tends to cause carbon repression which interferes with secondary metabolite formation (44). Similarly, other researchers have reported that *Amycolatopsis mediterranei* utilized glucose (1%) for rifamycin production however increase in concentration of glucose did not increase rifamycin production, which is attributable also to carbon catabolite repression (45).

Nitrogen plays an important role in biosynthesis of secondary metabolites. Nitrogen sources are classified into two types; inorganic nitrogen source which are regarded as the fast metabolizable nitrogen sources, wherein long-time accumulation of product does not occur, and the organic nitrogen sources are sustainable sources that are beneficial for steady accumulation of product (46). In the present study, organic nitrogen sources showed relatively higher

antimicrobial metabolite production and growth as compared to inorganic nitrogen sources. Peptone, an organic nitrogen source at a concentration of 0.8 % (w/v) in the basal medium was found optimum for the growth and antimicrobial metabolite production. The results agree with the earlier studies which suggest that low concentration of peptone (1%) greatly favored antimicrobial metabolite yield (32). The inorganic nitrogen sources such as ammonium nitrate and ammonium chloride supported growth to certain extent but did not allow production of antimicrobial metabolite. This might be due to presence of ammonium which inhibits the biosynthesis of antibiotics by decreasing the activities of enzymes involved in nitrogen metabolism (47). This is similar to the findings of the work done by Yu et al., (48).

Physiological factors such as pH and temperature have profound effects on the microbial growth and antimicrobial metabolite production. The results of our study indicated that growth and antimicrobial activity increased with gradual increase of pH from 5 to optimum pH of 6.5, and thereafter decrease in antimicrobial metabolite production and growth occurred. Actinobacteria have the ability to tolerate wide range of temperature. Temperature of $32\pm 1^\circ\text{C}$ was found optimal for growth as well antimicrobial metabolite production in our study, thus, confirming the organism as a strict mesophile. Similar studies conducted by other researchers have also confirmed pH and temperature as critical factors for growth and metabolism of actinobacteria (25, 41, 45, 49).

The variations in inoculum dose were also observed to have significant influence on growth and bioactive metabolite production in our study, with 11% (v/v) inoculum found optimum for antimicrobial metabolite production when tested in the range of 2 to 15 %. Other researchers have demonstrated inoculum volume effects on bioactive metabolite production, with lower levels of inoculum resulting in insufficient biomass leading to reduced product formation, while higher

level of inoculum may produce too much biomass leading to poor product formation (26, 48, 50).

The influence of incubation period and agitation has also been studied, as this forms an integral part of optimization. Krishna et al., observed that a period of 11 days and aeration of 50/250ml was optimum for the production of rifamycin by *Amycolatopsis mediterranei* (45) while Thakur et al., observed that *Streptomyces* sp. 201 required a maximum period of 6 days under shaking conditions for optimum antibiotic yield and biomass production (25). In the present study, maximum incubation period required for optimum growth and antibiotic yield was 11 days under shaking conditions, but under stationary condition, the growth and antimicrobial metabolite production were comparatively slower and the optimum production of antibiotics was on the day 17. Thus, agitation which provides proper oxygen transfer to the cells generally caused increase in growth of the organism.

Conclusion:

Our study reveals that a rare actinobacteria strain, *Amycolatopsis* sp. ST-28 isolated from tea garden soil produced maximum growth and bioactive metabolite with a medium supplemented with 1.5% mannose as carbon source, peptone at a concentration of 0.8% as nitrogen source, and other process parameters such as media pH of 6.5, incubation temperature of $32\pm 1^\circ\text{C}$, inoculum volume of 11% (v/v) and incubation period of 11 days under shaking condition. Therefore, crude extracts of *Amycolatopsis* sp. ST-28 has huge potential to be explored as antimicrobial agents for future development. Further characterization, purification and elucidation of the antimicrobial compounds present in the crude extracts are parts of an ongoing research.

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Original Article

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Seroprevalence of Toxoplasmosis and associated risk factors in pregnant women at the Protestant Hospital, Mbouo-Bandjoun, Cameroon

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Abstract:

Background: Toxoplasmosis is a common worldwide infection caused by the protozoan *Toxoplasma gondii*. In Cameroon, several recent studies have reported high seroprevalence of this parasitosis in various hospitals (Douala, Limbe, Njinikom and Yaoundé). The aim of this study was to determine whether this high prevalence of toxoplasmosis might occur in other regions of the country.

Methodology: Serological tests by the indirect Enzyme Linked Immunosorbent Assay (ELISA) for IgG and IgM were carried out on 200 pregnant women (36 HIV-positive and 164 HIV-negative) at the Protestant Hospital of Mbouo-Bandjoun in western Cameroon to determine the presence of *Toxoplasma gondii* infection and to identify the risk factors associated with seropositivity of the subjects.

Results: The overall seroprevalence of anti-*Toxoplasma* antibodies was 45.5%. The prevalence rate was 80.5% in the HIV-positive group (47.2% IgG, 22.2% IgM and 11.1% IgG + IgM) and 37.8% in the HIV-negative group (25.6%, 9.7% and 2.4% respectively). Using a multivariate logistic regression analysis, the secondary level of educational, presence of HIV infection, and frequency of close contacts with cats were significantly associated with the prevalence of IgG and/or IgM antibodies.

Conclusion: Compared with previous reports of human toxoplasmosis in Cameroon, the prevalence in our study showed a decrease in the disease occurrence. Further studies are needed to determine whether this decrease is localised to our study or a general phenomenon currently affecting the country.

Keywords: Cameroon, IgG, IgM, pregnant women, seroprevalence, *Toxoplasma gondii*

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Séroprévalence de la toxoplasmose et facteurs de risque associés chez des femmes enceintes à l'Hôpital Protestant de Mbouo-Bandjoun, Cameroun

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Résumé:

Contexte: La toxoplasmose est une infection répandue dans le monde entier et causée par le protozoaire *Toxoplasma gondii*. Au Cameroun, plusieurs études récentes ont signalé une forte séroprévalence de la parasitose dans divers hôpitaux (Douala, Limbé, Njinikom et Yaoundé). Le but de cette étude était de déterminer si cette forte prévalence de la toxoplasmose pouvait se produire dans d'autres régions du pays.

Méthodologie: Des tests sérologiques pour quantifier les IgG et IgM par dosage indirect immuno-enzymatique indirect selon la méthode ELISA ont été effectués sur 200 femmes enceintes (36 VIH-positives et 164 VIH-négatives) à l'hôpital protestant de Mbouo-Bandjoun, dans l'ouest du Cameroun, afin de déterminer la présence d'infection à *T. gondii* et d'identifier les facteurs de risque associés à la séropositivité des sujets.

Résultats: La séroprévalence globale des anticorps anti-*Toxoplasma* était de 45,5 %. Le taux de prévalence était de 80,5 % dans le groupe VIH-positif (47,2 % IgG, 22,2 % IgM et 11,1 % IgG + IgM) et de 37,8 % dans le groupe VIH négatif (25,6 %, 9,7 % et 2,4 %, respectivement). L'emploi d'une analyse de régression logistique multivariée a montré que le niveau secondaire d'éducation, la présence d'une infection par le VIH et la fréquence des contacts étroits avec les chats étaient significativement associés à la prévalence des anticorps IgG et/ou IgM.

Conclusion: Par rapport aux rapports précédents sur la toxoplasmose humaine au Cameroun, la prévalence dans notre étude a montré une diminution de la fréquence de la maladie. Des études complémentaires sont nécessaires pour déterminer si cette baisse est localisée dans notre étude ou est un phénomène général qui affecte actuellement le pays.

Mots clés : Cameroun, femmes enceintes, IgG, IgM, séroprévalence, *Toxoplasma gondii*

Introduction

Toxoplasmosis is a common world-wide infection caused by the protozoan *Toxoplasma gondii*. In immuno-competent adults and children, this infection is usually asymptomatic or causes symptoms such as fever or malaise that spontaneously resolve (1, 2). Sometimes, it can induce foetal complications in pregnant women (2) and also cervical lymphadenopathy and/or ocular disease in immuno-compromised patients infected with the human immunodeficiency virus (HIV) (3). This parasitosis can be contracted by ingestion of tissue cysts in undercooked contaminated meat, poorly washed raw vegetables or in contaminated drinking water (4). Cats and other members of the family Felidae are reported as the single definitive hosts because they excrete resistant oocysts in their faeces. Other mammals, including humans, serve as intermediate hosts in which the parasite can cause systemic infection that leads to the formation of tissue cysts (4).

Infection caused by *T. gondii* is considered a global zoonotic disease of public health importance. The distribution of this parasite depends on the countries and weather conditions where the oocysts survive in environment (4, 5). It is estimated that between 30% and 65% of all people in the world are infected with toxoplasma (6). Toxoplasmosis can be detected in these patients by the use of specific anti-*Toxoplasma* IgG and IgM antibodies. IgM antibodies increased from day 5 after an acute infection to reach a maximum after 1-2 months and then rapidly decreased to low or undetectable levels. In many cases, they persist for years after acute infection (7). IgG antibodies appeared later than IgM and are generally detectable within the first two weeks after infection. The peak levels of IgG occurred after a period ranging from 12 weeks to 6 months (7).

In Cameroon, several recent studies have reported high seroprevalence of toxoplasmosis in various hospitals. A prevalence of 69.9% (out of 133 men and women) was reported among HIV

patients at Yaoundé (8). The same study reported a rate of 70% among 110 pregnant women at Douala (9) within the same year. In the coastal region of Cameroon, a rate of 71.8% (out of 170 pregnant women) was reported in the health district of Limbe (10). Values close to the rates reported above were also recorded in 2016; 54.5% (out of 178 women of child-bearing age) at Njinikom, north-western region (11) and 78.6% (out of 327 pregnant women) in Douala (12). All these studies emphasize the need to sensitize pregnant women to the disease and its mode of transmission during their prenatal follow-up, in order to reduce the risk of first infection during their pregnancy.

In view of the results from these studies, it becomes necessary to investigate whether this high prevalence of toxoplasmosis might occur in other regions of Cameroon with the following two research questions; (i) what is the prevalence of this parasitosis in mountainous area located at 2000m altitude such as Western Highlands?, and what are the risk factors associated with this disease?

Materials and methods:

Study population

The study population consists of pregnant women attending the Protestant Hospital, Mbouo-Bandjoun (West Cameroon) for antenatal clinic and medical check-ups, enrolled by simple random sampling between June and September 2016. As about 20% of the women followed up in this hospital are HIV-positive (F. F. Djuikwo Teukeng, personal communication), patient enrolment was based on a sample size of 200 subjects in order to have a significant number of HIV-positive women ($n > 31$). No exclusion criteria were used in the choice of pregnant women recruited into the study.

The subjects were from Bafoussam ($n=103$), Bandjoun ($n=55$) and 11 other municipalities located in the West region ($n=19$). Nineteen others from Douala ($n=14$) or Yaoundé ($n=5$) have also consulted in this hospital. The detection of *T. gondii* antibodies was

carried out during the course of their first pregnancy for 40 of the subjects.

Study protocol

Each participant in this study completed a structured questionnaire. This questionnaire contained simple questions about known risk factors of *T. gondii* exposure in addition to clinical and socio-demographic information. Blood samples were collected from each participant into two pre-labelled tubes. The blood from the EDTA-containing tube was immediately tested for the presence of anti-HIV antibodies using test strips (Alere Determine™ HIV-1/2) according to the manufacturer's recommendations. The second blood sample was centrifuged at 3000 rpm for 15 minutes to obtain the serum. The serum was stored at -20°C and then tested for the presence of *T. gondii* antibodies using the indirect Enzyme Linked Immunosorbent Assay (ELISA) method and anti-*Toxoplasma* IgG and IgM antibody ELISA kits (Golden Bio Technologies Corporation, Upland, California, USA).

Ethical consideration

Ethical clearance was obtained from the Evangelical University of Bandjoun (2016/0017) and authorization to collect and analyse blood samples was also given by the Protestant Hospital administrative authorities. All participants were informed of the study goals, procedures and finality, and all signed the informed consent form. Informed consent was provided for subjects less than 21 years by their parents or guardians.

Sociodemographic and risk factors

Information about the main behavioural factors known for toxoplasmosis such as contact with cats and garden soil, consumption of undercooked and smoked meat sold along roads, raw unwashed vegetables and fruits, drinking unfiltered water, and hand washing practices (4), were collected into a structured questionnaire. Socio-demographic and clinical histories such as age, educational level, marital status, profession, place of residence, number of previous pregnancies,

gestational age of present pregnancy, and HIV status were also collected. The professions of the subjects were classified according to the criteria defined by the National Institute of Statistics in Cameroon (13).

Statistical analysis

The overall seroprevalence of toxoplasmosis took into account the different types of antibodies studied and was assessed using the ratio between the numbers of serologically positive anti-*T. gondii* samples and that of all samples. The same protocol was used to calculate the prevalence of infection for each antibody type (IgG, IgM or IgG + IgM). These prevalence rates were given with their 95% confidence intervals. The Pearson's Chi square test was used to establish levels of significance between HIV-positive and HIV-negative groups.

Univariate logistic regression was first used to test whether potential risk factors were associated with the prevalence of infection. Variables with a *p* value less than 0.25 in univariate analysis were integrated into a multivariate logistic regression model to

control the possible effect of confounding factors. The relative risk of infection was measured using odds ratios (OR) with 95% confidence intervals. All analyses were performed with R 3.3.0 software (14).

Results:

Seroprevalence of IgG & IgM antibodies

Of the 200 pregnant women involved in this study, 36 (18%) were HIV-infected while 164 (82%) were HIV-negative (Table 1). The table shows the distribution of the pregnant women in relation to HIV infection and the presence of anti-*Toxoplasma* antibodies. Overall seroprevalence rate of *T. gondii* infection was 45.5% (91/200). In the HIV-positive group, 29 of 36 (80.5%) were seropositive for toxoplasmosis; 17 (47.2%) for IgG, 7 (22.2%) for IgM and 4 (11.1%) for both antibody types. In the HIV-negative group, the seroprevalence rate was 36.5% (62 of 164) with 25.0% (42 of 164) for IgG, 9.1% (16 of 164) for IgM and 2.4% (4 of 164) for both antibody types.

Table 1: Seroprevalence of *Toxoplasma gondii* infections among pregnant women attending the Protestant Hospital, Mbouo-Bandjoun, Cameroon according to HIV status

Number of pregnant women	Population group: number of patients (prevalence in % (95% CI))		
	HIV-positive (n = 36)	HIV-negative (n = 164)	Total (n = 200)
Positive IgG	17 (47.22) (30.40-64.51)	42 (25.61) (19.10-33.00)	59 (29.50) (23.20-36.34)
Positive IgM	8 (22.22) (10.10-39.15)	16 (9.76) (5.67-15.36)	24 (12.00) (7.84-17.33)
Positive IgG + IgM	4 (11.11) (3.11-26.06)	4 (2.44) (0.67-6.13)	8 (4.00) (1.74-7.73)
Total of women with anti- <i>Toxoplasma</i> antibodies	29 (80.56) (63.90-91.81)	62 (37.80) (30.30-45.70)	91 (45.50) (38.40-52.67)

n = number of women per group; CI = confidence interval

Table 2 shows significant differences between the two groups of subjects. The number of women positive for IgG antibodies was significantly higher in the HIV-positive group than in HIV-negative patients ($p < 0.05$), and the same was observed for IgM and both IgG and IgM antibodies. In each group considered separately (Table 2), the number of IgG-positive women was significantly higher than those patients positive for IgM antibodies or both types of antibodies.

Fig 1 shows the distribution of the

91 seropositive women through their pregnancy period in relation to the type of antibodies. Women with positive IgG antibodies were more in the second and third trimesters (25 and 21 subjects respectively, compared to 15 in the first trimester). The distribution of the 23 women with positive IgM antibodies was roughly uniform during the three trimesters, and the same was noted for the 8 women with both types of antibodies.

Table 2: Differences between groups of subjects or antibody types (Chi² test)

Difference between	Parameter or group studied	χ^2 value	<i>p</i> value
HIV positive and HIV negative	IgG ⁺	6.63	< 0.05
	IgM ⁺	5.33	< 0.05
	IgG ⁺ , IgM ⁺	5.78	< 0.05
IgG ⁺ and IgM ⁺	HIV-positive	4.96	< 0.05
	HIV-negative	14.16	< 0.001
IgG ⁺ and IgG ⁺ , IgM ⁺	HIV-positive	11.36	< 0.001
	HIV-negative	36.51	< 0.001
IgM ⁺ and IgG ⁺ , IgM ⁺	HIV-positive	1.60	NS
	HIV-negative	7.66	< 0.01

NS = not significant

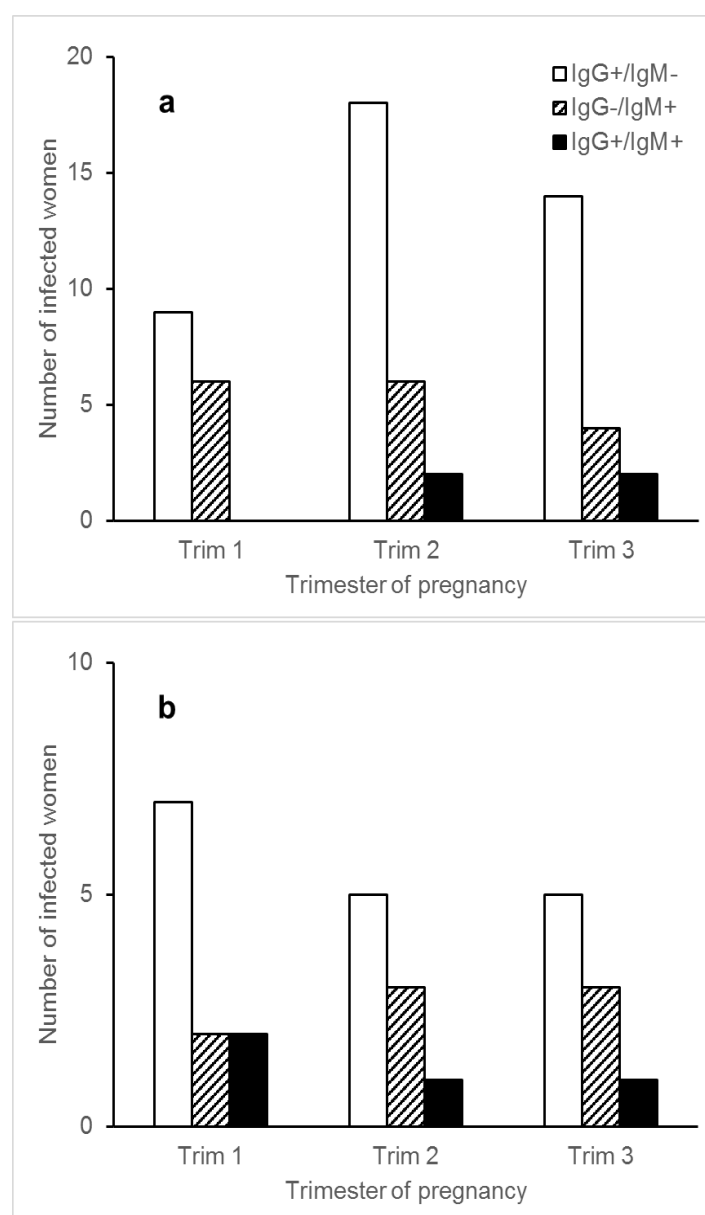


Fig 1: Distribution of 91 pregnant women infected with *Toxoplasma gondii* in relation to the antibody type: a = HIV-positive women; b = HIV-negative women

Factors associated with *Toxoplasma gondii* seropositivity

As shown in Tables 3 and 4, educational status at secondary level, HIV infection, and close contacts with cats were identified as possible risk factors associated with *T. gondii* infection. A multiple logistic regression analysis (Table 5) showed that these three variables were significantly associated with the prevalence of IgG and IgM antibodies in the subjects.

Compared to women without HIV infection, the HIV-infected women were 5.7 times more likely to develop *Toxoplasma* infection. Similarly, women with secondary level of education and those who had frequent close contacts with cats were, respectively 4.48 and 2.95 times more likely to be infected with this parasite when compared to women with a primary level of education and those without contacts with cats (Table 5).

Table 3: Univariate logistic regressions of the socio-demographic and clinical characteristics of pregnant women attending the Protestant Hospital, Mbouo-Bandjoun (West Cameroon)

Variable and group (total number of women per group)	Prevalence of toxoplasmosis in % (95% CI)	Odds ratio (95% CI)	p value
Age category			
20-25 years (52)	46.15 (32.20 - 60.53)		
26-30 years (82)	46.34 (35.20 - 57.70)	1.00 (0.50 - 2.03)	0.98
31-35 years (42)	45.24 (29.80 - 61.33)	0.96 (0.42 - 2.18)	0.92
≥ 36 years (24)	41.67 (22.10 - 63.36)	0.83 (0.30 - 2.20)	0.71
Level of education			
Primary (56)	55.36 (41.40 - 68.66)		
Secondary (68)	35.29 (24.00 - 47.83)	0.44 (0.21 - 0.90)	0.02 *
Higher (76)	47.37 (35.70 - 59.16)	0.72 (0.36 - 1.44)	0.36
Marital status			
Married (116)	47.41 (38.00 - 56.90)		
Single (84)	42.86 (32.10 - 54.13)	0.83 (0.47 - 1.46)	0.52
Occupation			
Artisans (43)	51.16 (35.40 - 66.70)		
Housewives (49)	46.94 (32.50 - 61.73)	0.84 (0.37 - 1.92)	0.69
Intermediate & higher professions (39)	41.03 (25.50 - 57.90)	0.66 (0.27 - 1.59)	0.36
Others (69)	43.48 (31.50 - 55.96)	0.73 (0.34 - 1.58)	0.43
Residence			
Bafoussam (106)	48.11 (38.30 - 58.03)		
Bandjoun (56)	48.21 (34.60 - 61.97)	1.00 (0.52 - 1.92)	0.99
Douala (14)	28.57 (8.38 - 58.10)	0.43 (0.11 - 1.38)	0.18
Other towns (24)	37.50 (18.70 - 59.41)	0.65 (0.25 - 1.58)	0.35
Number of previous pregnancies			
1 (41)	41.46 (26.30 - 57.89)		
2 (41)	56.10 (39.70 - 71.53)	1.80 (0.76 - 4.39)	0.19
3 (44)	54.55 (38.80 - 69.61)	1.69 (0.72 - 4.05)	0.23
4 (35)	31.43 (16.80 - 49.29)	0.65 (0.25 - 1.65)	0.37
≥ 5 (39)	41.03 (25.50 - 57.90)	0.98 (0.40 - 2.40)	0.97
Miscarriages			
No (135)	47.41 (38.70 - 56.18)		
Yes (65)	41.54 (29.40 - 54.44)	0.78 (0.43 - 1.42)	0.43
Gestational age (present pregnancy)			
Trimester 1 (55)	47.27 (33.60 - 61.20)		
Trimester 2 (83)	42.17 (31.40 - 53.51)	0.81 (0.41 - 1.62)	0.55
Trimester 3 (62)	48.39 (35.40 - 61.44)	1.05 (0.51 - 2.17)	0.90
Knowledge of toxoplasmosis			
No (130)	46.92 (38.10 - 55.87)		
Yes (70)	42.86 (31.00 - 55.25)	0.84 (0.47 - 1.52)	0.58
HIV infection			
No (164)	37.80 (30.80 - 45.70)		
Yes (36)	80.56 (63.90 - 91.81)	6.81 (2.96 - 17.76)	0.0001 ***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CI = confidence interval

Table 4: Univariate logistic regressions of behavioural risk factors of pregnant women attending the Protestant Hospital, Mbouo-Bandjoun (West Cameroon)

Variable and group (total number of women per group)	Prevalence in % (95% CI)	Odds ratio (95% CI)	p value
Presence of cats at home			
No (147)	40.82 (32.70 - 49.22)	2984,708 (0, NC)	0.98
Yes (53)	58.49 (44.10 - 71.86)		
Close contact with cats			
Never (96)	35.42 (25.90 - 45.84)	1.14 (0.52 - 2.45)	0.74
Rarely (39)	38.46 (23.30 - 55.38)		
Often (65)	64.62 (51.70 - 76.08)		
Consumption of undercooked meat			
Never (168)	44.64 (36.90 - 52.50)	1.24 (0.58 - 2.66)	0.58
Sometimes (32)	50.00 (31.00 - 68.11)		
Consumption of smoked meat sold along roads			
Never (76)	38.16 (27.20 - 50.02)	1.38 (0.67 - 2.86)	0.38
Rarely (50)	46.00 (31.80 - 60.68)		
Often (74)	52.70 (40.70 - 64.43)		
Consumption of raw and unwashed vegetables or fruit			
Never (157)	45.22 (37.20 - 53.35)	1.05 (0.53 - 2.07)	0.88
Sometimes (43)	46.51 (31.10 - 62.35)		
Consumption of water			
Forehole ¹ (18)	55.56 (30.70 - 78.47)	0.47 (0.14 - 1.57)	0.22
Source ¹ (27)	37.04 (19.40 - 57.63)		
Mineral water (15)	46.67 (21.20 - 73.41)		
Tap water (140)	45.71 (37.20 - 54.34)		
Hand washing			
Always (158)	43.67 (35.80 - 51.78)	1.42 (0.72 - 2.82)	0.31
Sometimes (42)	52.38 (36.40 - 68.00)		
Contact with garden soil			
No (9)	55.56 (21.20 - 86.30)	0.66 (0.16 - 2.55)	0.53
Yes (191)	45.03 (37.80 - 52.37)		

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ¹, Unfiltered water, CI = Confidence interval

Table 5: Multivariate logistic regression with selected variables of pregnant women attending the Protestant Hospital, Mbouo-Bandjoun (West Cameroon)

Variable and group (total number of women per group)	Odds ratio (95% CI)	p-value
Level of education		
Primary (56)	0.44 (0.20 - 0.97)	0.04 *
Secondary (68)		
Higher (76)		
Close contact with cats		
Never (96)	1.41 (0.62 - 3.19)	0.41
Rarely (39)		
Often (65)		
HIV infection		
No (164)	5.98 (2.50 - 16.07)	0.0001 ***
Yes (36)		

The prevalence rates and their confidence intervals (CI) are given in Tables 3 and 4

Discussion:

The present study shows significant difference between the overall seroprevalence of *Toxoplasma* infection among the HIV-positive group (77.7%)

and HIV-negative patients (36.5%). The high seroprevalence of 77.5% in the HIV-infected group in our study is in the range of 69.9% reported by Assob et al., (8) among 133 HIV-positive patients in Yaoundé and 77.7% reported by Wam et

al., (11) among 90 HIV-positive women in Njinikom, north-western Cameroon. However, these high *Toxoplasma* seroprevalence rates are not universal because the prevalence rate was only 27.8% among 72 HIV-positive women in Abuja, Nigeria (15).

The prevalence of 36.5% among HIV-negative women in our study however contrasts sharply the rates reported by other researchers in Cameroon. The prevalence rate of 70% (77 of 110 pregnant women) was reported in Douala (9), 71.8% (122 of 171) in the Limbe health district (10), 73.9% (54 of 73) in Njinikom (11) and 78.6% (257 of 327) by another study in Douala (12). Prevalence rates below 50% have also been reported for HIV-negative pregnant women in other African countries such as Nigeria with 44.5% (32 of 72) in Abuja (15), 29.9% (83 of 276) in Lagos (17), 48.9% (176 of 360) in Maiduguri (18), as well as 31.1% (98 of 316) in Bobo-Dioulasso (16).

Although *T. gondii* is an opportunistic pathogen that more readily develops when HIV infection is present (3, 19), the difference in the prevalence rates in our HIV-positive and HIV-negative women, and in other studies cannot be solely explained by immunosuppression induced by HIV infection. Other plausible explanation for this difference may be related to the variation which exists in geographical and climatic conditions between different countries. High seroprevalence rate is associated with warmer and wetter climates, conditions that are favourable for sporulation of *Toxoplasma* oocysts (20, 21).

In HIV-positive and HIV-negative groups, the women with positive IgG antibodies were significantly more than those with positive IgM antibodies, while those positive for both antibodies were fewer. Similar observations have been reported by other researchers in sub-Saharan Africa; Ndassi and Kamga (10) and Wam et al., (11) in Cameroon, and Nasir et al., in Nigeria (18). Compared with rates reported by these authors, the prevalence rates in the present study for all women, regardless of HIV infection, were lower; 29.5% for IgG, 11.1% for IgM, and 4% for both. Although this

difference can be interpreted by relating it to the heterogeneity in the geographical origin of the women in this study, another hypothesis based on a current decrease in seroprevalence of toxoplasmosis in the population living in the West region of Cameroon cannot be totally excluded.

The large proportion of infected women with positive IgG antibodies in the second and third trimesters of pregnancy is surprising. However, these results are consistent with the kinetics of these antibodies during toxoplasmosis. As IgM antibodies were negative in these patients, it indicates that the infection must have occurred more than six months before the date of antibody detection (22, 23). The presence of IgM without IgG in 24 pregnant women may actually mean a recent infection of the pregnant women because IgM is the first immunoglobulin to be produced against any new infection. However, the presence of both IgG and IgM in 4 HIV-positive and 4 HIV-negative women is more difficult to explain though it may indicate either an evolving infection or re-infection or re-activation of an old infection or changing from acute to chronic stage or antibody switching occurring at molecular level. A similar rate (10.8%) has already been noted by Assob et al., (8) among 93 HIV-positive women in Cameroon and these authors explained this result by the fact that most toxoplasma cases in these women would probably be due to the reactivation of a latent infection. In contrast, the occurrence of such rates in HIV-negative women has not yet been satisfactorily explained.

The review of literature shows the existence of variability in the risk factors associated with toxoplasmosis. The nature of these varies according to the country and the place where each study was conducted (6). In the present study, the frequent contact with cats was significantly associated with the prevalence of toxoplasmosis. This finding is consistent with the report by Nissapatorn et al., (24) in Malaysia but disagrees with reports of three others; Wam et al., in Cameroon (11), Uttah et al., in Nigeria (15) and Agmas et al., in Ethiopia (25), as these researchers did

not find any significant association between contact with cats and the disease.

On the other hand, rare contact or mere ownership of cats did not show any significant relationship with the prevalence of the disease in the our study, whereas several authors such as Njunda et al., (9), Nasir et al., (17) and Wam et al., (11) in Cameroon reported a significantly higher prevalence of toxoplasmosis in cat owners. This variability was also noted in European studies as Hofhuis et al., (26) in the Netherlands reported an increased risk of toxoplasmosis for cat owners, while Petersen et al., (27) did not observe such association. To explain the difference between our results and those of these authors, the frequency of close contact with this mammal, especially when raised as a pet, is probably the main cause. The other risk factors analyzed in this study did not show significant association with the prevalence of the disease, and this may be related to the women population in our study.

In conclusion, the seroprevalence of *T. gondii* infection among pregnant women population at the Protestant Hospital of Mbouo-Bandjoun, Cameroon was 45.0% with more infected women in the HIV-positive group than in HIV-negative women. The frequency of close contact with cats was significantly associated with the prevalence of the disease. Compared with previous reports of human toxoplasmosis in Cameroon, our study reported a decrease in the prevalence of the disease. Further studies are needed to determine whether this decrease is localized to our study or a general phenomenon that currently affect the West region or other regions in Cameroon.

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Competing interests:

No conflict of interest is declared

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Original Article

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Characterization of antibiotic-resistant *Staphylococcus aureus* from gills and gastro-intestinal tracts of catfish (*Clarias gariepinus*), and water samples from Jabi Lake, Abuja, Nigeria

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Abstract:

Background: The isolation of antibiotic resistant *Staphylococcus aureus* in freshwater fish poses a threat to public health because of the risk of human infections from consumption of such contaminated fish. Studies assessing antibiotic resistance of bacteria from body parts of fish and freshwater in Nigeria are sparse in the literature. This study therefore characterized *S. aureus* isolates from gills and gastrointestinal tract (GIT) of catfish (*Clarias gariepinus*), and water samples from Jabi Lake, Nigeria

Methodology: Over a period of three months (April to June 2018), gills and GIT samples of 30 fish, and water samples randomly collected from 6 sites of the Lake, were cultured on Mannitol Salt Agar (MSA) for the isolation of *S. aureus*. Standard biochemical tests were used for bacteria identification, and antibiogram of the isolates was determined by the disc diffusion method

Results: The bacterial colony count in the gills ($54.6 \pm 1.41 \times 10^5$ CFU/ml) and GIT ($54.3 \pm 1.31 \times 10^5$ CFU/ml) was significantly higher ($p < 0.05$) than the count from water sample ($27.7 \pm 2.85 \times 10^5$ CFU/mL). *S. aureus* was isolated from 53% (16 of 30) of the gills, 57% (17 of 30) of the GIT, and 33% (2 of 6) of the water samples ($p < 0.05$). Ninety four point one percent of *S. aureus* recovered from gills were resistant to ampicillin while 53.3% from the GIT were resistant to levofloxacin. *S. aureus* from water samples were resistant (100%) to ciprofloxacin, norfloxacin, gentamycin, amoxicillin, rifampicin, erythromycin, ampicillin and levofloxacin, and 50% were resistant to streptomycin and chloramphenicol

Conclusion: The presence of antibiotic resistant *S. aureus* in this study may be the result of selective antimicrobial pressure from anthropogenic activities as a result of abuse and overuse of antimicrobials leading to residual antibiotics in the aquatic environment

Keywords: *Clarias gariepinus*; gill, gastrointestinal tract; antibiotic; *Staphylococcus*

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Caractérisation de *Staphylococcus aureus* résistant aux antibiotiques à partir de branchies et du tractus gastro-intestinal de poisson-chat (*Clarias gariepinus*) et d'échantillons d'eau du lac Jabi, Abuja, Nigéria

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Abstrait:

Contexte: L'isolement de *Staphylococcus aureus* résistant aux antibiotiques chez des poissons d'eau douce constitue une menace pour la santé publique en raison du risque d'infections humaines résultant de la consommation de ce poisson contaminé. Les études évaluant la résistance aux antibiotiques de bactéries provenant de parties du corps de poissons et d'eau douce au Nigéria sont rares dans la littérature. Cette étude a donc caractérisé les isolats de *S. aureus* provenant des branchies et du tractus gastro-intestinal de poisson-chat (*Clarias gariepinus*), ainsi que des échantillons d'eau de Jabi Lake, au Nigeria. Méthodologie: sur une période de trois mois (avril à juin 2018), échantillons de branchies et de GIT. de 30 poissons et d'échantillons d'eau prélevés au hasard sur 6 sites du lac ont été cultivés sur gélose au sel de mannitol (MSA) afin d'isoler *S. aureus*. Des tests biochimiques standard ont été utilisés pour l'identification des bactéries et l'antibiogramme des isolats a été déterminé par la méthode de diffusion sur disque.

Résultats: Le nombre de colonies bactériennes dans les branchies ($54,6 \pm 1,41 \times 10^5$ UFC/ml) et le GIT ($54,3 \pm 1,31 \times 10^5$ UFC/ml) étaient significativement plus élevés ($p < 0,05$) que le nombre issu de l'échantillon d'eau ($27,7 \pm 2,85 \times 10^5$ UFC/mL). *S. aureus* a été isolé chez 53% (16 sur 30) des branchies, 57% (17 sur 30) du GIT et 33% (2 sur 6) des échantillons d'eau ($p < 0,05$). Quarante-deux pour cent des *S. aureus* récupérés des branchies étaient résistants à l'ampicilline, tandis que 53,3% des GIT étaient résistants à la lévofloxacine. *S. aureus* à partir d'échantillons d'eau était résistant (100%) à la ciprofloxacine, la norfloxacine, la gentmycine, l'amoxicilline, la rifampicine, l'érythromycine, l'ampicilline et la lévofloxacine, et 50% étaient résistants à la streptomycine et au chloramphénicol.

Conclusion: La présence de *S. aureus* résistant aux antibiotiques dans cette étude peut être le résultat d'une pression antimicrobienne sélective résultant d'activités anthropiques résultant d'un usage abusif et excessif d'antimicrobiens entraînant la présence d'antibiotiques résiduels dans le milieu aquatique.

Mots-clés: *Clarias gariepinus*; branchies, tractus gastro-intestinal; antibiotique; Staphylocoque

Introduction:

Lakes and dams are reservoirs containing water of great importance for fishing, agricultural, industrial and domestic usage. The reservoirs serve as regions of biodiversity and are also used as tourists, recreational and conservation locations (1). However, concerns of public interest have been raised because reservoirs are most often exposed to environmental pollutants from anthropogenic activities that may make the water unsuitable for aquatic flora and fauna.

Jabi Lake, which was formerly a small water body, is natively one of the largest freshwater bodies in the Jabi district of Abuja, Nigeria, which residents depended on for their domestic water needs. This lake was expanded for commercial and fishing purposes in 1993 (2). However as at present, the lake is surrounded by a big shopping mall and residential houses, and is subjected

to recreational activities. It is thus considered as a close basin for different types of drainages in the city of Abuja. The lake is also an important source of fishing activities in the district but subject to high levels of pollution from domestic drainage and raw domestic sewage.

The drainage of contaminated wastewater into the lake is a great health risk due to the possibility of large number of pathogens and antibiotic products being discharged into the water body daily. Recently, detection of *Staphylococcus aureus* from fish, *Oreochromis niloticus*, and wastewater samples from Qarun Lake, with a prevalence of 81.5%, suggests that this pathogen may be abundant in lakes (3). Many species of staphylococci are capable of releasing enterotoxin that can cause gastroenteritis in humans when foods contaminated by these species are consumed (4).

Water drainages containing antimicrobial agents, metals and biocides

are also diverse sources for antimicrobial resistance in water bodies (5). The presence of antibiotic resistant microorganisms in fish has been documented in several lakes around the world (6, 7). However, there are no documented studies on antibiotic resistance and detection of *S. aureus* in Jabi Lake. Hence, the objective of this study was to determine the prevalence of antibiotic resistant *S. aureus* in catfish (*Clarias gariepinus*) and water samples from Jabi Lake.

Materials and Methods:

Study location and condition

This study was conducted on Jabi Lake, Abuja, Nigeria. This lake is natural water located in Jabi district of Abuja with 9.0771°N and 7.4233°E coordinates. The lake experiences two weather conditions annually, a warm humid rainy season and a dry season (8). At the bank of the lake are a recreational park and a shopping mall, and fishing activities usually takes place inside the lake throughout the year.

Samples collection

Thirty cat fish (*C. gariepinus*) were randomly collected from Jabi Lake in order to harvest their gills and intestine, while water samples were randomly collected from 6 locations in the lake between April and June 2018. The gills of the fish were selected because of their perennial contact with water while the intestines were selected because of their ability to absorb food and the tendency to harbor microorganisms. The fishes were killed by destruction of the brain with a needle, and the microbial load on the skin was reduced by washing the fish with 70% ethanol (9). After dissection with sterile scissors, the gills and the intestine were separated for analysis.

Water samples from the lake were collected into sterile glass bottles (500 mL) at a depth of 20 cm below the water surface from randomly selected locations (middle, east and western) of the lake. The container was immediately labeled and kept in ice chest box, and conveyed

to the laboratory of the Department of Biological Sciences, Baze University, Abuja, Nigeria, within 6 hours for culture and isolation of *S. aureus*.

Isolation, identification and characterization of *S. aureus*

One milliliter of water sample (collected with a pipette) was serially (5 folds) diluted with 9 ml sterile distilled water. One milliliter of each serial dilution was added to 20 ml of sterile Mannitol Salt Agar (MSA) plate using pour plate method, and this was thoroughly mixed and allowed to set. The plates were incubated at 37 °C but briefly opened for 30 seconds to remove condensed water on the agar surface. The plates were then covered, inverted and incubated at 37°C for 48 hours. Suspected colonies of *S. aureus* based on their size, shape, color, pigmentation, consistency, and Gram reaction, were sub-cultured on nutrient agar plates to obtain pure cultures (10).

One gram of the gills and intestine were cut out, transferred and crushed with a sterile pestle in a sterile mortar containing 9 ml of sterile distilled water. A serial dilution (5 folds) of each homogenate was prepared prior to inoculation. A 1 mL aliquot of the serially diluted homogenate from the gills and intestine was incubated at 37°C for 24 hour on MSA plates by pipetting 0.1 ml of 10⁻⁴ dilution aseptically unto the surface of the agar using a sterile bent glass rod to spray the homogenate. After incubation, the colonies were counted using a colony counter (Brunswick Scientific C-110). Yellow colonies presumptively identified as *S. aureus* were then sub-cultured onto nutrient agar slants, following which they were Gram stained and tested for catalase, coagulase and deoxyribonuclease (11).

Antibiotic susceptibility testing

Antibiotic sensitivity test was performed on each *S. aureus* isolate by the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) on Mueller-Hinton (MH) agar (12) using the following antibiotic

discs; ciprofloxacin (10 µg), norfloxacin (10 µg), gentamycin (10 µg), amoxicillin (20 µg), streptomycin (30 µg), rifampicin (20 µg), erythromycin (30 µg), chloramphenicol (30 µg), ampicillin (20 µg) and levofloxacin (20 µg). The MH plates were incubated at 37°C for 24 hours, and the zone of inhibition around the disc was interpreted according to CLSI guideline (12).

Statistical analysis

One way analysis of variance (ANOVA) and Chi square test were performed using Origin 8.1 Lab pro software for windows.

Results:

Colony count

Fig 1 shows the bacterial colony count in the gills and GIT of fish, and water samples. The mean count was highest in the gills with 54.64×10^5 CFU/ml followed by the GIT with 54.38×10^5 CFU/ml. The water sample had the lowest colony count of 27.78×10^5 CFU/ml ($p < 0.05$)

Isolation of *S. aureus*

Fig 2 shows percentage of *S. aureus* isolated from the gills and GIT of fish, and water samples. *S. aureus* was isolated from 53% (16 of 30) of the gills, 57% (17 of 30) of the GIT, and 33% (2 of 6) of the water samples ($p < 0.05$).

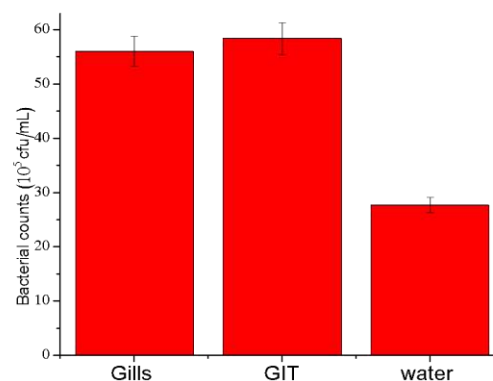


Fig.1: Bacterial count in the gills and gastrointestinal tracts of fish, and water samples ($F=120.56$, $p=0.0013$)

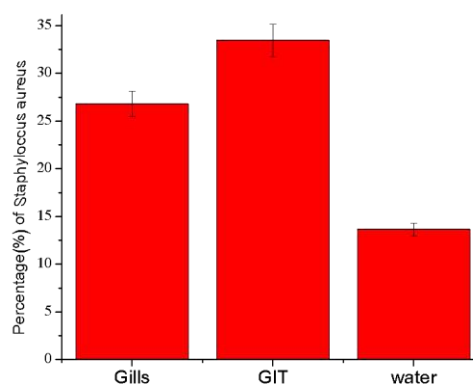


Fig. 2: Percentage isolates of *S. aureus* in the gills and GIT of fish, and water samples ($X^2 = 13.26$, $p = 0.0013$)

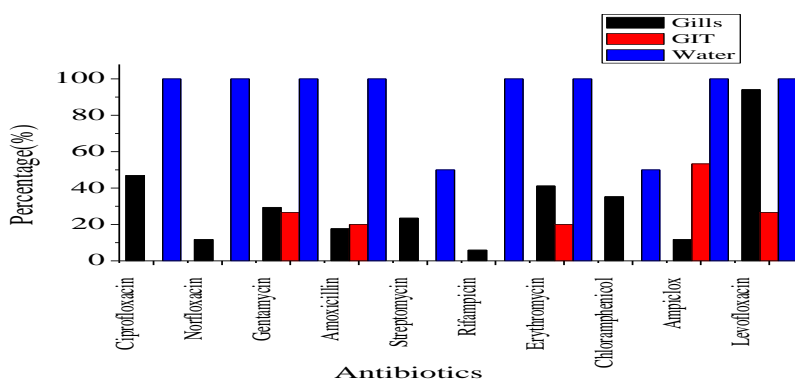


Fig 3: Antimicrobial susceptibility of *Staphylococcus aureus* from gills and gastrointestinal tracts of fish, and water samples

Antimicrobial susceptibility of *S. aureus*

Ninety four point one percent (94.1%) of *S. aureus* recovered from gills were resistant to ampicillin while 53.3% of those recovered from the GIT were resistant to levofloxacin. *S. aureus* recovered from water samples were resistant (100%) to ciprofloxacin, norfloxacin, gentamycin, amoxicillin, rifampicin, erythromycin, ampicillin and levofloxacin, and 50% were resistant to streptomycin and chloramphenicol (Fig 3)

Discussion:

The findings of this study indicated that the gills and GIT of the fish, and the water samples from Jabi Lake were contaminated with *S. aureus*. This may be due to the introduction of biological pollutants containing pathogenic microorganisms such as *S. aureus*, through human activities around the lake. The presence of *S. aureus* in the lake in both the fish and water, make this a reservoir for this pathogenic bacterium. This agrees with the findings of Gono et al., in Zimbabwe who isolated *S. aureus* from edible freshwater fish, *Tilapia rendalii* and *Oreochromis niloticus* (13).

Our study also showed that the counts of *S. aureus* in the gills and GIT of the fish were higher than from the water itself. This may sometimes be due to the large accumulation of bacteria in the gut of fish from the raw open sewage that is discharged directly into the lake. The potential danger of human infection by these pathogenic microorganisms from consumption of improperly cooked contaminated fish remains very considerable. The highest colony (54.68×10^5 CFU/ml) of bacteria in this study was found in the gills, a count that is close to 10^6 bacteria count per gram, a level reported to be unsuitable for human consumption (14).

Antibiotics are important for treatment of infectious diseases of man however, microorganisms are capable of developing resistance especially following misuse of antimicrobials, making it

possible for microorganisms that have not been exposed to antibiotic acquire resistance. In this study, a high prevalence of *S. aureus* resistant to ampicillin, ciprofloxacin and erythromycin was observed in the gills of fish, compared to the GIT, which may due to genetic mutations as reported by Toroglu et al. (15). The recovery of *S. aureus* resistant to multiple antibiotics in this study may be attributed to the effect of polluted run-off water generated by anthropogenic activities, and discharged into the lake, with high tendency for transfer of antimicrobial resistant determinants to the aquatic environment and the fish in the lake (15).

Conclusion:

The results from this study revealed that *C. gariepinus* from Jabi Lake harbored high number of *S. aureus* resistant to multiple antibiotics in the gills and GIT of fish, as well as high prevalence in the lake water samples. Therefore water from this lake must be tested to determine bacterial load and antibiotic resistance in contaminating microorganisms. In order to reduce lake pollution, wastewater discharged into the lake must be treated before discharge. For safe consumption, fish from this lake must be properly cooked to prevent development of infections from *S. aureus* or other pathogenic microorganisms that might have contaminated the lake.

Conflict of interest:

Authors declare no conflict of interest

Acknowledgements:

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Original Article

Open Access

Seroprevalence of microbial organisms during routine infertility evaluation at University of Benin Teaching Hospital, Benin-City, Nigeria

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Abstract:

Background: The association of genital microorganism with infertility has been documented but no consensus exists. Understanding their prevalence amongst infertile clients may assist in facilitating better screening protocols. The objective of this study is to determine the prevalence of microorganisms routinely screened among women undergoing infertility evaluation at the University of Benin Teaching Hospital.

Methods: A three year (January 2015 to December 2017) retrospective survey of all patients evaluated for infertility at the assisted reproduction unit of the hospital was undertaken. *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, cytomegalovirus (CMV), hepatitis B (HBV), hepatitis C (HCV) virus and the human immunodeficiency virus (HIV) were microorganisms serologically assayed at the unit. We analyzed data containing patients' demography and results of serological assay of these microorganisms.

Results: There were 576 patients (288 couples) who completed their microbiological evaluation during the study period. The mean age (years) of female partners was 38.2 ± 5.7 , while the mean age of the male partners was 42.7 ± 6.1 . The frequency of CMV positive assay for infertile couples was 129 (22.4%); *C. trachomatis* 125 (21.7%); *M. hominis* 92 (15.9%) and *U. urealyticum* 76 (13.2%). Overall, more women (50.7%) were seropositive compared to men (26%). HIV was positive in 10 patients (1.73%) with 60% being women. HBV was seropositive in 8 (1.4%) (women 62.5% and men 37.5%) while HCV was positive in only 2 (0.3%) patient. Majority (over 80%) of couples were sero-discordant with 20% (2) concordance rate for HIV and 12.5% (1) for hepatitis B.

Conclusion: Despite a relatively high seroprevalence rate of the studied microorganisms, the documented uncertainty on their association with infertility or its treatment limits justification for incorporation of routine screening of microbiological organisms into standard protocols for evaluation of infertile couples. A robust study on the impact of genital microorganism on specific infertility variables with comparison to fertile controls is recommended.

Keywords: microorganism, viruses, infertility, assisted reproduction, serological assay, screening

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Séroprévalence d'organismes microbiens au cours de l'évaluation de routine de l'infertilité à l'hôpital universitaire de Benin, Benin-City, Nigéria

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Abstrait:

Contexte: L'association d'un microorganisme génital à l'infertilité a été documentée mais il n'y a pas de consensus. Comprendre leur prévalence chez les clients infertiles peut aider à faciliter de meilleurs protocoles de dépistage. L'objectif de cette étude est de déterminer la prévalence des micro-organismes régulièrement dépistés chez les femmes subissant une évaluation de la stérilité à l'hôpital universitaire de Bénin.

Méthodes: Une enquête rétrospective de trois ans (de janvier 2015 à décembre 2017) sur tous les patients évalués pour l'infertilité dans l'unité de procréation assistée de l'hôpital a été entreprise. *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, cytomégalovirus (CMV), l'hépatite B (VHB), le virus de l'hépatite C (VHC) et le virus de l'immunodéficience humaine (VIH) étaient des microorganismes testés sérologiquement à l'unité. Nous avons analysé les données contenant la démographie des patients et les résultats du dosage sérologique de ces micro-organismes.

Résultats: 576 patients (288 couples) ont terminé leur évaluation microbiologique au cours de la période d'étude. L'âge moyen (en années) des partenaires féminins était de $38,2 \pm 5,7$ ans, tandis que l'âge moyen des partenaires masculins était de $42,7 \pm 6,1$. La fréquence du test CMV-positif pour les couples infertiles était de 129 (22,4%); *C. trachomatis* 125 (21,7%); *M. hominis* 92 (15,9%) et *U. urealyticum* 76 (13,2%). Dans l'ensemble, plus de femmes (50,7%) étaient séropositives que d'hommes (26%). Le VIH était positif chez 10 patients (1,73%), dont 60% de femmes. Le VHB était séropositif chez 8 (1,4%) (les femmes 62,5% et les hommes 37,5%), tandis que le VHC était positif chez seulement 2 patients (0,3%). La majorité (plus de 80%) des couples étaient sérodiscordants avec un taux de concordance de 20% pour le VIH et de 12,5% (1) pour l'hépatite B.

Conclusion: Malgré un taux de séroprévalence relativement élevé des microorganismes étudiés, l'incertitude documentée de leur association à l'infertilité ou à son traitement limite la justification de l'incorporation du dépistage systématique des organismes microbiologiques dans les protocoles standard d'évaluation des couples infertiles. Une étude robuste sur l'impact des microorganismes génitaux sur des variables spécifiques d'infertilité comparées aux témoins fertiles est

Mots-clés: microorganisme, virus, infertilité, procréation assistée, test sérologique, dépistage

Introduction:

Genital microbial infections can influence the fertility potential of a couple. Common pathogenic species implicated in both genital infections and infertility are *Chlamydia trachomatis*, Genital ureaplasmas (*Ureaplasma urealyticum* and *Ureaplasma parvum*) and mycoplasmas (*Mycoplasma genitalium* and *Mycoplasma hominis*) as well as viruses such as cytomegalovirus, human immunodeficiency virus and hepatitis viruses. The causal role of *C. trachomatis* in infertility is reasonably defined, as it may cause

pelvic inflammatory disease with resultant tubal factor infertility and ectopic pregnancy (1, 2).

Chronic viral infections have been found to be a risk during infertility treatment (3, 4). In a previous work, hepatitis B was reported as the most prevalent of the viral infection detected amongst infertile couple (5). Another research showed that hepatitis C virus (HCV) seropositive women had decreased response during ovarian stimulation at assisted reproduction treatment cycles (4). Mycoplasma and Ureaplasma species

have been associated with increased risk of genitourinary tract infections and recurrent pregnancy losses (6, 7). Their role in the aetiology of infertility is still speculative but they may impair fertilization or implantation. *Ureaplasma urealyticum* and *M. hominis* have been demonstrated to affect semen quality of infertile men, with recovery of the organisms in 7–14% of semen samples of infertile men (8, 9).

Human Cytomegalovirus (CMV) also known as human herpesvirus-5 (HHV-5) is the most studied of all CMVs. Human CMV infection is typically non pathogenic but can be life-threatening for the immuno-compromised patients such as organ transplants recipients, newborn, infants or HIV-infected persons. Some studies have shown that CMV may play a significant role in male infertility, and its early detection will permit successful antiviral therapy, to increase the fertility potential of the individual (10, 11).

It is with this foregoing that, some guidelines have recommended that women should be screened for *C. trachomatis* or given appropriate antibiotic prophylaxis, before any uterine instrumentation takes place with regard to infertility patients receiving *in vitro* fertilization (IVF) treatment (12, 13). For this reason many centres with assisted reproduction units have evolved guidelines for evaluation of the infertile couple to include detailed history, physical examination and laboratory analyses. However the genital microbial infection screening protocol still varies for different institutions. Hence the prevalence of these infections in the couples undergoing infertility evaluation is still uncertain.

The University of Benin Teaching Hospital (UBTH) is a public tertiary healthcare centre offering assisted reproduction services in a population characterized by deepening economic and resource constraints, and poor health seeking behavior. The benefit of screening for the aforementioned micro-organisms while evaluating infertile couple in the overall interest of their reproductive health is underscored in the goal of

improving success of infertility treatment as well as opportunistic detection of these infections in apparently healthy individuals.

In sub-Saharan Africa, reports of prevalence and significance of these key micro-organisms that may influence overall reproductive health of the infertile couple are limited in the literature. This study aims to determine the prevalence of *C. trachomatis*, *U. urealyticum*, *M. hominis*, cytomegalovirus (CMV), hepatitis B (HBV) and C (HCV) virus and the human immunodeficiency virus (HIV) among women undergoing infertility evaluation.

Methods:

Study design and setting:

A retrospective survey of all patients evaluated for infertility at Human Reproduction and Research Programme (HRRP) unit of University of Benin Teaching Hospital (UBTH) was undertaken between January 2015 and December 2017. The HRRP is a dedicated infertility treatment unit of the hospital with services for *In vitro* Fertilization (IVF) and other assisted reproduction techniques (ART). Evaluation of infertility at the unit is in line with global standard guideline on the investigation of infertile couples.(12) In addition routine screening for micro-organism is done specifically for *C. trachomatis*, *U. urealyticum*, *M. hominis*, cytomegalovirus (CMV), hepatitis B (HBV) and C (HCV), and human immunodeficiency virus (HIV) infections.

Serological methods:

Rapid test strip was the principal method for microorganism detection in this study. Colloidal Gold Immuno-filtration (GIFA) Assay was used to qualitatively detect *U. urealyticum*, *M. genitalium* and *C. trachomatis* antibody in human serum. Qualitative detection of viral antibodies in serum or plasma was with Rapid test chromatographic immuno-assay. Those with positive serological results were counseled and managed as appropriate. Approval for the study was obtained from the institutions Ethics and

Research Committee.

Case files of patients who presented for initial infertility evaluation during the selected study period were retrieved. All clients with completed laboratory work-up results were included for analysis. The data extracted were analyzed using InStat Graph Pad and presented in the form of frequency tables and descriptive statistics. Chi square test was used to test for statistical difference where appropriate and *p* value of less than 0.05 was considered significant.

Results:

Overall, 576 patients (288 couples) completed their microbiological evaluation during the study period and these were extracted for analysis. The age (years) of female partners ranged from 23 to 56 and the mean was 38.2 ± 5.7 , while the mean age of the male partners was 42.7 ± 6.1 and a range of 31 to 63. On the whole seropositive detection rate was 76.7% (442/576) with 292 (50.7%) being seropositive female and 150 (26.0%) for male. Overall sero-concordance rate was 10.8% (62/576). It is pertinent to note that some patients were seropositive for more than one microbial organism.

Cytomegalovirus was the commonest organism detected amongst infertile couples (Table 1) with frequency of seropositivity for immunoglobulin G of

129 (22.4%); female 88 (68.2%) and male 41 (31.8%). One hundred and twenty-five (21.7%) were seropositive for *C. trachomatis*; 81 (64.8%) female and 44 (35.2%) male. Ninety-two (15.9%) were seropositive for *M. hominis*; female 64.1% and male 35.9%, and 76 (13.2%) were seropositive for *U. urealyticum* (female 68.4% and male 31.6%).

Also shown in Table 1 is the frequency of couples with concordant positive test; 5.3%, 5.4%, 19.4% and 20.0% for ureaplasma, mycoplasma, cytomegalovirus, and chlamydia respectively. Other organisms screened for routinely are shown; HIV was positive in 10 patients (1.73%) with 6 (60%) female and 4 (40%) male. Hepatitis B was seropositive in 8 (1.4%) with females 62.5% (5) and male 37.5% (3); hepatitis C was positive in only 2 (0.3%) patients. Majority of couples were sero-discordant with 20% (2) concordance rate for HIV and 12.5% (1) for hepatitis B.

Comparison of microorganism seroprevalence rate between female and male patients (Table 2) showed that for Chlamydia, Ureaplasma, Mycoplasma and Cytomegalovirus, significantly more females were infected compared to males ($p < 0.05$), however there was no statistical difference between female and male seroprevalence for HIV, HBV and HCV ($p > 0.05$).

Table 1: Seroprevalence of microorganisms in subjects evaluated for infertility at UBTH Benin between January 2015 and December 2017

Microorganism	Subjects (%) (n=576)	Women (%) (n=288)	Men (%) (n=288)	Sero-concordance No of couples
<i>Ureaplasma urealyticum</i>	76 (13.2)	64 (69.6)	28 (30.4)	4 (5.3)
<i>Mycoplasma hominis</i>	92 (15.9)	56 (73.7)	20 (26.3)	5 (5.4)
<i>Chlamydia trachomatis</i>	125 (21.7)	88 (66.7)	44 (33.3)	25 (20.0)
Cytomegalovirus	129 (22.4)	116 (69.1)	52 (30.9)	25 (19.4)
Human immunodeficiency virus	10 (1.7)	6 (2.1)	4 (1.4)	2 (20)
Hepatitis B Virus	8 (1.4)	5 (1.7)	3 (1.0)	1 (12.5)
Hepatitis C Virus	2 (0.3)	1 (0.3)	1 (0.3)	0
Total	442 (76.7)	*292 (50.7)	150 (26.0)	62 (10.8)

n = number of subjects screened, * = some subjects were seropositive for more than one organism

Table 2: Comparison of microorganism seroprevalence rate between female and male patients

Microorganism	Female n=288	Male n=288	p value	Relative risk (confidence interval)
<i>Ureaplasma urealyticum</i>	52 (18.1)	24 (8.3)	0.001*	1.45 (1.21-1.73)
<i>Mycoplasma hominis</i>	59 (20.5)	33(11.5)	0.004*	1.36 (1.13-1.62)
<i>Chlamydia trachomatis</i>	81 (28.1)	44 (15.3)	0.001*	1.41 (1.19-1.66)
Cytomegalovirus	88 (30.6)	41 (14.2)	0.0001*	1.52 (1.30-1.78)
Human immunodeficiency virus	6 (2.1)	4 (1.4)	0.749	1.2 (0.72-2.01)
Hepatitis B Virus	5 (1.7)	3 (1.0)	0.721	1.25 (0.72-2.16)
Hepatitis C Virus	1 (0.3)	1 (0.3)	1	1 (0.25-4.12)

n = number of subjects screened, * = significant difference $p < 0.05$

Discussion:

The association of genital microbial infection and infertility is still not explicit. Hence there is no clear consensus guideline with regard to screening for these microorganisms during infertility evaluation. In this study, the high seroprevalence rate of microorganisms of over 75% apparently supports the call for routine screening for genital microbial infection at the infertility clinic. However, this finding cannot be generalized or equated to individual patients as some were seropositive for multiple microorganisms. With regards to specific genital microorganism in this study, the most frequent organisms (cytomegalovirus and chlamydia) had seroprevalence rate of about 22%. This finding is similar to those of Rodriguez and co workers (14) who reported a seroprevalence rate of 23.5% for the most common microorganism detected (ureaplasma) although the organism was different from those from this study.

Our findings are also comparable to reported seroprevalence rate of 19.2% and 15.2% respectively for genital mycoplasma and ureaplasma among infertile male patients (15). Similar studies in southern Nigeria have reported prevalent rates among patients with infertility (16, 17) but while the rate in our study is higher than some previously reported in this environment, it is lower

than others (8, 18, 19). Although, the exact role of genital microbial infection in infertility is not clearly defined, previous researchers have documented microbial infections of the genital tract or semen as causes of male infertility (16, 20). Another study that investigated the association between genital ureaplasmas and mycoplasmas and risk of infertility showed a significantly higher positive rate of *U. urealyticum* and *M. hominis* in the infertile compared to control group but there was no significant causal association with male infertility (21).

The seroprevalence was similar to that observed in this study although we did not compare with fertile controls. Evidently *C. trachomatis* infection in women has been implicated in tubal factor infertility and tubal ectopic pregnancy but its sequelae in men, is still unclear (2). Similarly, the role of cytomegalovirus in infertility is also doubtful. Some researchers reported that while seroprevalence rate and shedding of cytomegalovirus in the genital tracts of infertile patients are relatively high, this seem to have no significant role on infertility (11). Also there was no difference in sperm count and motility between those with and without cytomegalovirus infections (11, 24). Notwithstanding, the high seroprevalence rate reported is consistent with the finding of cytomegalovirus being the commonest detected microorganism in our study.

The seroprevalence of other viruses (HIV, HBV and HCV) studied were low and comparable to previous research on viral infections amongst infertile couples (25). HCV seroprevalence in our study is lower compared to a previous study in our centre which investigated HCV prevalence amongst antenatal clinic attendees (26). A higher HCV rate of 3.2% amongst infertile couples has been reported in Brazil (27). The implications of these transmissible viral infections (HIV, HBV and HCV) on infertility management at assisted reproduction are quite established and unambiguous. These include issues of cross-contamination in the laboratory and vertical transmission with possible deleterious consequences. Also, HCV infection has been reported to negatively affect ovarian response during stimulation (3, 13, 27).

From the foregoing screening for genital microbial infections at infertility evaluation is important. Our finding of a relatively high seroprevalence and the negative influence of these microorganisms on an individuals' fertility potential supports the consideration for incorporating genital microorganism screening into routine infertility evaluation protocol. This may facilitate better screening protocols and improve infertility treatment outcome. In addition it will afford the opportunity for patients to be referred to appropriate caregivers for other treatment and follow-up. Although guidelines for routine screening for transmissible viruses such as HIV, HBV and HCV are well established, there is still no consensus recommendation for routine screening for other genital microorganisms during infertility evaluation. This is largely due to the uncertainty regarding the role and association of these microorganisms and infertility.

In conclusion the observed seroprevalence of microbial organisms amongst infertile couples in this study is in line with global average. However while the utility of routine screening of a population seeking infertility treatment is not in doubt, there is no consensus on relevance and effect of these genital

microorganisms on infertility or its treatment. Furthermore since this study focused on prevalence and not the effect of the organisms, justification and generalization on screening protocols for the aforementioned organisms during infertility evaluation is limited.

Conflict of interest:

No conflict of interest is declared

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ORIGINAL ARTICLE

OPEN ACCESS

Co-infection of Parvovirus B19 and *Plasmodium falciparum* among Sick Cell Disease Patients in Benin City, Nigeria

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Abstract:

Background: Infections by parasites, bacteria, viruses such as human parvovirus B19 amongst others, have been widely reported as contributing to high prevalence of anaemia in many populations. This study was conducted to determine the co-infection of *Plasmodium falciparum* and human parvovirus B19 among sickle cell disease (SCD) patients in Benin City, Edo State, Nigeria.

Methodology: A total of 400 participants consisting 300 SCD patients (134 males, 166 females) and 100 (38 males, 62 females) apparently healthy subjects with haemoglobin AA (which served as control) who were contacted in homes, schools and offices, were enrolled for the study. The age of the participants ranged from 1 to 54 years. Venous blood was collected for detection of *P. falciparum* using Giemsa stain while parvovirus B19 was detected with enzyme linked immunosorbent assay (ELISA). Full blood count was estimated using Sysmex KX-21N haematology auto-analyzer.

Results: An overall prevalence of parvovirus B19 and *P. falciparum* co-infection observed among SCD patients in this study was 3.0% while single infection was 14.0% for *P. falciparum* and 26.7% for parvovirus B19. Religion was associated with 0 to 22 fold increased risk of acquiring co-infection of *P. falciparum* and parvovirus B19. Gender was significantly associated with *P. falciparum* infection ($p=0.0291$) while tribal extraction, platelet index and seasonal variation were significantly associated with single parvovirus B19 or co-infection of *P. falciparum* and parvovirus B19 ($p<0.05$).

Conclusion: The provision of strict regulatory policy concerning the screening of whole blood or pooled plasma before the use of blood products and transfusion of SCD patients is advocated.

Keywords: parvovirus B19, Benin City, *P. falciparum*, sickle cell disease

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Co-infection par le parvovirus B19 et *Plasmodium falciparum* chez des patients atteints de drépanocytose à Benin City, au Nigéria

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Abstrait:

Contexte: Il a été largement rapporté que les infections par des parasites, des bactéries, des virus tels que le parvovirus humain B19, contribuent à la prévalence élevée de l'anémie dans de nombreuses populations. Cette étude visait à déterminer la co-infection de *Plasmodium falciparum* et du parvovirus humain B19 chez des patients atteints de drépanocytose à Benin City, dans l'État d'Edo, au Nigéria.

Méthodologie: Un total de 400 participants comprenant 300 patients atteints de MCA (134 hommes, 166 femmes) et 100 (38 hommes et 62 femmes) des sujets apparemment en bonne santé avec l'hémoglobine AA (qui servait de contrôle) qui ont été contactés à la maison, dans les écoles et au bureau inscrit à l'étude. L'âge des participants allait de 1 à 54 ans. Le sang veineux a été recueilli pour la détection de *P. falciparum* à l'aide de la coloration de Giemsa, tandis que le parvovirus B19 a été détecté par un test d'immunosorbant lié à une enzyme (ELISA). La numération globulaire totale a été estimée à l'aide de l'auto-analyseur d'hématologie Sysmex KX-21N.

Résultats: La prévalence globale de la co-infection au parvovirus B19 et à *P. falciparum* observée chez les patients atteints de MCS dans cette étude était de 3,0%, tandis que l'infection simple était de 14,0% pour *P. falciparum* et de 26,7% pour le parvovirus B19. La religion était associée à un risque accru de contracter la co-infection à *P. falciparum* et au parvovirus B19 de 0 à 22 fois plus élevé. Le sexe était significativement associé à l'infection à *P. falciparum* ($p = 0,0291$), tandis que l'extraction tribale, l'indice plaquettaire et la variation saisonnière étaient significativement associés à un parvovirus simple B19 ou à une co-infection à *P. falciparum* et au parvovirus B19 ($p < 0,05$).

Conclusion: La mise en place d'une politique réglementaire stricte concernant le dépistage du sang total ou du plasma réuni avant l'utilisation du produit sanguin et la transfusion de patients atteints de MCS est recommandée.

Mots-clés: parvovirus B19, Benin City, *Plasmodium falciparum*, drépanocytose

Introduction:

Sickle cell disease (SCD) is known to consist of several disorders characterized by the presence of sickle haemoglobin (1). An estimated 300,000 children are born annually with SCD worldwide. This constitutes about 1% of the global population of SCD with over 75% in sub-Saharan Africa (2, 3). The high birth rate of SCD has highlighted the burden of SCD as a public health priority (3). However, there is a dearth of information on the burden of SCD to healthcare system and the significance on individual health (4).

Infection with Parvovirus B19 is common and can lead to a variety of clinical manifestations based on the immunological and haematological status of patients (5). Parvovirus B19 belongs to the family *Parvoviridae* which is subdivided into *Parvovirinae* and *Densovirinae* depending on the type of the infected host (6). Parvovirus B19 has specific tropism

for erythroid progenitor cells and is capable of causing temporary infection of the bone marrow resulting in transient arrest of erythropoiesis (7). In patients with underlying haemolysis or haematological disorders such as sickle cell disease, acute B19 infection may cause transient aplastic anaemia, erythema infectiosum, hydrops fetalis, abrupt and severe anaemia due to failure of red blood cell production (8, 9, 10). This virus is transmitted mainly via respiratory droplets but can be spread by contaminated blood, organ transplantation and transmission from mother to foetus (11).

Malaria is one of the major causes of morbidity and mortality in tropical and sub-tropical countries and is caused by the protozoan parasites of the genus *Plasmodium* with *P. falciparum* being the most virulent species (12). Malaria causes over 200 million cases of febrile illness out of which over a million children living in sub-Saharan Africa die annually (13, 14).

It is widely seen as a major health challenge in Africans with SCD (15).

Parvovirus B19 infection can cause significant drop in haemoglobin concentration and reticulocyte count, conditions that could have serious consequences in patients particularly children with underlying malaria or those in malaria endemic regions (16). There are a number of studies that have emphasized the importance of co-infection with Parvovirus B19 in the etiology and pathogenesis of malaria in adults and children in non-sickle cell disease subjects (17-22). It is recognized that interactions between SCD and other infectious agents influence the health status of SCD patients. Parasites, bacteria, human parvovirus B19, and other infectious agents have been widely reported as important factors contributing to the high prevalence of anaemia in many populations (23, 24, 25, 26).

There is however a dearth of information on the co-infection of *P. falciparum* and Parvovirus B19 among SCD patients in our environment. Against this background, this study was conducted to determine the co-infection of these pathogens among SCD patients in Benin City, Edo State, Nigeria.

Materials and methods:

Study population

The study was conducted between September 2017 and July 2018 at the Sickle Cell Center, Benin City, Edo State. The Sickle Cell Center has a referral status for the management of SCD patients Edo, Delta, and other neighbouring states. A total of 400 participants consisting of 300 SCD patients (134 males and 166 females) and 100 (38 males and 62 females) apparently healthy subjects with haemoglobin AA that were contacted in homes, schools and offices (served as control), were enrolled for the study. The age of the participants ranged from 1 and 54 years.

A well-structured questionnaire was administered to collect bio-data and other demographic information from the participants. Informed consent was

obtained from all subjects or the parents or guardians in the case of children prior to specimen collection. The protocol for this study was approved by the Ethics and Research Committee of the Ministry of Health, Edo State, Nigeria.

Specimen collection and processing

Venous blood sample of about 8 ml was collected from each participant, out of which 4.5 ml was dispensed into ethylene diamine tetraacetic acid (EDTA) bottle and thoroughly mixed. The remaining 3.5 ml sample was dispensed into plain container, allowed to clot, and serum separated for Parvovirus B19 analysis. *Plasmodium falciparum* was detected using a previously described method (27). Briefly, both thick and thin blood films were made from each blood specimen and allowed to air-dry. The blood films were stained in 3% Giemsa stain for 30 min, rinsed in tap water and allowed to air dry. The thick film was examined microscopically for presence of malaria parasite while the thin film was used to detect the species of *Plasmodium* using the oil immersion lens. A total of 200 fields per film were examined.

Full blood count was analyzed using a Sysmex KX-21N haematology auto-analyzer (Sysmex Corporation, Japan). Whole blood specimen dispensed into EDTA container was used. Anaemia was defined using the WHO criteria as haemoglobin concentration <13 g/dl for males and <12 g/dl for females (28). Parvovirus B19 was detected using enzyme-linked immunosorbent assay (ELISA) technique (Serion classic Parvovirus B19 IgG/IgM Wuzburg, Germany). Briefly, each sample was assayed according to the manufacturer's instruction using peroxidase-labeled rabbit anti-human IgM as the secondary antibody, tetramethyl benzidine as a substrate, and 1M H₂SO₄ as a stop solution. The absorbance was read at 450 nm using a spectrophotometer. Index value between 10 and 15 was taken as reference value, with samples below the index range taken as negative while value

above this range was taken as positive for IgM.

Statistical analysis

The data generated were analyzed using Chi square (χ^2) test for frequency data whereas the odd ratio was calculated for each potential risk factor. The statistical software used was INSTAT (GraphPad Software Inc, La Jolla, CA, USA).

Results:

The prevalence of 26.7% for parvovirus B19, 14.0% for *P. falciparum* and 3.0% for co-infection of both pathogens were reported among the SCD patients, while prevalence of 4.7% for B19 infection only was observed in the control subjects. Gender was not significantly associated with prevalence of B19 infection or co-infection of B19 and *P. falciparum* ($p>0.05$) (Table 1). However, gender was significantly associated with the prevalence of *P. falciparum* infection among the SCD patients (OR=0.445; 95%

CI=0.2186, 0.9095; $p=0.0291$). The age of SCD patients was not associated with single infection as well as co-infection of B19 and *P. falciparum* in the study ($p>0.05$). Educational status and religion were also not significantly associated with single and co-infection of B19 and *P. falciparum* ($p>0.05$).

Tribal extraction was significantly associated with single and co-infection of B19 and *P. falciparum* with the Etsako subjects (Edo State) being the most infected (53.9%) by B19 ($p=0.0065$), Yoruba tribe had the highest prevalence of *P. falciparum* infection (45.5%) ($p=0.0137$) while the Hausa tribe had the highest prevalence (20.0%) of coinfection of B19 and *P. falciparum* among the SCD patients ($p=0.0012$). Seasonal variation was not significantly associated with prevalence of co-infection of B19 and *P. falciparum* ($p>0.05$). However, rainy season significantly influenced the prevalence of B19 infection among SCD patients (OR=2.077; 95% CI=1.171, 3.684; $p=0.0144$).

Table 1: Factors associated with infection of parvovirus B19 and *Plasmodium falciparum* among sickle cell disease patients in Benin-City, Nigeria

Factors	No tested	No infected (%)	OR	95%CI	p value
Gender					
Parvovirus B19					
Male	134	36 (26.9)	1.019	0.6088,1.704	1.000
Female	166	44 (67.7)			
<i>P. falciparum</i>					
Male	134	12 (9.0)	0.4459	0.2186,0.9095	0.0291
Female	166	30 (18.1)			
Co-infection					
Male	134	5 (3.7)	1.570	0.4130, 0.5967	0.5194
Female	136	4 (2.4)			
Age group (years)					
Parvovirus B19					
1-10	89	14 (15.7)			0.0746
11-20	122	37 (30.3)			
21-30	53	16 (30.2)			
31-40	29	11 (37.9)			
41 & above	7	2 (28.6)			
<i>P. falciparum</i>					
1-10	89	14 (15.7)			0.0654
11-20	122	20 (16.3)			
21-30	53	4 (7.5)			
31-40	29	2 (6.9)			
41 & above	7	2 (28.6)			
Co-infection					
1-10	89	2 (2.2)			0.2357
11-20	122	3 (2.5)			
21-30	53	3 (5.7)			

31-40	29	0			
41 & above	7	1 (14.3)			
Educational status					
Parvovirus B 19					
Primary	120	21 (17.5)			
Secondary	98	32 (32.7)			
Tertiary	82	27 (32.9)			
<i>P. falciparum</i>					
Primary	120	16 (13.3)			
Secondary	98	19 (19.4)			
Tertiary	82	7 (8.5)			
Co-infection					
Primary	120	2 (1.7)			
Secondary	98	4 (4.1)			
Tertiary	82	3 (3.7)			
Religion					
Parvovirus B19					
Christian	282	74 (26.2)	0.7115	0.2577, 1.964	0.5828
Muslim	18	6 (33.3)			
<i>P. falciparum</i>					
Christian	282	39 (13.8)	1.246	0.3447, 4.505	0.7257
Muslim	18	3 (16.7)			
Co-infection					
Christian	282	9 (3.2)	1.285	0.7191, 22.969	1.000
Muslim	18	6 (33.3)			
Tribe					
Parvovirus B 19					
Igbo	48	14 (29.2)			0.0065
Yoruba	11	4 (36.4)			
Ibibio	21	8 (38.1)			
Bini	164	31 (18.9)			
Esan	20	7 (35.0)			
Etsako	26	14 (53.9)			
Hausa	10	2 (20.0)			
<i>P. falciparum</i>	48	4 (8.3)			0.0137
Igbo	11	5 (45.5)			
Yoruba	21	2 (9.5)			
Ibibio	164	19 (11.6)			
Bini	20	4 (20.0)			
Esan	26	6 (23.1)			
Etsako	10	0			
Hausa					
Co-infection					
Igbo	48	2 (4.2)			0.0012
Yoruba	11	2 (18.2)			
Ibibio	21	0			
Bini	164	2 (1.2)			
Esan	20	0			
Etsako	26	1 (3.85)			
Hausa	10	2 (20.0)			
Season					
Parvovirus B19					
Rainy	190	60 (31.6)	2.077	1.171, 3.684	0.0144
Dry	110	20 (18.2)			
<i>P. falciparum</i>					
Rainy	190	31 (16.3)	1.755	0.8436, 3.650	0.1669
Dry	110	11 (10.0)			
Co-infection					
Rainy	190	7 (3.7)	2.066	0.4213, 10.127	0.4939
Dry	110	2 (1.8)			

Table 2: Effect of haematological factors on co-infection of Parvovirus B19 and *Plasmodium falciparum* among Sickle Cell Disease patients in Benin-City, Nigeria

Factors/Patients	No tested	No infected	OR	95% CI	p value
Transfusion					
Parvovirus B19					
Yes	176	46 (26.1)	0.9367	0.5577, 1.573	0.8946
No	124	34 (27.4)			
<i>P. falciparum</i>					
Yes	176	26 (14.8)	1.170	0.5985, 2.287	0.7363
No	124	16 (12.9)			
Co-infection					
Yes	176	6 (3.4)	1.424	0.3490, 5.806	0.7406
No	124	3 (2.4)			
Anaemia					
Parvovirus B19					
Anaemia	292	76 (26.0)	0.3519	0.08584, 1.442	0.2160
No anaemia	8	4 (50.0)			
<i>P. falciparum</i>					
Anaemia	292	40 (13.7)	0.4762	0.09282, 2.443	0.3110
No anaemia	8	2 (25.0)			
Co-infection					
Anaemia	292	8 (2.7)	0.1972	0.02162, 1.798	0.2186
No anaemia	8	1 (12.5)			
Platelet count (cells/ μ L)					
Parvovirus B19					
< 150,000	24	6 (25.0)	0.9099	0.3478, 2.381	1.000
\geq 150,000	276	74 (26.8)			
<i>P. falciparum</i>					
< 150,000	24	4 (16.7)	1.253	0.4058, 3.866	0.7576
\geq 150,000	276	38 (13.8)			
Co-infection					
< 150,000	24	4 (16.7)	10.840	2.696, 43.580	0.0031
\geq 150,000	276	5 (1.8)			

History of blood transfusion was not significantly associated with the prevalence of single or co-infection of B19 and *P. falciparum* among the SCD patients ($p>0.05$) (Table 2). Anaemia was also not significantly associated with single or co-infection of B19 and *P. falciparum* ($p>0.05$). However, platelets count was significantly associated with co-infection of B19 and *P. falciparum* among the SCD patients especially with platelet count of <150 cells/ μ L (OR 0.840, 95%CI 2.696, 43.580, $p=0.0031$).

Discussion:

Sickle cell disease runs a variable clinical course ranging from mild disease to severe life threatening complications (29). Individuals with SCD are known to be susceptible to infectious agents (30, 31). This study examined parvovirus B19 and *P. falciparum* infections in SCD patients in our locality. To our knowledge,

this is the first study on this in Edo State. It has been hypothesized that depression of cell-mediated immunity in *P. falciparum* infection might favour co-infection with opportunistic pathogens including Parvovirus B19 (32). An overall prevalence of parvovirus B19 and *P. falciparum* co-infection observed in this study was 3.0% whereas the single infection was 14.0% for *P. falciparum* and 26.7% for B19. The prevalence of co-infection of B19 and *P. falciparum* observed in our study is lower than the 14.21% observed in non-SCD patients in Gabon (33). This difference in prevalence rates may be related to population studied, geographical location and seasonal variation.

Gender was not significantly associated with co-infection of *P. falciparum* and B19 although it was significantly associated with *P. falciparum* infection among the SCD patients. Similarly, age was not significantly associated with single infection or co-

infection of B19 and *P. falciparum* among the SCD patients. These observations in our study may indicate adherence of SCD patients or their parents or guardians or relatives to health information that can aid quality of life of SCD patients, usually provided by their clinicians.

Patients or individuals living in malaria endemic regions are known to be at increased risk of serious complications with co-infection of B19 and *P. falciparum* (34). In individuals with SCD who have tolerated chronic anaemia, there could be rapid worsening of the anaemia, which can present as an emergency (4). Under these circumstances, anaemia becomes life threatening and requires prompt treatment with blood transfusion to reduce the deleterious effects of haemoglobin S and improve outcome (4). Parvovirus B19 and *P. falciparum* are easily transmitted by blood transfusion and transfusion with plasma derived products (35). SCD patients are known to be at high risk of transfusion-transmissible infections since they receive frequent, often unplanned, emergency blood transfusion (36, 37). Surprisingly, history of blood transfusion was not significantly associated with single and co-infection of B19 and *P. falciparum* among our SCD patients. The reason for this finding is unclear.

In this study, religion and educational status of our SCD patients were not significantly associated with single or co-infection of B19 and *P. falciparum*. However, tribal extraction was significantly associated with single or co-infection of B19 and *P. falciparum* among the SCD patients, with the SCD patients of Etsako tribe in Edo State having the highest prevalence (53.9%) of B19 infection, the Yoruba tribe had the highest prevalence (45.5%) of *P. falciparum* while the Hausa tribe had the highest prevalence of co-infection (20.0%) of B19 and *P. falciparum*. The reasons for these tribal differences remain to be elucidated. Seasonal variation in prevalence of malaria is well established with highest prevalence during the rainy season (38, 39). Surprisingly, seasonal variation was

not significantly associated with prevalence of *P. falciparum* malaria and co-infection of B19 and *P. falciparum* in our SCD patients, but was significantly associated with the prevalence of B19 infection, with highest prevalence (31.6%) in the raining season compared to the dry season (18.2%) ($p=0.0144$).

Both immunological and non-immunological destructions of platelets have been implicated to cause thrombocytopenia, resulting from consumptive coagulopathy, platelet sequestration in spleen, antibody mediated platelet destruction and oxidative stress. Platelet may also act as cofactor to trigger severe malaria, and abnormalities in platelet structure and function have been described as a consequence of malaria and in rare instances, platelets can be invaded by malaria parasites (40, 41, 42). Previous studies have indicated the involvement of white cells and platelets in single infection and co-infection of B19 and *P. falciparum* (43, 44, 45, 46). In our study, platelet index was not significantly associated with prevalence of single infection of B19 or *P. falciparum*. However, platelet count of <150 cells/ μ L was a risk factor as it was associated with a 2 to 43 fold increased risk of acquiring co-infection of B19 and *P. falciparum* among our SCD patients. In addition, platelet index was significantly associated with the prevalence of co-infection of B19 and *P. falciparum* among the SCD patients. Our findings are in agreement with the previous report of Girei *et al.* among SCD patients with B19 infection in Jos (46).

Parvovirus B19 and *P. falciparum* co-infections have been reported to cause severe anaemia, which can be fatal particularly among SCD patients (11, 47, 48). Parvovirus B19 causes anaemia because it selectively inhibits and lyse actively replicating erythroid progenitor cells (9, 49) which are targets of *P. falciparum*, co-infection of the two pathogens therefore result in severe anaemia (19, 50, 51). Surprisingly, anaemia was not significantly associated with single infection or co-infection of

parvovirus B19 and *P. falciparum* among our SCD patients. Our finding is consistent with the previous study of Toan *et al.* (33) who also did not observe significant difference in haemoglobin concentration among non-SCD patients with co-infections of B19 and *P. falciparum*. The reason for this finding remains unclear.

Conclusion:

An overall prevalence of parvovirus B19 and *P. falciparum* co-infection of 3.0% was observed in our SCD patients in this study, and single infection of 14.0% for *P. falciparum* and 26.7% for B19 were similarly reported. While gender was significantly associated with *P. falciparum* infection among our SCD patients, tribal extraction, platelet index and seasonal variation were significantly associated with single parvovirus B19 infection or co-infection of B19 and *P. falciparum*. The provision of strict regulatory policy concerning the screening of whole blood or pooled plasma before transfusion of blood or blood products in SCD patients is advocated.

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Short Communication

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Comparison of two diagnostic techniques to determine the prevalence of *Schistosoma mansoni* infections in Cameroonian school children

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Abstract:

Background: The Kato-Katz technique is recommended for diagnosis of *Schistosoma mansoni* infection by the World Health Organization. However, egg counts are subject to variability. The aim of this study is to determine the prevalence of *S. mansoni* infection in school children using two different techniques and to recommend the technique that should be routinely used in the diagnosis of this infection.

Methodology: Field investigations on faecal samples from 299 Cameroonian school children were carried out in 2016 to compare the effectiveness of the Kato-Katz and Formalin-ether techniques in diagnosis of *S. mansoni* infections.

Results: Schistosome eggs were detected in 37 (12.3%) samples with the Kato-Katz technique and 61 (20.4%) samples with the Formalin-ether technique. The difference between the prevalence observed for the two techniques was significant in males and age group 10 - 12 years ($p < 0.5$).

Conclusion: The Formalin-ether technique was more sensitive than the Kato-Katz method for detecting *S. mansoni* eggs in faecal matter. Despite its cost, the Formalin-ether technique can be routinely used in the laboratory for epidemiological studies of intestinal schistosomiasis

Key words: Formalin-ether, Kato-Katz *Schistosoma mansoni*, school children

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Comparaison de deux techniques de diagnostic pour déterminer la prévalence d'infections à *Schistosoma mansoni* chez des écoliers camerounais

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Résumé:

Contexte: La méthode Kato-Katz est recommandée par l'Organisation mondiale de la santé pour le diagnostic de l'infection à *Schistosoma mansoni*. Cependant, le nombre d'œufs est sujet à une variabilité. Le but de cette étude est de déterminer la prévalence de l'infection à *S. mansoni* chez les écoliers en utilisant deux techniques différentes et de recommander la technique à utiliser systématiquement pour le diagnostic de cette infection.

Méthodologie: Des enquêtes sur le terrain sur des échantillons de selles provenant de 299 écoliers camerounais ont été menées en 2016 pour comparer l'efficacité des techniques de Kato-Katz et au formol-éther dans le diagnostic des infections à *S. mansoni*.

Résultats: Des œufs de schistosomes ont été détectés dans 37 échantillons (12,3%) avec la technique de Kato-Katz et 61 échantillons (20,4%) avec la technique au formol-éther. La différence entre la prévalence observée pour les deux techniques était significative chez les hommes et chez les 10-12 ans ($p < 0,5$).

Conclusion: La technique au formol-éther était plus sensible que la méthode de Kato-Katz pour détecter les œufs de *S. mansoni* dans les matières fécales. En dépit de son coût, la technique formol-éther peut être utilisée systématiquement en laboratoire pour des études épidémiologiques sur la schistosomiase intestinale.

Mots-clés: formol-éther, Kato-Katz, *Schistosoma mansoni*, écoliers

Introduction:

Human schistosomiasis is a disease transmitted by freshwater snails and caused by blood flukes of the genus *Schistosoma*. This parasitic infection is widespread in tropical and subtropical areas and has been reported in 78 countries (1-2). About 218 million people needed preventive treatment in 2015, however, this preventive chemotherapy is only required in 52 countries with moderate to high endemicity (3). The prevalence of schistosomiasis is still high in sub-Saharan Africa, as approximately 120 million individuals manifest symptoms of schistosomiasis (4). The main burden of this disease in sub-Saharan Africa is usually attributed to two species: *Schistosoma mansoni* and *Schistosoma haematobium*, which are designated as the major human schistosomes (5).

In Cameroon, intestinal schistosomiasis is endemic in the North and far North of the country (6) and more localized in the Southern part (7-9). A large-scale survey performed on 12,594 school children revealed that *S. mansoni*

was the most prevalent schistosome species in the regions of Centre, East and West (9). Infected children were found in 60 of 244 schools investigated, with an average prevalence of 5.5% and a range of 0% to 66.3% in the three regions. In the regions of Littoral, North-West, South and South-West regions, the prevalence of *S. mansoni* infection among 4,130 school-aged children was lower, with an average prevalence of 3% and a range of 0% to 86% (10).

Stool examination is the main method for the diagnosis of suspected *S. mansoni* infections and the Kato-Katz technique is the most commonly used (11-12). This technique is recommended for surveillance and epidemiological field surveys of soil-transmitted helminthic diseases because of its ease of use in the field and the relatively low cost (13). In addition, it provides satisfactory results in case of mass investigations (13). However, the amount of faecal matter (20-50 mg) used for diagnosis is low, which causes a great variability in the number of eggs identified (14). On the other hand, the formalin-ether technique is time consuming and requires several

materials. It is however recommended as the best overall method for concentrating parasite eggs in faeces (15). Reliable diagnosis of intestinal schistosomiasis requires a rapid, cheap, and less laborious method as well as water-based techniques.

As the treatment of *S. mansoni* is dependent on the presence of eggs in faeces, the aim of the present study is to evaluate the effectiveness of two methods used to determine the prevalence of this disease. The study was conducted among school children in Penja, Cameroon, because Penja is known for its high endemicity of intestinal schistosomiasis (10).

Methodology:

The Penja municipality is located in the Department of Mounjo and the region of Littoral. It covers the territory of the district of Njombe-Penja (arrondissement). Its surface area is 260km². The municipality had 36,287 inhabitants at the 2011 census. Its tropical climate and volcanic soil are at the origin of many ponds and small rivers which are regularly frequented by the population.

Faecal samples were collected in April 2016 from 299 children aged 4 to 16 years in three randomly selected schools in the town of Penja. Children were eligible if their parents or guardians gave written consent for the study and children have not received anti-helminthic drugs prior to the time of stool collection. Each child was given an identification number and children were treated by local doctors if *S. mansoni* eggs were detected in their faeces.

In each school, faecal specimens were collected in the morning, placed in 60 mL screw-cap vials and transported in a portable cooler to the Laboratory at the Université des Montagnes for examination on the same day. The consistency and colour of each sample were first noted before subjecting them to the two diagnostic techniques. The first technique was the Kato-Katz technique (16). Briefly, 3 g of faeces

were pressed through a sieve and 20 to 50mg of sieved materials was transferred to a slide. A piece of cellophane soaked in glycerin was placed on these materials. Glycerin eliminates faecal debris, allowing eggs to be seen and counted 45 min or more after preparation. The other technique was the Ritchie's formalin-ether technique (16). About 1 g of faeces was emulsified in a solution of formalin and water. The suspension was sieved and ether was then added. After centrifugation of the mixed suspension, the sediment was examined for coccidian oocysts and helminth eggs and/or larvae.

Differences between the prevalence obtained with the two techniques, and the relationship between prevalence, gender and age group of the children were compared using Fisher's exact test, with *p* value less than 0.05 considered significant. The children were stratified into four age groups; < 7 years, 7-9 years, 10-12 years, and > 12 years. All analyses were performed using software R 3.3.0 (17).

Results:

Table 1 shows the distribution of the school children in relation to gender. Of the 299 faecal samples collected, 90 (30.1%) were diarrheic in consistency while 209 were formed stool. One hundred and ten of the formed stool (36.7%) were brown in colour while 99 (33.1%) had blood stain.

Table 2 shows the prevalence of *S. mansoni* in relation to gender and age group of the children. Of the 299 children, schistosome eggs were recovered in 37 faecal samples (12.3%) with the Kato-Katz technique and 61 (20.4%) with the formol-ether technique. The difference between these techniques was significant (*p*<0.05). The Formalin-ether technique detected more eggs in the males which was significantly more than the values from the females, and also in children aged 10-12 years than from children in other age groups (*p*<0.05).

Table 1: Distribution of schoolchildren in relation to gender in Penja, Cameroon

School	Gender		Total
	Male	Female	
Public primary school 1	45	41	86
Public primary school 2	85	71	156
Bilingual public School	35	22	57
Total	165	134	299

Table 2: Prevalence of *Schistosoma mansoni* infection in schoolchildren at Penja, Cameroon

Gender and age group	Number of children	Number of positive faecal samples with <i>Schistosoma mansoni</i> (prevalence in %)	
		Kato-Katz technique	Formalin-ether technique
Gender of children			
Male	165	19 (11.5)	34 (20.6)*
Female	134	18 (13.4)	27 (20.1)
Age group (years)			
4-6	20	2 (10.0)	4 (20.0)
7-9	75	11 (14.6)	19 (25.3)
10-12	166	19 (11.4)	31 (18.6)*
> 12	38	5 (13.1)	7 (18.4)
Total	299	37 (12.3)	61 (20.4)*

* $p < 0.05$

Discussion:

Contradictory results have been reported in the literature on the effectiveness of Kato-Katz and Formol-ether techniques in the detection of gastrointestinal parasites in faeces. Some authors have noted that the Kato-Katz technique was better and significantly more sensitive than the other method (18-19) while others have reported that the Formol-ether technique had a better sensitivity than the Kato-Katz method (20-21). According to Knight et al., (22), the concentration of eggs in the faeces enabled the detection of more patients infected with *S. mansoni* when the Formol-ether technique was used. The results reported in the present study are consistent with the reports of these last authors because 20% of children infected with *S. mansoni* were detected with the Formalin-ether technique (compared to 12% with the other method).

Although the Formol-ether technique is more expensive and labour intensive to perform than the Kato-Katz technique, its current use in parasitology

laboratory would provide better results in the diagnosis of gastrointestinal infections. Although there was significant difference in the prevalence of *S. mansoni* infections detected by the two techniques among the male children in our study, other authors did not find clear difference in prevalence with respect to gender (23-24). The possible explanation for the difference observed in our study may be related to size and composition of our samples. Conversely, the difference in prevalence according to age group of children is consistent with reports of other authors (23-24).

In conclusion, the Formol-ether technique was clearly more sensitive than the Kato-Katz method for detecting *S. mansoni* eggs in faeces in this study. Despite its cost, this technique is recommended for routine use in the laboratory for epidemiological studies of intestinal schistosomiasis.

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Competing interest:

No competing interest is declared

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