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**Review Article****Open Access****Artemisinin drug resistance and monitoring: a narrative review**

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**Abstract:**

Artemisinin drug resistance is one of the major reasons for malaria treatment failures in the sub-Saharan African countries where artemisinin-based combination therapy (ACT) is the first-line treatment for uncomplicated malaria. The occurrence of single nucleotide polymorphisms (SNPs) is found to correlate with antimalarial drug resistance. With artemisinin, the SNPs occurs at the Kelch 13-propeller gene locus on chromosome 13. The artemisinin drug resistance surveillance strategy involves continuous monitoring of Kelch 13-propeller biomarker to detect emergence of mutations which could herald drug resistance in the region. In this narrative review paper, we examined existing literature to bridge the knowledge gap and accentuate the importance of routine surveillance for artemisinin resistance in sub-Saharan Africa. We conducted our search on PubMed database and Google Scholar to identify peer-reviewed articles, reports, and abstracts on artemisinin drug resistance using the following keywords; 'artemisinin drug resistance', 'antimalarial drug resistance', 'artemisinin-based combination therapy', 'Kelch 13-propeller', 'K13-propeller gene', and 'K13 molecular marker'. The review provided pertinent information on artemisinin derivatives, artemisinin-based combination therapy, molecular action of artemisinin, definition of artemisinin resistance, genetic basis of artemisinin drug resistance and discovery of Kelch 13, and the importance of artemisinin resistance surveillance. Molecular surveillance can provide healthcare policy makers a forecast of impending threats to malaria treatment. This is more so when drugs are in combination therapy, for instance, molecular surveillance can give a hint that one drug is failing despite the fact that in combination, it is still apparently clinically effective.

**Keywords:** malaria; artemisinin; resistance; molecular marker; Kelch 13-propeller gene

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**Résistance aux médicaments à base d'artémisinine et surveillance: un examen narratif**

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**Résumé:**

La résistance aux médicaments à base d'artémisinine est l'une des principales raisons des échecs du traitement du paludisme dans les pays d'Afrique subsaharienne où la polythérapie à base d'artémisinine (ACT) est le traitement de première intention du paludisme simple. L'apparition de polymorphismes mononucléotidiques (SNP) est corrélée à la résistance aux médicaments antipaludiques. Avec l'artémisinine, les SNP se produisent au locus du gène Kelch 13-propeller sur le chromosome 13. La stratégie de surveillance de la résistance aux médicaments à base d'artémisinine

implique une surveillance continue du biomarqueur Kelch 13-propeller pour détecter l'émergence de mutations qui pourraient annoncer une résistance aux médicaments dans la région. Dans cet article de revue narrative, nous avons examiné la littérature existante pour combler le manque de connaissances et accentuer l'importance de la surveillance de routine de la résistance à l'artémisinine en Afrique subsaharienne. Nous avons effectué notre recherche sur la base de données PubMed et Google Scholar pour identifier des articles, des rapports et des résumés évalués par des pairs sur la résistance aux médicaments à base d'artémisinine en utilisant les mots-clés suivants; «résistance aux médicaments à base d'artémisinine», «résistance aux médicaments antipaludiques», «thérapie combinée à base d'artémisinine», «Kelch 13-propeller», «gène K13-propeller» et «marqueur moléculaire K13». L'examen a fourni des informations pertinentes sur les dérivés de l'artémisinine, la polythérapie à base d'artémisinine, l'action moléculaire de l'artémisinine, la définition de la résistance à l'artémisinine, la base génétique de la résistance aux médicaments à base d'artémisinine et la découverte de Kelch 13, ainsi que l'importance de la surveillance de la résistance à l'artémisinine. La surveillance moléculaire peut fournir aux responsables des politiques de santé une prévision des menaces imminentes pour le traitement du paludisme. C'est d'autant plus vrai lorsque les médicaments sont en thérapie combinée, par exemple, la surveillance moléculaire peut donner un indice qu'un médicament échoue malgré le fait qu'en combinaison, il est toujours apparemment cliniquement efficace.

**Mots clés:** paludisme; artémisinine; résistance; marqueur moléculaire; gène Kelch à 13 hélices

## Introduction:

Malaria, caused by *Plasmodium falciparum*, places immense burden on resource-poor countries, particularly those from sub-Saharan Africa. Nigeria is known to contribute the highest burden to global malaria morbidity and mortality. The world recorded an estimated 227 million cases of malaria in 85 malaria endemic countries in 2019 (1). Malaria cases rose to 241 million in 2020, an additional increase of 14 million, from the previous year. Similarly, in 2020, there was an estimated malaria deaths of 627 million, a 12% (69, 000 deaths) increase from 2019 report. Majority of the 29 of the 85 endemic countries which accounted for the 96% of malaria cases and deaths are from sub-Saharan Africa (1). There is need for sustained, effective malaria control programme, to stem the tide of morbidity and mortality arising from malaria, especially in all the 85 endemic countries.

Effective malaria control programme incorporates different kinds of malaria surveillance methods to its control programme scheme. Some of such malaria surveillance schemes include, active case detection for case investigation (reactive), active case detection of febrile cases at community level (pro-active), mass screening, uncomplicated *P. falciparum* cases routinely admitted, and case reporting from private sector. Outside these surveillance schemes, there is also need to monitor susceptibility of the mosquito vector to the common insecticides and the response of the *Plasmodium* spp to the current antimalarial drugs in use.

Molecular markers associated with antimalarial drug resistance are being used to monitor and map the extent of spread of the resistance and hence plan effectively for necessary containment programmes. Molecular markers are excellent tools for surveillance of antimalarial drug resistance. They have been useful as predictors of emerging or existing levels of

antimalarial drug resistance in many malarial endemic countries. Three of the molecular markers often used to monitor resistance to ACTs include the *P. falciparum* multidrug resistance 1 (PfMDR1), *P. falciparum* Ca<sup>2+</sup>-ATPase (PfATP6) and Kelch-13 propeller domain (PfK13) marker.

Perhaps, if there had been an effective surveillance on the spread of chloroquine and sulfadoxine/pyrimethamine resistance to other malaria endemic nations early enough, followed by containment programmes, the global resistance spread experienced with those erstwhile first-line drugs would have been averted. In Nigeria, chloroquine and sulfadoxine/pyrimethamine antimalarial drugs were used as first-line of drugs for treatment of malaria. However, the result of the 2002 and 2004 antimalarial efficacy studies showed that chloroquine and sulfadoxine/pyrimethamine were no longer adequate for national first line use (2). The attendant rising cases of treatment failures with these drugs, strengthened by the recommendation of the World Health Organization (WHO), led to the Federal Ministry of Health in Nigeria proscribing their use as first-line for treatment of malaria. The need to move from monotherapy antimalarial regime to more effective combination therapy rose, and with further efficacy trials in 2004, two artemisinin-based combination therapies were recognized. Nigeria, thus moved from the use as first-line, the failing chloroquine and sulphadoxine/pyrimethamine antimalarial drugs to artemisinin combination therapy (ACT) in 2005 (2).

There has been considerable progress in combating the malaria burden globally. Mortality rates arising from malaria was estimated to have declined by 62% globally between 2000 and 2015 and by 29% between 2010 and 2015. Seventeen countries eliminated malaria (attained zero indigenous cases for 3 years or more) (3). In sub-Saharan African countries, the number of people infected with malaria parasites has

declined from 131 million in 2010 to 114 million in 2015 (3). All these were made possible by adherence to WHO-recommended intervention policies and strategies: vector control, larval control, intermediate preventive therapy (IPT) in infants, children and pregnant women, improved and accessible diagnostic tools, and prompt treatment of cases.

The introduction of highly effective ACTs in 2005 by the WHO, particularly added to the gains (4). However, the gains made so far, seem to be threatened by the recent emergence in South East Asia (Cambodia, Myanmar, Thailand, Vietnam, and Laos) of *P. falciparum* resistant to ACTs (5,6,7). South East Asia has been noted as the centre hub for previous development and subsequent spread of *P. falciparum* strains resistant to erstwhile effective and potent antimalarial drugs.

#### Artemisinin derivatives:

Artemisinin was discovered in China from medicinal plant, *Artemisia annua*, locally known as Qinghao (8). The discovery of artemisinin as an antimalarial drug is tied to the urgent military need that came during the Vietnam War in 1960s and 1970s. The war witnessed huge loss of military personnel on both warring sides. To assuage further loss and deal with the effect of chloroquine-resistant *P. falciparum*, the Chinese government responded to the request of the North Vietnamese by researching to develop new antimalarial drugs. It was this research that led to the discovery of artemisinin (8,9).

At the Academy of Chinese Medical Sciences in Beijing, a team of researchers found that wormwood extract was 100% effective against rodent malaria, although the outcome was inconsistent. The inconsistency in result was later discovered to be due to the use of the traditional method of extraction which was destroying the pharmacologic constituent of the extracts. This understanding paved way for the development of an alternative extraction method of using cool ether. The problem of inadvertently damaging the pharmacologic constituent was solved and with further researches, they were able to purify the effective component and determine the structure to be an endoperoxide located within a sesquiterpene backbone (10,11).

Artemisinin is a sesquiterpene lactone, and since its initial discovery, pharmacologists/pharmacists have tried to modify the original structure at C10 position to produce more potent derivatives such as artesunate, artemether, artemotil, arteether, and dihydroartemisinin (DHA). Originally, artemisinin and its derivatives were used to treat malaria, however activities against cancer (12), parasites such as *Toxoplas-*

*ma* (13), *Clonorchis* (14), *Schistosoma* (15), *Leishmania* (16), and viruses (17) have recently been documented. The artemisinin prototype and its derivatives possess important pharmacological qualities that heighten their antimalarial activity such as rapid onset of action, short half-life and activity against different stages in the life cycle of the malaria parasite (18). They are also known to kill the sexual stages (gametocytes) of malaria parasites in the circulation (19).

#### Artemisinin-based combination therapy (ACT): What it is!

The idea of combining an artemisinin with another structurally unrelated antimalarial drug was to optimize malaria treatment. This was seen as the best therapeutic strategy to improve treatment efficacy and slow down the development of resistance to the individual drugs in the combination (20,21). The multidrug concept of treating infectious diseases has been applied in the treatment of human immunodeficiency virus (HIV) infection and tuberculosis. It is believed that the combination therapies which involve the use of drugs with different targets and mechanism of action, decreases the chance of emergence of drug resistance.

The treatment success achieved from combination therapy is underpinned by the assumption that drug resistance depends on DNA mutation. The assumption maintains that provided that the constituent drugs in the combination have different modes of action, the probability of a parasite developing resistance to both drugs simultaneously will be highly reduced compared to developing resistance to one drug (22). Most artemisinins combined treatments contain one of the three artemisinin derivatives including artemether, artesunate or dihydroartemisinin (DHA). Blood esterases and hepatic cytochrome P450 enzymes respectively convert oral artesunate and artemether to DHA, but they are active themselves (23). Although artemisinins have short elimination half-life of about 1 hour, the time is ample for maximal effects against the different life-cycle stages of the malaria parasites. However, to make-up for the rapid elimination of the active metabolite (DHA) from the blood stream, the administration of artemisinin with long-acting agents is required (24).

Two prominent pharmacodynamic properties of artemisinin determine how best they are used. One is their ability to kill both ring stage and more mature trophozoites rapidly, a property that underlie their importance in life-saving efficacy in severe disease and in cure (25). The other property is that patients treated with artemisinin always have a subpopulation of

artemisinin-treated ring-stage parasites enter a state of dormancy, avoiding being killed outrightly. This subpopulation of parasites resume growth a few days or weeks after stoppage of therapy (26). This is seen as the reason for the approximately 10% failure rates observed with the use of artemisinin monotherapy in malaria treatment (27). It was for this phenomenon that artemisinins are best used in combinations with a long-acting partner drug. In this way, the artemisinin derivative kills the most of the parasites within a few days, while the few remaining parasites are killed by the long-acting partner drug (28).

Five of the combined formulations currently recommended by the WHO for the treatment of uncomplicated *P. falciparum* malaria are; artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine/pyrimethamine, dihydroartemisinin-piperaquine and artemether-lumefantrine (29). Artesunate-amodiaquine, artesunate-mefloquine and artemether-lumefantrine fixed-dose ACTs have been implemented as first-line treatment for uncomplicated malaria in most malaria endemic countries (30). Piperaquine was originally added as partner drug to ACTs for the first-line treatment of uncomplicated confirmed-malaria cases in Cambodia, Vietnam, Myanmar and China (31). A new ACT recently introduced into the market is artesunate-pyronaridine (32). The WHO has recommended ACTs since 2005, and treatment with these drugs provide excellent cure rates, above the minimum acceptable threshold of 90% (23).

#### Molecular action of artemisinin:

The knowledge of exactly how artemisinin and its derivatives work at the molecular and cellular level to bring about the cellcidal effect has been elusive for a while. Several models from *in vitro* medicinal chemical studies have tried to explain this at one or the other, however, it seems consensus has not been reached yet. A known basic feature of artemisinin is that they are unstable in the presence of alkaline or acidic milieu, and they react in the presence of peroxide with certain reducing agents such as  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$  and haeme. The endoperoxide bridge has been shown to be critical to artemisinin's antimalarial and anti-cancer properties. The pharmaceutical properties of artemisinin are thought to be associated with their reductions, though the mechanisms that bring about this reduction is still uncertain (33,34).

Four models considered to be possible modes of action of artemisinin include; haeme-detoxification pathway interference, induction of alkylation of translationally controlled tumour protein, interference with mitochondrial function,

and inhibition of the sarco/endoplasmic reticulum membrane calcium transporting ATPase 6. A study by Sun et al., (35), using the baker's yeast (*Saccharomyces cerevisiae*) demonstrated the molecular and cellular properties of artemisinin. In the model, they proposed that the anti-mitochondrial and haeme-mediated reactive oxygen species (ROS)-generating properties constitute two cellcidal actions of artemisinin.

#### Definition of artemisinin resistance:

Adopting a global consensus definition of artemisinin resistance has been a big challenge. However, a proposal was made some-times ago that a clinical case of artemisinin resistance would have the following attributes (36); (i) persistence of parasites on the 7<sup>th</sup> day after the start of treatment or re-emergence of parasites within 28 days after the start of treatment; (ii) adequate plasma concentrations of dihydro-artemisinin, a major artemisinin metabolite; (iii) prolonged parasite clearance time; and (iv) reduced *in vitro* susceptibility of the parasite.

A study carried out at different sites in Western Cambodia in 2007-2008 reported a significant delay in parasite clearance time following treatment with artesunate monotherapy and with an ACT, when compared with that conducted in eastern Thailand (37). In any patient from South Asia with uncomplicated *P. falciparum* malaria, and a starting parasite count of 10,000 parasites/ $\mu\text{l}$  of blood, artemisinin resistance is defined as a parasite clearance half-life 5 hours following treatment with an artesunate monotherapy or an ACT (38). In the study done by Witkowski et al., (39) in 2013, they defined artemisinin resistance in *P. falciparum* as a parasite survival rate 1% in the ring-stage survival assay ( $\text{RSA}^{0-3\text{h}}$ ) *in vitro*. The assay involved adapting clinical parasite isolates to culture, and synchronizing them at the early-ring stage (0-3 hr post-invasion of red blood cells), exposing them to a pharmacologically-relevant dose of DHA for 6 hours and then culturing for 66 hours further. The survival rates were assessed by counting the proportion of viable parasites that developed into second-generation rings or trophozoites with normal morphology at 66 hours ( $\text{RSA}^{0-3\text{h}}$ ), after drug removal.

#### Genetic basis of artemisinin drug resistance and discovery of Kelch 13:

The slow parasites clearance rates experienced with administration of artemisinin prompted many genotype-phenotype research works that were aimed at unravelling the phenomenon. A region on *P. falciparum* chromosome 13 was found to be strongly associated with slow clearance of the parasites in genome-wide stu-

dies (40). The major breakthrough in the search of genetic basis of the reduced susceptibility of the *P. falciparum* parasites to artemisinin came in 2013 when parasites subjected to artemisinin pressure, were cultured for 5 years and then sequenced (41). A mutation was found close to the chromosome 13 region in the genome association studies (40,42). Arley et al, (41) by comparing the whole-genome sequences of drug-selected and unselected parasite lines, identified on chromosome 13, a single-nucleotide polymorphism (SNP) in the *PF3D7\_1343700* gene that encodes a M476I substitution in the propeller domain of a kelch protein. This kelch protein which contains a 'kelch' motif is now generally being referred to as K13.

The use of K13-propeller polymorphism as a molecular marker of artemisinin resistance in Cambodia was validated after demonstrating that 17 different K13 mutations were present in parasites from this country and that the predominant C580Y mutation had increased in prevalence especially in areas where artemisinin resistance was so common. Also, that the common mutations; C580Y, R539T, and Y493H were associated with prolonged clearance half-lives and elevated RSA<sup>0-3h</sup> survival rates (43). Many more researchers have since conducted more studies establishing the fact that K13-propeller polymorphism is a marker for artemisinin resistance

even in other Great Mekong Regions in South-east Asian countries including Thailand, Vietnam, China and Myanmar, with the K13-propeller mutation findings being also associated with slow parasite clearance (44,45).

In Africa, some studies have reported dozens of K13-propeller mutations, many of which are different from those found in SE Asia (46-48). These African variants of mutations are still at low frequency, and are yet to be associated with artemisinin resistance in patients and *in vitro* in 17 countries (49-51). However, more recently, validated Pfk13-propeller mutations have been reported in India, South America, and on the African continent, in Uganda and Rwanda (52-57). Also in Nigeria, V692G, B664I, Q661H, and C469C mutations detected in a study by Fehintola et al., (58) were consistent with delayed parasitological clearance. Few other studies conducted in Nigeria, demonstrated presence of Pfk13 propeller gene mutations, however, they could not be linked to ACT failure or delayed clearance (59,60).

Different Pfk13 mutations produce varying effects on the clearance phenotype. Box 1 below shows the WHO list of candidate/associated and validated markers of artemisinin partial resistance and the criteria for the classification. The list of candidate and validated markers is updated continually (Table 1).

Box 1: WHO list of candidate/associated and validated markers of artemisinin partial resistance and the criteria for classification (61)

Candidate or associated Pfk13 markers of artemisinin partial resistance	<p>A statistically significant association (<math>p &lt; 0.05</math>) between a Pfk13 mutation and clearance half-life <math>&gt; 5</math> hours or day 3 parasitaemia via a chi-squared test or appropriate multivariable regression model on a sample of at least 20 clinical cases</p> <p>OR</p> <p>Survival of <math>&gt; 1\%</math> using the RSA0-3h in at least five individual isolates with a given mutation or a statistically significant difference (<math>p &lt; 0.05</math>) in the RSA0-3h assay between culture-adapted recombinant isogenic parasite lines, produced using transfection and gene editing techniques, which express a variant allele of Pfk13 as compared with the wild-type allele</p>
Validated Pfk13 markers of artemisinin partial resistance	Both requirements 1 and 2 are met



Table 1: Pfk13-propeller gene markers of artemisinin partial resistance (61)

Validated markers		Candidate or associated markers
F446I	P574L	P441L
N458Y	C580Y	G449A
C469Y	R622I	C469F
M476I	A675V	A481V
Y493H		R515K
R539T		P527H
I543T		N537I/D
P553L		G538V
R561H		V568G

### Why surveillance?

Artemisinin combination therapies are currently critical in the management of uncomplicated *P. falciparum* malaria in all sub-Saharan African nations, including Nigeria. No new drugs of equivalent efficacy have been developed to replace them and even if there are plans to produce more potent drugs, it will take some years. The emergence of artemisinin resistance in South East Asian countries is thus, a threat to the global malaria control and elimination action plans.

There is the premonition that artemisinin-resistant parasites will behave the same way they did with chloroquine and later pyrimethamine, spread westward to reach Africa. If this should happen, this would add to already high burden of malaria in this region. Emergence of chloroquine resistance was estimated to have caused the death of millions African children (62). This trend should not be allowed to repeat itself. There is therefore need for a surveillance scheme to be put in place in order to monitor the emergence and spread of artemisinin-resistance to other malaria endemic regions which should prompt drastic containment measures.

### Conclusion:

The emergence and subsequent spread of antimalarial drug resistance has been one of the main challenges to malaria containment in many malarial-endemic areas such as sub-Saharan African countries. Detection and characterization of resistance to chloroquine and sulfadoxine/pyrimethamine that were once used as first-line in the treatment of malaria occurred after resistance had already spread globally, hence there was no opportunity for containment.

With the emergence of artemisinin drug resistance in Southeast Asia, and in line with the WHO Global Plan for Artemisinin Resistance Containment (GPARC), there is need to proactively avert the spread of artemisinin resistance to sub-Saharan Africa by constantly monitoring its spread and instituting containment measures.

### Contributions of authors:

AAI and GIO conceptualized this study. AAI, EM, and PAO conducted the literature searches, collated articles, and drafted this paper. PCE and GEN, and ABO reviewed, edited, and contributed to the final version of the manuscript.

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### Conflict of interest:

Authors declare no conflict of interest.

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## Review Article

## Open Access

# A review of the current diphtheria outbreaks

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## Abstract:

*Corynebacterium diphtheriae* is responsible for both endemic and epidemic diphtheria. The predisposing factor for this disease is the failure to immunize during childhood. Humans are the only hosts of the organism and is present in the upper respiratory tract. The organism is transmitted via airborne route and can cause respiratory obstruction and heart failure because of the exotoxin it produces. There is presently a resurgence of diphtheria outbreaks in Nigeria. The Nigeria Center for Disease Control (NCDC) was notified of suspected diphtheria outbreaks in Lagos and Kano States, Nigeria, in December 2022 and has been issuing monthly reports since that time. This review of the diphtheria outbreaks following online database searches on PubMed and Google Scholar as well as the NCDC/WHO websites and grey literatures, describes the current trend of the outbreaks globally, elucidated the different strains of *Corynebacterium* responsible for the outbreaks, identified the recent vaccine formulation developed to tackle the outbreaks, and provide information on vaccine delivery and efficacy studies in the country and globally.

**Keywords:** Corynebacteria; outbreaks; DPT; immunization coverage

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## Un examen des épidémies actuelles de diphtérie

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## Résumé:

*Corynebacterium diphtheriae* est responsable à la fois de la diphtérie endémique et épidémique. Le facteur prédisposant à cette maladie est l'absence de vaccination pendant l'enfance. Les humains sont les seuls hôtes de l'organisme et sont présents dans les voies respiratoires supérieures. L'organisme est transmis par voie aérienne et peut provoquer une obstruction respiratoire et une insuffisance cardiaque en raison de l'exotoxine qu'il produit. Il y a actuellement une recrudescence des épidémies de diphtérie au Nigeria. Le Centre Nigérian de Contrôle des Maladies (NCDC) a été informé des épidémies présumées de diphtérie dans les États de Lagos et de Kano, au Nigéria, en décembre 2022 et publie depuis lors des rapports mensuels. Cet examen des épidémies de diphtérie à la suite de recherches dans les bases de données en ligne sur PubMed et Google Scholar ainsi que sur les sites Web et les littératures grises du NCDC/OMS, décrit la tendance actuelle des épidémies dans le monde, a élucidé les différentes souches de *Corynebacterium* responsables des épidémies, identifié les récentes la formulation de vaccins développée pour lutter contre les épidémies et fournir des informations sur l'administration des vaccins et les études d'efficacité dans le pays et dans le monde.

**Mots clés:** Corynébactéries; les épidémies; TPD; couverture vaccinale

## Introduction:

Corynebacteria are aerobic Gram-positive non-encapsulated, non-sporulating, and non-motile, pleomorphic granular rods, arranged in palisades. Many non-pathogenic species are members of the indigenous flora of the skin, oropharynx, urogenital and intestinal tracts where they are collectively known as diphtheroids (1). They produce polyphosphate volutin (metachromatic) granules, which stain red with Albert or methylene blue stain. The most pathogenic species, *Corynebacterium diphtheriae* has three strains identifiable on tellurite agar; the gravis (3-5mm), mitis 2-4 mm and intermedius (1-2mm) strains. The gravis strain produces frosted metallic grey colonies with strained margins ("daisy head" colonies). On blood agar, some gravis and mitis strains are haemolytic, while the intermedius strain is non-haemolytic (1,2).

*Corynebacterium diphtheriae* produce diphtheria toxin, which is a lethal exotoxin produced under the influence of a temperate bacteriophage. The exotoxin is a heat-labile coagulable protein sensitive to denaturation by solutions at pH < 6, and by moderate heat. The addition of formalin will turn the toxin into a toxoid which is highly immunogenic (1,2). Only three *Corynebacterium* species (*C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans*) are known to produce the lethal exotoxin called diphtheria toxin. The diphtheria toxin gene (*tox*) is donated by corynebacteriophages and are produced by the bacteria through lysogenisation, which allows the corynebacteriophage carrying the *tox* gene to be stably integrated into the chromosome (2). During the diphtheria epidemic in Eastern Europe in the 1990s, a new strain called non-toxigenic *tox* gene-bearing (NTTB) strain, was identified (2,3). They however did not express the gene protein, but are believed to be potentially toxigenic. It is therefore possible that the new resurgent outbreaks of diphtheria in different parts of the world could be due to these NTTB strains of *C. diphtheriae*.

The clinical spectrum of infection includes nasal diphtheria, anterior nasal diphtheria, "bull-neck" diphtheria, severe pharyngeal diphtheria, laryngeal diphtheria, tracheobronchitis and cutaneous diphtheria (1). The manifestations of *C. diphtheriae* infection are influenced by the anatomic site of infection, the immune status of the host, and the production and systemic distribution of toxin (4). Diphtheria toxin is easily absorbed in the surrounding tissues of the patient's throat, where it induces local inflammatory reaction in the nasopharynx and larynx. There is a serocellular exudate which forms a grayish membrane across the larynx, causing severe acute respiratory obstruction.

*Corynebacterium diphtheriae* is responsible for both endemic and epidemic diseases, and is communicable for 2-6 weeks without antibiotic treatment (4). The predisposing factor for this disease is the failure to immunize during childhood. Humans are the only hosts of the organism and are present in the upper respiratory tract. The organisms are transmitted via airborne droplets (4,5). Blockage of the recurrent laryngeal nerve by exotoxin is an important part of the pathogenesis of diphtheria because it leads to respiratory failure. The toxin also blocks the bundle of His, leading to atrio-ventricular block and heart failure. Patients could quickly die from acute respiratory obstruction, and/or heart failure (1, 4,5).

There is presently a resurgence of diphtheria outbreaks in Nigeria. The Nigeria Center for Disease Control (NCDC) was notified of suspected diphtheria outbreaks in Kano and Lagos States on December 1, 2022. From January to March 2023, the NCDC reported a total of 733 suspected cases, including 89 deaths, with children between the ages of 5 and 18 years mostly afflicted, with overall case fatality rate (CFR) of 12.3%. There have been reports that *Corynebacterium ulcerans* and some other species are now capable of elucidating exotoxin (2,6).

The aims of this review therefore are to; (i) provide information on the epidemiology and current trend of diphtheria outbreaks globally and in Nigeria; (ii) elucidate the different strains of *Corynebacterium* responsible for the outbreaks; (iii) describe the response and identify the recent vaccines developed to tackle the outbreaks; (iv) provide information on diphtheria, pertussis and tetanus (DPT) vaccine delivery and DPT efficacy studies in the country; and (v) to suggest recommendations on how the current diphtheria outbreaks can be speedily controlled.

## Methodology and Results:

Electronic databases including PubMed and Google Scholar were searched for primary source articles including original reports, case series studies, case reports, seroprevalence studies and epidemiologic investigation reports on diphtheria in Nigeria from 2015 to 2023. The websites of the NCDC and World Health Organization (WHO), including other grey literatures, were equally searched for information relevant to diphtheria outbreaks. Secondary search was also conducted using references of primary articles reviewed.

Inclusion criteria for selecting studies for the review were studies that provided information on strains of *C. diphtheria* responsible for outbreaks, recent diphtheria vaccines developed, DPT vaccine delivery, and DPT effi-

cacy studies in Nigeria. Search words used include "diphtheria" AND/OR "*Corynebacterium diphtheria*" AND/OR "*C. diphtheria*" AND "diphtheria outbreak in Nigeria" AND "*C. diphtheria* vaccine" AND/OR "DPT vaccine" AND "DPT vaccine delivery" AND "DPT efficacy". Publications on systematic and narrative reviews on diphtheria were excluded.

Using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guide, initial search produced 2240 articles from Google Scholar and 1139 articles from PubMed. Following de-duplication, 495 article titles and abstracts were screened, followed by assessment of 70 full articles to determine eligibility, and 53 articles were further excluded. Following primary and secondary searches, 23 articles were included (17 from primary search and 6 from secondary search), in addition to 16 publications from the grey literatures (Fig 1).

## Discussion:

### Global epidemiology of diphtheria:

According to the WHO data, there has been a gradual rise in the global diphtheria cases from 4,535 in 2015 to almost 23,000 in 2019. There was a decline in reported cases in 2020 (10,137 cases) possibly due to the COVID-19 pandemic but the downward trend continued in 2021 with just over 8,500 cases reported (5). Ethiopia accounts for the highest number of cases with over 50% of reported cases. Other countries with a high number of

cases include India, Nigeria and Yemen (7). The incidence rate in recent times was lowest in 2015 at 0.7/1,000,000 population and highest in 2019 at 3.4/1,000,000 population. The incidence rate currently stands at 1.3/1,000,000 population (7).

Global diphtheria mortality rate has been reported to range between 5-10% with vaccination coverage being a major determinant of mortality rate. Children under five years and adults above 40 years may have higher death rates, which can be as high as 20% (8). Diphtheria is known as a childhood disease, most common in children below 15 years, but with waning immunity and increase in unvaccinated individuals in countries with low diphtheria prevalence, there has been an increase in case detection among people above 40 years (5). Diphtheria is not associated with any race. Incidence is similar in both males and females, with some studies showing higher incidence in males below 15 years and adult females over 40 years (9). Mortality may be higher among females in endemic regions as vaccination is higher in male children in these regions.

Global DPT 1<sup>st</sup> dose vaccine coverage has remained high. It increased by 1% in 2016 and reached its peak coverage of 90% where it remained till 2020 when it declined by 3% and by 2021, it declined to 86%. DPT 3rd dose coverage also followed a similar trend from 86% in 2016 down by 3% in 2020 and at 81% in 2021 (10).

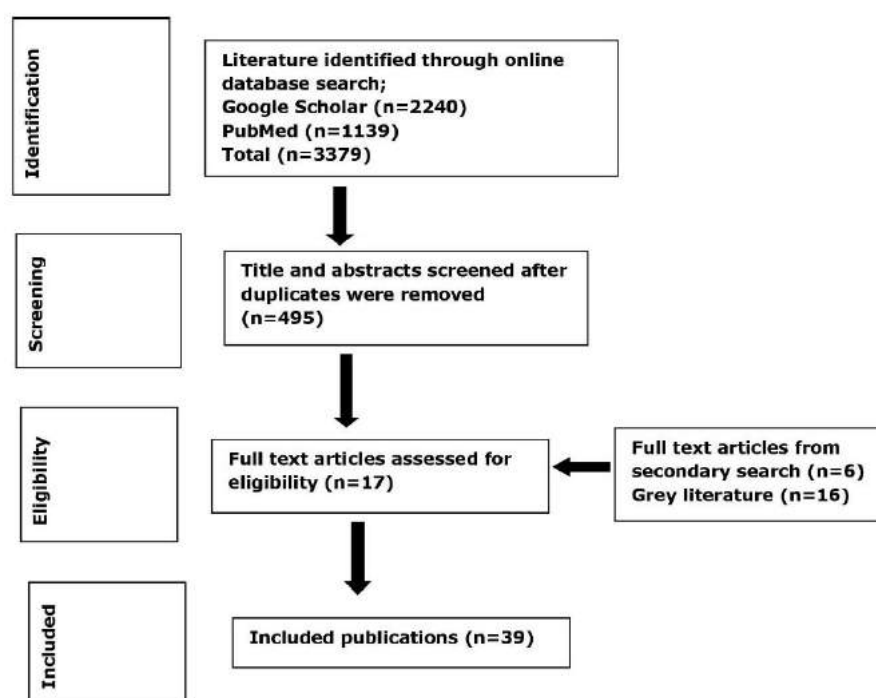


Fig 1: Process of selection of publications (PRISMA guide) used for the review

### **Epidemiology of diphtheria in Nigeria from 2015 to 2023:**

The diphtheria outbreak in Nigeria in 2023 has been concerning, with 216 reported cases and 40 deaths across four States as of February 2023 (11). The outbreak is believed to have started in late December in Kano State, but cases have also been reported in Lagos, Osun, and Yobe States (12-15). Reports had Kano with 172 of 216 cases (91.0%), Katsina with 9 cases (4.8%), and Lagos with 8 cases (4.2%). Among the suspected cases that were reported, 111 cases, accounting for 42.1% of the total, were confirmed. Out of these confirmed cases, 8 were confirmed through laboratory testing, while 103 were determined to be clinically compatible with the disease. Additionally, 18 cases (7.1%) were discarded as not being related to the disease, while 40 cases (15.3%) are pending classification, and 84 cases (33.2%) had unknown status.

Notably, the majority of confirmed cases (91.9%) occurred in the age group of 2 to 14 years. Sadly, a total of 22 deaths were recorded among the confirmed cases, resulting in a case fatality rate (CFR) of 19.8%. It is concerning to note that only 12 out of the 111 confirmed cases (10.8%) had received full vaccination with a diphtheria toxin-containing vaccine (11,15). All confirmed cases were reported from nine local government areas (LGAs) in Kano State, and four deaths were recorded among these confirmed cases, resulting in CFR of 7.4% (11,13,14).

There were no reported cases of diphtheria in 2022 although in general, data are sparse with many years not having records of diphtheria officially reported (7). According to the NCDC, there were 160 reported cases of diphtheria in Nigeria in 2021. Most of the cases were reported in the northern part of the country, with Kano, Bauchi, and Yobe States having the highest number of cases. Other States with reported cases include Borno, Gombe, Jigawa, Kaduna, Katsina, Kebbi, Sokoto, and Zamfara (11,16,17). In Kano State, majority of the cases were reported in the Kumbotso, Gwale, and Tarauni LGAs. In Bauchi State, majority of the cases were reported from Bauchi, Ganjuwa, and Tafawa Balewa LGAs, and in Yobe State, majority of cases were reported from Damaturu, Fika, and Potiskum LGAs.

In 2020, there were 245 reported cases from 25 States of the country (11,18), the highest number of cases were reported in the northwest region of Nigeria, with Katsina and Kano States recording the highest number of cases. Other States with significant number of cases included Jigawa, Sokoto, and Zamfara. However, cases were reported across the country, including the southern States.

In 2019, a total of 2,289 cases were reported, of which 157 were confirmed (11,

18). The outbreak was reported in 24 States in the country, with the highest number of cases reported in Kano (41 cases) followed by Zamfara (33 cases). Other affected States in 2019 included Kaduna, Plateau, Bauchi, Jigawa, and Lagos, among others. At the LGA level, 80 LGAs in the affected States reported cases of diphtheria.

In 2018, Nigeria experienced a large outbreak of diphtheria, with a total of 1,870 suspected cases reported (7). Of these, 176 were confirmed, and 22 were fatal. The outbreak was mainly concentrated in four States; Adamawa, Bauchi, Borno, and Kano, which accounted for more than 80% of the total suspected cases. The outbreak affected several LGAs, including Gombi, Yola North, Yola South, Jama'are, Katagum, and Damban in Bauchi State; Jere, Konduga, Maiduguri Metropolitan, and Mafa in Borno State; Nasarawa, Tarauni, and Kano Municipal in Kano State; and Song in Adamawa State.

In 2017, there were 782 suspected cases of diphtheria reported in Nigeria, with 213 confirmed cases and 22 deaths. Reports were from 24 of the 36 States of the Federation (11,19), with the highest number of cases reported in Kano State, accounting for 36% of the confirmed cases. A total of 80 LGAs were affected with the highest number reported in Kano Municipal LGA, followed by Gwale LGA, Tarauni LGA, and Dala LGA.

In 2016, there were 29 confirmed cases of diphtheria reported, a significant increase from the 12 cases reported in the previous year. The cases were reported from 11 States across the country including Bauchi, Borno, Kano, Katsina, Sokoto, Yobe, Zamfara, Delta, Ebonyi, Enugu, and Rivers. Bauchi State had the highest number of reported cases (10 cases), followed by Kano State (6 cases). The cases were reported from 16 LGAs across the 11 States, with the highest number of cases reported from Bauchi LGA (6 cases), followed by Kano Municipal LGA (4 cases).

In 2015, there were 5,959 suspected cases of diphtheria with a case fatality rate of 7.5% (7). The outbreaks were reported in 16 States of the country, with the highest number of cases recorded in Kebbi, Sokoto, and Zamfara States. The other States include Bauchi, Borno, Gombe, Jigawa, Kaduna, Kano, Katsina, Nasarawa, Niger, Plateau, Taraba, Yobe, and the Federal Capital Territory. The outbreaks occurred in several local government areas in the affected States. The overall cases reported by Nigeria officially to the WHO are shown in Table 1.

### **Case fatality rate of diphtheria in Nigeria:**

The CFR for diphtheria in Nigeria has shown significant variability in recent years (20). In 2016, there were four reported deaths,



Table 1: Diphtheria cases in Nigeria reported to the World Health Organization

Period	Diphtheria cases reported to WHO by Nigeria
2015-2019	4159
2010-2014	0
2005-2009	312
2000-2004	7253
1995-1999	2724
1990-1994	9479
1985-1989	11551
1980-1984	2144
1975-1979	2144
1970-1974	129
<b>Total</b>	<b>39895</b>

with a CFR of 13.8%. The CFR decreased in 2017 to 10.3%, although males were slightly more affected, accounting for 54% of the confirmed cases. In 2018, out of the 2,360 suspected cases, 22 were fatal, resulting in a CFR of 0.93%. However, in 2019, the number of deaths increased significantly to 21, with a CFR of 13.4%. In 2020, CFR was not reported, while in 2021, it was reported as 5%. These trends suggest that while efforts to combat

diphtheria in Nigeria have been effective in reducing the CFR, there is still a need for continued vigilance to prevent and manage outbreaks. Fig 2 shows the CFR trend over this period.

#### Age group and gender distribution of diphtheria in Nigeria:

The diphtheria trend in 2020 and 2021 showed that the highest number of reported diphtheria cases in Nigeria occurred in children under 15 years of age, with the most affected age group being children between 5 and 14 years, accounting for 54% of the reported cases. In 2019, the age distribution of confirmed cases showed that 61% were between the ages of 5 and 14 years, followed by those aged 15-24 years, which accounted for 23% of the cases.

There was equal distribution of diphtheria cases among both sexes in 2020 and 2021. In 2019, however, males accounted for the majority (56%) of reported cases (18). Similarly, in 2018, a slightly higher proportion of males (53%) were affected compared to females (47%). In 2015, there was a slightly higher number of diphtheria cases reported among males compared to females. Fig 3 shows the general trend as reported by WHO for diphtheria cases in Nigeria.

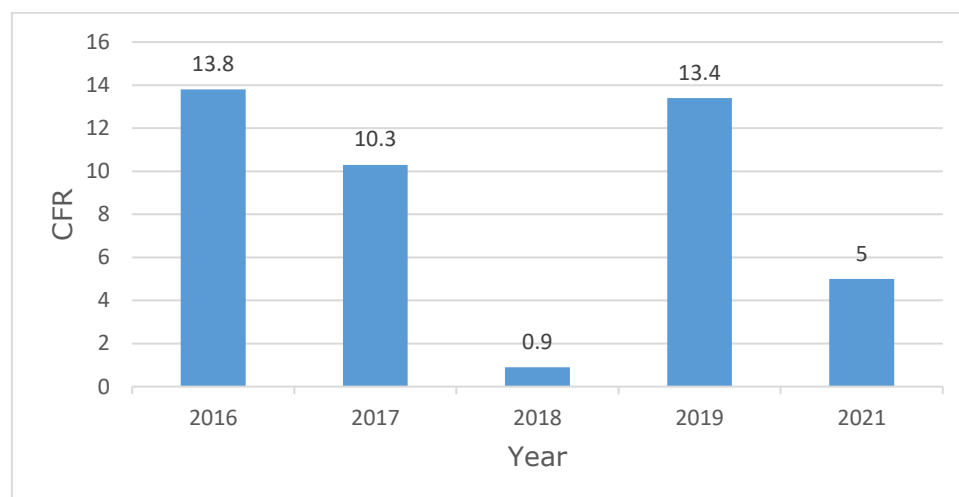


Fig 2: Case fatality rate (CFR) of diphtheria in Nigeria over 5 years



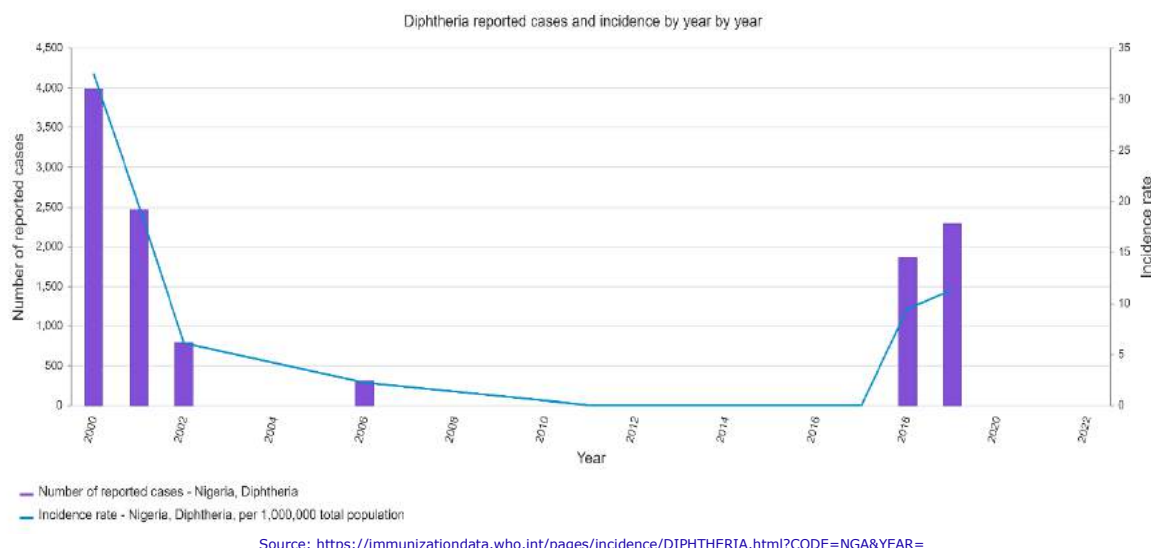


Fig 3: Trend of diphtheria cases in Nigeria as reported to the World Health Organization

#### Laboratory diagnosis and strains of corynebacteria responsible for the outbreaks:

In general, advancements in laboratory diagnostics have revolutionized the diagnosis of diphtheria. Serological diagnosis, utilizing the detection of specific antibodies in the patient's blood serum against the diphtheria toxin, remains a critical tool in confirming cases, assessing immune status, and monitoring vaccine effectiveness especially in low-and-middle-income-countries (LMICs) because of its relatively lower cost. The classic Elek test is one such method, known for its simplicity, affordability, and suitability for resource-limited settings, it has been widely utilized for serological diagnosis (21). It does have the limitation of only being useful for diagnosis and not strain typing. However, newer serological methods such as enzyme-linked immunosorbent assay (ELISA) have emerged, offering improved sensitivity and specificity (22,23).

The biochemical differentiation of *C. diphtheriae* strains into biovars have been relegated to historical significance only due to its lack of reliability, low reproducibility, false-negative results due to variations in biological expression, and its moderate to high complexity (24). In addition to serological methods, various molecular genotyping methods such as ribotyping, amplified fragment length polymorphism (AFLP) or random amplified polymorphic DNA (RAPD), pulse-field gel electrophoresis (PFGE), clustered regularly interspaced short palindromic repeat (CRISPR)-based spoligotyping, and multilocus sequence typing (MLST), have been utilized to investigate the molecular epidemiology and diversity of *C. diphtheriae* (25,26).

In particular, the MLST scheme has shown clinical correlation with severity of disease with most of the >300 strains identified up to date being clinically and epidemiologi-

cally relevant (26,27). The MLST strains causing outbreaks are largely country specific with the only literature report from Africa being the MLST ST-375 which caused the diphtheria epidemic of 2015 in South Africa (28).

The molecular approach provides valuable insights into the genetic characteristics and population dynamics of the bacterium, allowing for a better understanding of its transmission patterns, evolution, and spread in different populations and geographic regions (22). Of these advanced methods, PCR is the most available in developing economies. PCR allows for rapid and highly sensitive detection of *C. diphtheriae* and its toxin gene, enabling faster turnaround times and more accurate identification of toxigenic strains. Other cutting-edge technologies like whole-genome sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry have been used in limited settings but have also shown promise in enhancing the accuracy and speed of diphtheria diagnosis (29).

A relatively new *C. diphtheriae* strain called non-toxigenic tox gene-bearing (NTTB) strain, has been identified (30). Molecular characterization of the NTTB strain revealed that it carries the *tox* gene, which traditionally encodes for the diphtheria toxin, but lacks the ability to produce the toxin. These strains are genotypically *tox*-positive but do not express the toxin. However, the level of their involvement in current outbreaks has not yet been investigated. The significance of the NTTB strain has been highlighted in numerous reported cases, and its epidemic potential even in countries with high vaccination rates has been well-documented (30). While there is at yet no reported NTTB strain in West-Africa, the strain has been linked to severe clinical manifesta-

tions, including myocarditis, polyneuritis, and bacteraemia (30). The MLST-212 has been one of the more common NTTB strains reported globally (31).

Surveillance data have shown that the NTTB strains have been identified in both toxigenic and non-toxigenic strains, and its presence has been associated with variable clinical manifestations (5). While traditionally diphtheria cases present with respiratory symptoms, including sore throat, difficulty breathing, and hoarseness, cases caused by the NTTB strain have shown a wider spectrum of clinical presentations, ranging from mild respiratory symptoms to severe systemic manifestations, including myocarditis and neuritis (31,32). This has therefore posed challenges in clinical diagnosis and management of diphtheria caused by the NTTB strain.

Limited data suggest that similar risk factors associated with diphtheria outbreaks caused by toxigenic strains, such as low vaccination coverage, poor sanitation and hygiene practices, overcrowded living conditions, and limited access to healthcare services, may also play significant roles in the transmission of the NTTB strains (5). However, further research is needed to better understand the unique epidemiology and risk factors associated with this strain. The advancements in laboratory diagnostics play a pivotal role in confirming diphtheria cases, identifying outbreaks, monitoring vaccine effectiveness, and guiding timely intervention strategies to prevent the spread of diphtheria. They offer improved accuracy, efficiency, and rapidity, empowering healthcare providers to promptly diagnose and manage diphtheria cases, and ultimately contribute to better patient outcomes.

However, there continue to be gaps in laboratory confirmation of diphtheria as well as other diseases of public health importance in Africa. These gaps include limited laboratory capacity to determine toxigenicity, challenges in availability of culture media, and difficulties in accessing diphtheria antitoxin. As a result, only a limited number of African countries report national, case-based surveillance for diphtheria with laboratory confirmation, indicating the need for increased laboratory capacity to effectively detect and manage diphtheria outbreaks in the region.

#### **Response to diphtheria outbreaks:**

The NCDC, Primary Health Care Development Agency (PHCDA), and WHO collaborated to respond to the 2019 diphtheria outbreak in Nigeria. They provided technical support for case management, laboratory testing, and active surveillance, while also improving routine immunization coverage and surveillance systems. In addition, they conducted public awareness campaigns to educate the

populace about the disease and the importance of vaccination. While the response helped control the outbreak, sustained efforts are needed to improve routine immunization coverage and disease surveillance in Nigeria. The NCDC is supporting States in increasing diagnostic capacity and supply of antitoxins for the treatment of diphtheria.

#### **Diphtheria vaccine delivery and coverage:**

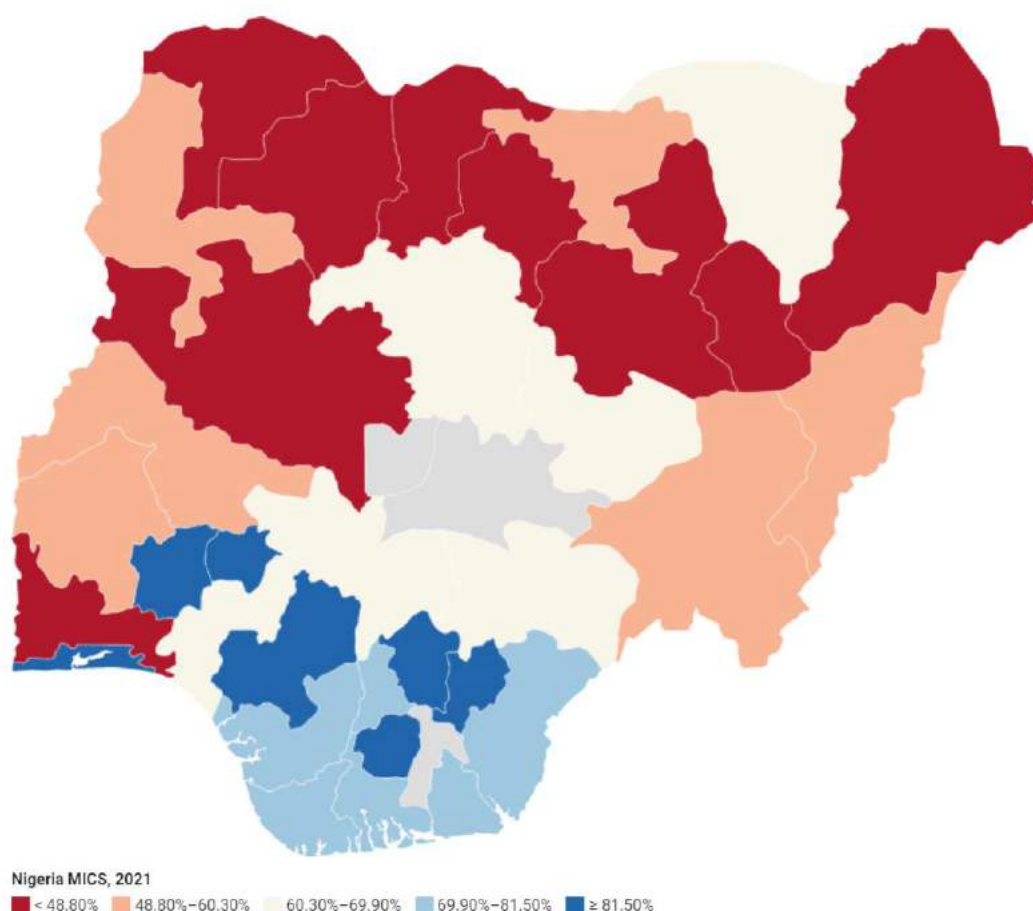
According to recent data, immunization rate for diphtheria was reported to be lowest in the northeast region of Nigeria, with only 41.7% of children receiving the vaccine. In the northwest region, the rate was slightly higher at 42.9%, while the north-central region had the highest rate at 57.2%. When considering urban versus rural areas, a higher percentage of children living in urban areas (72.7%) had received the third dose of the diphtheria, pertussis, and tetanus (DPT) vaccine compared to those in rural areas (47.5%). Additionally, vaccination rates were found to be higher (88.9%) among children whose mothers had higher or tertiary education compared to those whose mothers had no education (36.2%). In terms of household income, the data showed that diphtheria vaccination rates were higher (84.6%) among children from households considered to be rich compared to those in the poor households (38.7%). Fig 4 shows the vaccination coverage rates of diphtheria in Nigeria.

#### **Efficacy studies on diphtheria vaccine platform in Nigeria and globally:**

The pentavalent (D, P, T, Hib, HepB) vaccine is now replacing the DPT vaccine in Nigeria and indeed, globally. The DPT and pentavalent vaccines have demonstrated equal efficacy in inducing IgG antibodies in vaccinated children (33,34). The pentavalent vaccine has been shown to be as effective as the previous individual DPT, Hib, and HepB, with associated fewer injections and similar tolerability (35,36). A meta-analysis by Truelove et al., (37) reported that receiving a full vaccination of at least three doses of the DTP vaccine is associated with 87% effectiveness in preventing symptomatic diphtheria.

On the other hand, they demonstrated that incomplete vaccination with just one or two doses is still associated with 71% effectiveness in preventing symptomatic disease (37). Very importantly, they demonstrated that full vaccination provided 93% protection against death and partial vaccination provided 68% protection against death (37). These findings highlight the importance of completing the recommended vaccination schedule to maximize protection against diphtheria and safeguard against its harmful effects.

Administering diphtheria antitoxin after



Source: <https://africacheck.org/fact-checks/factsheets/factsheet-what-you-should-know-about-diphtheria-outbreak-nigeria>

Fig 4: Diphtheria vaccination coverage rate (%) of Nigerian children aged 12–23 months by geographical regions of Nigeria

infection has been shown to significantly reduce mortality by 76%. However, it is important to note that the effectiveness of the antitoxin depends on prompt administration, as it only neutralizes circulating and not intracellular toxin. Delay in administering the antitoxin can have serious consequences, as mortality rate doubles with each day of delay (37), underscoring the critical importance of timely intervention to prevent adverse outcomes in diphtheria cases. The efforts towards ensuring zero missed dose of childhood vaccinations in Nigeria have been largely successful although there are still significant gaps especially in northern Nigeria where the overall vaccination rates are lowest (38,39).

### Recommendations for control of diphtheria outbreaks:

Ensuring that laboratories have the necessary resources and capabilities for diagnosis of diphtheria is of utmost importance. Laboratory testing plays a critical role in confirming cases of suspected diphtheria, tracking the circulation of toxin-producing strains, and

evaluating the effectiveness of vaccines. The availability and functionality of reliable laboratory surveillance system in place enables generation of timely and reliable data to make informed decision for appropriate treatment, and implementation of public health interventions. It is therefore vital to prioritize and invest in strengthening laboratory diagnostics for diphtheria, ensuring that laboratories have the necessary tools and support to effectively contribute to diphtheria control and prevention efforts.

We recommend that pentavalent (DPT, HiB and HepB) vaccine be continued as the primary childhood vaccination schedule for diphtheria in Nigeria, and in other priority settings where diphtheria is a public health issue. The advantages of the pentavalent vaccine, such as immune-protection, fewer injections and similar tolerability, can potentially help to streamline the vaccination process and increase vaccine coverage, leading to better protection against diphtheria. Furthermore, the importance of completing the full vaccination schedule for diphtheria cannot be over emphasized. Full vaccination provides higher protec-

tion against symptomatic diphtheria and its complications compared to partial vaccination. Thus, healthcare providers and policy makers should prioritize efforts to ensure that children receive the full complement of diphtheria-containing vaccines according to the recommended schedule, and address any barrier to completion.

In addition, timely administration of diphtheria antitoxin is crucial in cases of suspected or confirmed diphtheria. Healthcare providers should be educated about the importance of early recognition of diphtheria symptoms and immediate administration of antitoxin to neutralize the circulating toxin and prevent severe outcomes. Strengthening the healthcare system to ensure availability of diphtheria antitoxin and improving surveillance and reporting of diphtheria cases can aid in timely intervention and reduce mortality associated with diphtheria.

Finally, continued research are essential in guiding vaccination policies and strategies. Regular monitoring of diphtheria epidemiology, vaccine coverage and effectiveness can provide valuable insights. Further research can also assess the long-term impact of pentavalent vaccine implementation on diphtheria incidence, evaluate vaccine safety and effectiveness in different populations, and identify strategies to improve vaccine uptake and completion rates.

## Contributions of authors:

AB conceived the review, designed the outline, wrote the introduction, efficacy of vaccines and recommendations, and reviewed and edited the manuscript; AO reviewed articles, wrote the introduction, and reviewed the drafts; TMB reviewed articles, wrote the global diphtheria and reviewed the manuscript; MN wrote the epidemiology of diphtheria in Nigeria, strains of diphtheria, and vaccine coverage, and reviewed the manuscript; MB reviewed the manuscript; and AR supplied some data from NCDC.

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## Conflict of interest:

Authors declare no conflict of interest.

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Copyright AJCEM 2023: <https://dx.doi.org/10.4314/ajcem.v24i2.3>**Review Article****Open Access****A review of the recent advances on Lassa fever with special reference to molecular epidemiology and progress in vaccine development**<sup>1</sup>Medugu, N., <sup>\*1</sup>Adegboro, B., <sup>2</sup>Babazhitsu, M. S., <sup>3</sup>Kadiri, M., and <sup>4</sup>Abanida, E. A.<sup>1</sup>Departments of Medical Microbiology and Immunology, Nile University of Nigeria, Abuja, Nigeria<sup>2</sup>Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences,  
College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria<sup>3</sup>Department of Medical Microbiology and Parasitology, University of Ilorin Teaching Hospital, Ilorin, Nigeria<sup>4</sup>Epidemiology Unit, World Health Organization, Harare, Zimbabwe\*Correspondence to: [boazadegboro@gmail.com](mailto:boazadegboro@gmail.com)**Abstract:**

Lassa fever, a viral hemorrhagic fever caused by the Lassa virus (LASV), is endemic in West Africa and is associated with high morbidity and mortality. At least three of the four proposed seven lineages of LASV are found in Nigeria, where the multimammate rat, *Mastomys natalensis*, serves as the primary reservoir. Endemic countries report approximately 200,000 infections and 5,000 deaths annually, with Nigeria experiencing thousands of infections and hundreds of deaths including healthcare workers. The aim of this review is to provide scientific information for better understanding of the evolutionary biology, molecular epidemiology, pathogenesis, diagnosis, and prevention of Lassa fever in Nigeria and other endemic regions worldwide, which can lead to improved control efforts and reduce morbidity and mortality from recurrent epidemics. To achieve this aim, observational studies such as case series, cross-sectional and cohort studies published between December 2017 and September 2022 were searched for on various online databases including Google Scholar, Africa Journals Online (AJOL), Research Gates, PubMed, PMIC, NCDC, and WHO websites. Although the origin and evolutionary history, and the transmission dynamics of Lassa virus have been revealed through recent molecular epidemiological studies, the factors that drive the evolution of the virus remain unclear. Genetic changes in the viral genome may have enabled the virus to adapt to humans. Diagnosis of Lassa fever has also advanced from basic serological tests to more sophisticated methods such as quantitative real time polymerase chain reaction (qRT-PCR) and sequencing, which are particularly useful for identifying outbreak strains. Several vaccines, including recombinant vesicular stomatitis virus (rVSV), virus-like particle (VLP), and DNA-based vaccines, have shown promise in animal models and some have progressed to phase 2 clinical trials. Preventing and controlling Lassa fever is critical to safeguard the health and well-being of affected communities. Effective measures such as rodent control, improved sanitation, and early detection and isolation of infected individuals are essential for reducing transmission. Ongoing research into the genetic and ecological factors that drive the evolution of Lassa virus is necessary to reduce the impacts of Lassa fever.

**Keywords:** Lassa fever; recent advances; molecular epidemiology; evolutionary history; vaccine

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Copyright 2023 AJCEM Open Access. This article is licensed and distributed under the terms of the Creative Commons Attribution 4.0 International License [<a rel="license" href="http://creativecommons.org/licenses/by/4.0/">](http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium, provided credit is given to the original author(s) and the source. Editor-in-Chief: Prof. S. S. Taiwo**Une revue des avancées récentes sur la fièvre de la Lassa avec une référence particulière à l'épidémiologie moléculaire et aux progrès du développement des vaccins**<sup>1</sup>Medugu, N., <sup>\*1</sup>Adegboro, B., <sup>2</sup>Babazhitsu, M. S., <sup>3</sup>Kadiri, M., et <sup>4</sup>Abanida, E. A.<sup>1</sup>Département de Microbiologie Médicale et d'Immunologie, Université du Nil du Nigéria, Abuja, Nigéria<sup>2</sup>Département de Microbiologie Médicale et de Parasitologie, Faculté des Sciences Cliniques de Base, Collège  
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## Résumé:

La fièvre de Lassa, une fièvre hémorragique virale causée par le virus de Lassa (LASV), est endémique en Afrique de l'Ouest et est associée à une morbidité et une mortalité élevées. Au moins trois des quatre lignées proposées de LASV se trouvent au Nigeria, où le rat multimammaire, *Mastomys natalensis*, sert de réservoir principal. Les pays endémiques signalent environ 200,000 infections et 5,000 décès par an, le Nigeria connaissant des milliers d'infections et des centaines de décès, y compris des travailleurs de la santé. L'objectif de cette revue est de fournir des informations scientifiques pour une meilleure compréhension de la biologie évolutive, de l'épidémiologie moléculaire, de la pathogenèse, du diagnostic et de la prévention de la fièvre de Lassa au Nigeria et dans d'autres régions endémiques du monde, ce qui peut conduire à des efforts de contrôle améliorés et réduire la morbidité et la mortalité des épidémies récurrentes. Pour atteindre cet objectif, des études observationnelles telles que des séries de cas, des études transversales et de cohorte publiées entre décembre 2017 et septembre 2022 ont été recherchées sur diverses bases de données en ligne, notamment Google Scholar, Africa Journals Online (AJOL), Research Gate, PubMed, PMIC, Sites Web du NCDC et de l'OMS. Bien que l'origine et l'histoire évolutive, ainsi que la dynamique de transmission du virus de Lassa aient été révélées par des études épidémiologiques moléculaires récentes, les facteurs qui déterminent l'évolution du virus restent flous. Des modifications génétiques du génome viral pourraient avoir permis au virus de s'adapter à l'homme. Le diagnostic de la fièvre de Lassa est également passé des tests sérologiques de base à des méthodes plus sophistiquées telles que la réaction quantitative en chaîne par polymérase en temps réel (qRT-PCR) et le séquençage, qui sont particulièrement utiles pour identifier les souches épidémiques. Plusieurs vaccins, y compris le virus recombinant de la stomatite vésiculeuse (rVSV), les particules pseudo-virales (VLP) et les vaccins à base d'ADN, se sont révélés prometteurs dans des modèles animaux et certains ont progressé vers des essais cliniques de phase 2. La prévention et le contrôle de la fièvre de Lassa sont essentiels pour préserver la santé et le bien-être des communautés touchées. Des mesures efficaces telles que le contrôle des rongeurs, l'amélioration de l'assainissement et la détection et l'isolement précoces des personnes infectées sont essentielles pour réduire la transmission. Des recherches continues sur les facteurs génétiques et écologiques qui déterminent l'évolution du virus de Lassa sont nécessaires pour réduire les impacts de la fièvre de Lassa.

**Mots-clés:** Lassa fever; avancées récentes; épidémiologie moléculaire; histoire évolutive; vaccin

## Introduction:

Lassa virus (LASV), the causative agent of Lassa fever, belongs to the family Arenaviridae. Arenaviruses are rodent-borne viruses which are classified as segmented negative-sense RNA (nsRNA) viruses and are phylogenetically closely related to other segmented nsRNA viruses belonging to Bunyaviridae and Orthomyxoviridae (1). These three virus families are basically similar in terms of intracellular replication. This is based on serological cross-reactivity, phylogenetic relatedness, and geographical distribution (1,2) All arenaviruses are further sub-divided into the Old World and New World virus complexes. The New World arenavirus complex comprises viruses that circulate in North America i. e. Whitewater Arroyo (WWAV), Tamiami (TAMV), and Bear Canyon (BCNV) viruses, and South America i. e. Tacaribe (TACV), Junin (JUNV), Machupo (MACV), Guanarito (GTOV), and Sabia (SABV) viruses. The Old-World complex includes arenaviruses that circulate in Africa, Europe, and Asia i. e. lymphocytic choriomeningitis (LCM) and LASV viruses (3).

The Lassa virus uses various methods to attach to and infect particular cells, while avoiding the host immune system. The observed bleeding diathesis is due to the effects of proinflammatory cytokines and capillary endothelial injury. Severe manifestations of the disease are also caused by cardiovascular, renal, and central nervous system involvement (4). Mortality is highest in pregnant women and children (5). Sequelae include

deafness, alopecia, and pericarditis.

Current diagnostic techniques for Lassa fever include serological tests such as ELISA which is highly sensitive and specific but cannot be used for detection of current acute infection. The reverse transcriptase polymerase chain reaction (RT-PCR) is currently the acceptable 'gold standard' test, which can detect low levels of viraemia, and is useful for follow-up of patients and early detection of outbreaks (6). Virus isolation is also a recognized 'gold standard' test but is very expensive to set-up especially in endemic regions where the burden is highest. Currently, there are about 34 different vaccine candidates in various stages of development with the vector-based recombinant vesicular stomatitis virus (rVSV), virus-like particle (VLP) based, and DNA-based vaccines showing the greatest promise (7-11).

The objective of this review is to examine the latest advancements in the biology, epidemiology, clinical characteristics, diagnosis, management, and vaccines for Lassa fever. This will assist in enhancing our efforts to manage the disease, resulting in decreased morbidity and mortality rates from repeated outbreaks of Lassa fever.

## Methodology and Results:

We performed online literature searches for studies referring to the epidemiology, biology, pathogenesis, diagnosis, and prevention of Lassa fever in Nigeria primarily, then in other endemic regions, and lastly globally.

The search engines used include Google Scholar, Africa Journals Online (AJOL), Research Gates, PubMed/PMIC, Nigeria Center for Disease Control (NCDC), and WHO websites. We searched for observational studies such as case series, cross-sectional and cohort studies published between December 2017 and September 2022, using keywords and Boolean search terms; 'Lassa virus', 'Lassa fever', 'recent advances on Lassa fever', 'Lassa fever vaccines', and 'diagnosis of Lassa fever'.

Information obtained was summarized for relevant key points and findings were included in this review. The total number of full-text and peer-review publications on Lassa fever cases were 1385 articles. After deduplication, review of articles for eligibility, and secondary search, 128 articles were considered eligible for full text revision (Fig 1).

## Discussion:

### Biology of Lassa virus

Lassa virus is an enveloped, single-stranded, negative-sense, bi-partite ribonucleic acid (RNA) virus which belongs to the family Arenaviridae (12,13). The virus is spherical with an average diameter of 110-130 nanometers, and in cross-section, they show 'grainy particles' (ribosomes acquired from host cells) and thus the Latin name "arena", which means "sandy". The RNA genome of the virus has four encoded proteins; nucleoprotein (NP) and glycoprotein precursor (GP) on the Small (S) segment, and RNA-depend-

ent RNA-polymerase (L) and matrix RING Zinc-finger protein (Z) on the Large (L) segment (13,14). Through nucleic acid sequencing of Lassa virus, identification and molecular characterization of seven LASV strains were possible. These include strain Josiah, originating from Sierra Leone (15), strain Nigeria (16) and strain LP (6,17) both from Nigeria, and strain AV imported into Germany by a traveller who had visited Ghana, Côte D'Ivoire, and Burkina Faso (18).

Three strains are found in Nigeria, with the fourth found in Guinea, Liberia, and Sierra Leone. The prototype LP strain isolated by Buckley and Casals in 1969 from Eastern Nigeria occupied the most basal lineage I. Strains isolated from Southern Central and Northern Central Nigeria were placed in lineage II and III, respectively, and the largest group of strains from Guinea, Liberia, and Sierra Leone occupied lineage IV (19). A fifth lineage, which falls between III and IV, has been proposed for the AV strain isolated from a patient that was infected (presumably) in Ghana or Ivory Coast (18,19). However, more recent lineage VI, originating from Togo (20). A new lineage has been discovered in the recent Nigerian Lassa fever outbreak and if confirmed will make a total of 7 lineages (21). The understanding of genetic variability of LASV is important for the designation of diagnostic molecular assays and more importantly for the development of universally acceptable vaccines for Lassa fever for use in different geographical settings.

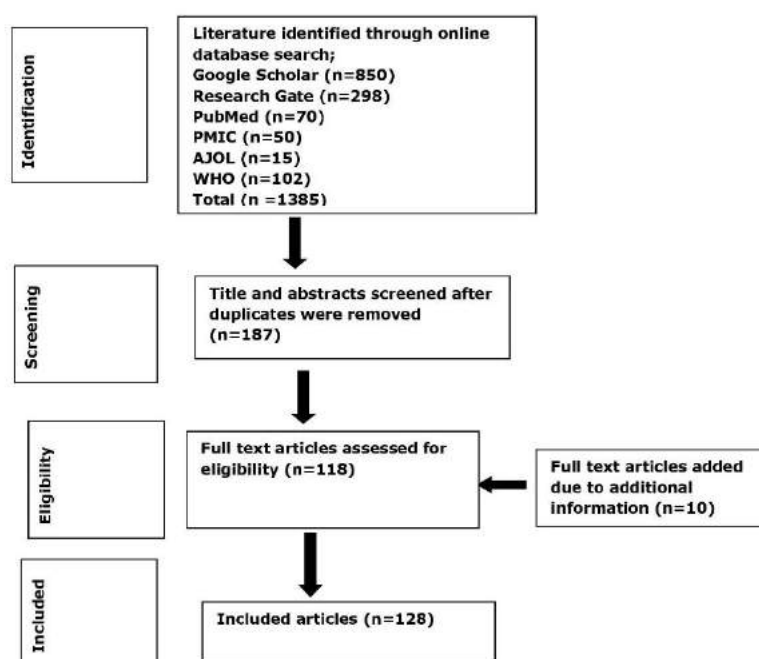


Fig 1: Process of selection of publications (PRISMA guide) used for the review





Fig 2: Pictures of the multimammate rat *Mastomys natalensis*

### Mode of transmission of Lassa virus

The “multimammate rat” *Mastomys natalensis* has been considered to be the animal reservoir for LASV (Fig 2). This rodent is abundant in West Africa and breeds productively (22). The infection of these rats is in-utero which remain infected throughout their life. They do not become ill, but the virus is being shed in their urine and faeces. Other rodent reservoirs discovered include *Mastomys erythroleucus* and *Hylomyscus pamfi* (23).

Primarily, transmission of infections to humans occurs from direct or indirect contact with LASV-infected rodents (24). Those at the greatest risk of acquiring LASV infection are those living in rural areas where the *Mastomys* rodents are usually found, especially in communities with poor sanitation or crowded living conditions (25-27). The *Mastomys* rodents invade homes of humans during the dry season in search of food. Direct contact with urine, faeces, blood or meat from LASV-infected *Mastomys* rodents can lead to infection (27). The infection is usually spread through the respiratory or gastrointestinal systems after direct or indirect contact

with animal excrements. Inhalation of microscopic infectious particles (aerosol) is thought to be the most common mode of acquiring infection (4).

The disease has been proven to spread from person to person, posing a threat to healthcare personnel (28). Although it has been observed that the virus is present in the semen, transmission by sexual routes remains debatable (29). Lassa fever has been reported from sexual transmission months after recovery from acute disease (30). Although no study has established the transmission by breast milk, the high level of viremia suggests its possibility (31).

### Epidemiology of Lassa fever

Lassa fever is a zoonotic acute viral illness is endemic in parts of West Africa including Sierra Leone, Liberia, Guinea and Nigeria. Neighboring countries are also at risk because the animal vector lives throughout the region. Although Lassa fever is endemic in the above countries, isolated cases occur in Cote D'Ivoire, Burkina Faso, Mali and Benin Republic. Peak incidence occurs during dry season (November-April) (Fig 3).



Fig 3: Geographical distribution of Lassa fever in West Africa  
Map available at: <https://www.afro.who.int/health-topics/lassa-fever> (33)



Fig 4: Regions and areas with major outbreaks of Lassa fever (33)

The number of LASV infections per year in West Africa is estimated at 100,000 to 300,000, with approximately 5,000 deaths. Unfortunately, such estimates are crude, because surveillance for cases of the disease is not uniformly performed. In some areas of Sierra Leone and Liberia, it is known that 10%-16% of people admitted to hospitals every year have Lassa fever, which indicates the serious impact of the disease on the population of this region (32).

The prevalence of antibodies to the virus in the population is 8-52% in Sierra Leone (33), 4-55% in Guinea (34), and 21% in Nigeria (35). Seropositivity has also been found in the Central African Republic, Democratic Republic of the Congo, Mali, and Senegal (Fig 4) (32). Staff from the UK Department for International Development, the International Committee of the Red Cross, and the United Nations Mission in Sierra Leone have succumbed to the disease. Cases have occurred in travelers returning to Britain, the Netherlands, Germany, and export to countries outside Africa such as UK, US, Germany and Netherlands (18,36-38).

#### Epidemiology of Lassa fever in Nigeria:

Lassa fever surveillance in Nigeria is conducted through the Integrated Disease

Surveillance and Response (IDSR) platform. Information on Lassa fever flows from the health facilities, through the ward focal persons to the Local Government Area (LGA) Disease Surveillance and Notification Officers (DSNOs), to the State DSNOs, to the State Epidemiologist and then to the NCDC and Federal Ministry of Health (FMOH). All States in Nigeria including the Federal Capital Territory (FCT) report through the IDSR (39). Weekly reports on number of confirmed cases and deaths from Lassa fever are published in the Lassa fever weekly epidemiological reports by the NCDC (Table 1).

Historically, outbreaks of Lassa fever occur during the dry season (November to April), however, in recent years, cases have also occurred during the rainy season. Each year, Nigeria records dozens of confirmed cases and deaths including those of health care workers (HCWs). In Nigeria, majority of the cases (>70%) are from three States of Ondo, Edo, and Bauchi. Other States affected are Benue, Taraba, Kogi, Oyo, Ebonyi, Kaduna, Plateau, Cross River, Katsina, Nasarawa, Gombe, Enugu and Delta (34).

In 2018, the NCDC reported the largest ever number of cases in Nigeria with over 600 confirmed cases and over 170 deaths (Table 1). Analysis of 77 LASV genomes from

Table 1: Reported cases of Lassa fever in Nigeria from 2017-2022 (35)

Year	Suspected cases	Confirmed cases	Probable cases	Deaths (confirmed cases)	CFR (%)	HCW
2017	733	143	-	-	-	-
2018	3498	633	20	171	27.0	45
2019	5057	833	19	174	20.9	20
2020	6791	1189	14	244	20.5	47
2021	4654	510	6	102	20.0	10
2022	3542	681	28	127	18.6	51

CFR: Case fatality rate; HCW: Healthcare worker

patients suggests that the surge was due to cross-species transmission from local rodents carrying various viral variants. The outbreak was not caused by a single virus variant or sustained by human-to-human transmission. Major rivers appeared to act as barriers to the migration of the rodent reservoir, leading to significant viral diversity based on geographic location (21).

A phylogenetic analysis of 14 genomes from the 2018 outbreak showed that the strains responsible were not novel and no clustering, indicating there were several sources from which zoonotic transmission occurred and were not due to a surge in human-to-human transmission (21). In 5 of the 14 sequenced LASV, intra-host single nucleotide variants (SNVs) were detected at a minor allele frequency >5%, suggesting that the virus underwent intra-host *de novo* mutations (21). When LASV from the northern, south-western, and eastern Nigeria were compared using phylogenetic analysis, the patterns from the three regions remained very distantly related suggesting the hosts in those regions were geographically confined (21). Since then, cases had been on the increase with 2020 being the worst hit within the period under review, with 1189 confirmed cases and 244 deaths, including over 45 healthcare workers (35).

#### **Morbidity and mortality of Lassa fever**

Sensorineural hearing deficit is a feature of Lassa fever, which occur in 29% of hospitalized confirmed cases compared with 0% in febrile controls (8,36). In the general population, 81% of those who experienced sudden deafness had antibodies to LASV versus 19% of matched controls. There is no apparent relation between the severity of viral illness, initial hearing loss, or subsequent recovery (40).

Although the actual incidence of Lassa fever in Nigeria is unknown, the case fatality rate, which is the number of deaths divided by the number of confirmed cases multiplied by a factor of 100 (41), ranges from 3% to 42%, and has over the last two years remained between 20%-25% (33,35,39) with 15-20% case fatality rate among hospitalized patients. There are 300,000 to 500,000 cases per year, with approximately 5,000 deaths (42-45).

#### **Pathogenesis of Lassa fever**

The incubation period of Lassa fever ranges from 6 to 21 days in roughly 80% of persons who are infected. The condition is asymptomatic or moderately symptomatic, while 20% of infected persons will have severe multisystem disease (46,47). The antigen-presenting cells are the primary target of the

virus upon entering into the host cells (48) but the virus infects most tissues in humans culminating in multi-systemic malfunctions and immunosuppression of host innate interferon (IFN) response through inhibition of interferon regulatory factor-3 (IRF-3) expression (49) through its nucleoprotein. The virus employs alpha-dystroglycan in establishing itself into targeted cells mostly macrophages, dendritic cells, and endothelial cells which are the points of commencement of its replication (50). In addition to preventing IFN production by infected cells, LASV inhibits the cells of the immune system, preventing the secretion of pro-inflammatory cytokines including tumor necrosis factor (TNF)- $\alpha$ , IL-6, and IL-1 $\beta$ , unlike the symptoms observed in other haemorrhagic fevers (50). It has been reported that LASV has exonuclease activity only targeted at double-stranded RNAs, which mostly inhibits the responses of IFN. This is achievable via assimilation of pathogen-associated molecular patterns (PAMPs), which help LASV to circumvent the immune response of the host (51).

The blood vessels are the tissues mostly afflicted and LASV replicates in the endothelial cells of blood vessels culminating in capillary injury. Bleeding might be observed in many organs such as hepatocyte, intestine, myocardium, lungs as well as the brain (52). Unregulated expression of cytokine referred to as "cytokine storm", similar to what is seen in SARS-CoV-2 and sepsis, could be another possible mechanism of Lassa fever pathogenesis.

There is paucity of autopsy findings due to religious, societal stigma and practices associated with the dead in many regions. However, few pathologic investigations of reported cases in human subjects after post-mortem examination shows injury in the hepatocyte, adrenal glands as well the spleen (46). Other studies on the analysed histologic results of the hepatocyte revealed marked necrotic eosinophils and parenchymal cells accompanied by penetration of eosinophils in the sinusoids, while analysis of spleen samples shows necrosis of eosinophils, depletion of lymphoid, deposition of fibrin, and shrinking of the white pulp as well as infiltration of mononuclear cells and lymphocytes (46).

#### **Clinical features of Lassa fever**

The clinical disease begins as a flu-like illness characterized by fever, general weakness, and malaise, which may be accompanied by cough, sore throat, and severe headache (4). Gastrointestinal manifestations such as nausea, vomiting, and diarrhoea are also common (4). Although, haemorrhagic manifestations are not significant feature of Lassa fever, perturbation of vascular function

is likely to be central to Lassa fever-associated pathobiology, since the signs of increased vascular permeability, such as facial oedema and pleural and pericardial effusions, indicate a poor prognosis for the disease outcome. Recovery from Lassa fever generally begins within 8 to 10 days of disease onset (4). In severe cases, the condition of the patient deteriorates rapidly between the 6th and 10th day of illness with severe pulmonary oedema, acute respiratory distress, clinical signs of encephalopathy, sometimes with coma and seizures, and terminal shock. Bleeding from mucosal surfaces is often observed; however, it is usually not of a magnitude to produce shock by itself (53). Sensorineural deafness is commonly observed in patients in the late stages of disease or in early convalescence in survivors (54).

In severe cases, cardiovascular symptoms appear including mucosal bleeding, melaena, and signs of vascular permeability such as oedema of the face and neck, pleural effusions, ascites, and shock accompanied by a sudden fall in blood pressure, hypovolaemia, vasoconstriction of peripheral blood vessels, and a decrease in urinary output. Some patients develop neurological symptoms including meningeal irritation, convulsions, disturbances of consciousness, and ataxia. Patients that are likely to die go into shock between the 7<sup>th</sup> and 14<sup>th</sup> day of illness. Death usually occurs during the second or third week of illness (4,55-57).

Lassa fever is severe in pregnancy, causing fetal death in about 80% of pregnant women. It is characterized by a high maternal mortality rate (up to 30%) and more than 90% fetal mortality rate during the third trimester (5,58). Lassa fever is a significant cause of paediatric hospitalizations in some areas of West Africa. The disease in children is often characterized by enlargement of the liver and the spleen, vomiting, convulsions, cough and malaise. It has been associated with the "swollen baby" syndrome in children under 2 years of age in Liberia, a presentation that is characterized by generalized edema, abdominal distension, and bleeding (55, 59). Deafness has been described in 20% of patients with Lassa fever. It develops during the second week of illness and involves the 8<sup>th</sup> cranial nerve, and may be unilateral or bilateral, temporary or permanent (4,54). Minor complications of Lassa Fever occur in some patients who develop pericarditis, which resolves within 7 to 10 days, while others develop transient alopecia during convalescence (57,60).

### Laboratory diagnosis of Lassa fever

The symptoms of Lassa fever can be nonspecific and are similar to other viral

haemorrhagic fevers, such as Ebola, and can include fever, headache, muscle pain, weakness, diarrhoea, vomiting, and abdominal pain (29,60,61). In some cases, the disease can also lead to severe bleeding and death. Accurate diagnosis is thus key to management. There are numerous methods used for the diagnosis of Lassa fever as enumerated below.

#### Lassa fever serological test:

This involves testing a patient's blood for antibodies against LASV. Immunofluorescence was the more widely used test before enzyme-linked immunosorbent assay (ELISA) (34). ELISA is now more commonly used for this purpose, and antibodies detected can be used to confirm a past or current infection (62,63). The ELISA test uses specific viral antigens to detect the presence of antibodies in the blood of patients who have been infected with LASV (64). ELISA test is highly sensitive and specific, which makes it useful for detecting early infections. However, it is important to note that a positive ELISA test does not necessarily indicate an active infection (54,65). This is because antibodies can persist in the blood for months or even years after a person has recovered from an infection, therefore a positive result may indicate a past infection rather than a current one (65).

Another serological test that can be used to diagnose Lassa fever is the indirect fluorescent antibody (IFA) test (66). The IFA fell into disuse largely because of the low specificity in certain populations (67). This test is similar to the ELISA test in that it uses a viral antigen to detect the presence of antibodies in a patient's blood. However, instead of using enzymes to detect the binding of antibodies to the antigen, the IFA test uses a fluorescent dye. This makes it more sensitive (although less specific) than the ELISA test and allows for the detection of low levels of antibodies in the blood (65).

A combination of tests and clinical presentation is needed for the final diagnosis (68,69). In addition, the timing of testing is important. The ELISA test, for example, is useful for early detection of Lassa fever, but it may not be positive during the first week of symptoms (70). The antibody response of the patient may not have risen enough to be detectable, therefore, it should be combined with other methods such as PCR. Serological tests do not necessarily indicate active infection and should be accompanied by other diagnostic methods and clinical presentation for accurate diagnosis (69).

#### Antigen detection:

This involves testing for the presence of specific viral antigens in the blood or other

body fluids of a patient, which can be used to diagnose Lassa fever (70,71). There are different types of antigen detection methods, but one common type is the rapid diagnostic test (RDT), which is simple and easy to perform at the point of care such as hospitals or clinics, rather than in specialized laboratories (68). One example of RDT for Lassa fever is the Lassa antigen rapid diagnostic test (LASV Ag-RDT), which is a lateral-flow based assay that detects LASV antigens in the blood of suspected patients (68). It is an easy to use, point-of-care diagnostic test that provides results in less than 30 minutes.

The LASV Ag-RDT test strip or cassette contains a sample pad, a test line, and a control line. To use the test, a small sample of the patient's blood is collected and applied to the sample pad. The blood then flows along the strip or cassette, passing over the test line and the control line. The advantage of antigen detection is that it can be done quickly at point-of-care, and it can be used as a screening test in areas where Lassa fever is prevalent. However, antigen detection tests are less sensitive than other diagnostic methods such as PCR and ELISA, and they may not be able to detect the virus in patients who are in the early stages of infection or have low levels of the virus in their blood (69,72). It should be noted that positive antigen detection results should be confirmed by a more specific and sensitive methods such as RT-PCR or virus isolation. Also, it is important to consider the clinical presentation along with the laboratory results for accurate diagnosis. A combination of tests and clinical presentation is needed for the final diagnosis.

#### **Reverse transcriptase polymerase chain reaction (RT-PCR) assay:**

The reverse transcriptase polymerase chain reaction (RT-PCR) is a technique that can be used to diagnose Lassa fever by detecting the genetic materials of LASV in a patient's blood, urine, or other body fluids. The starting material for diagnosing Lassa fever by RT-PCR is the patient's blood, urine, or other body fluid, and isolating the viral RNA using automated or manual genome extraction techniques. Following extraction, the viral RNA is converted into cDNA using reverse transcriptase, following which the cDNA is amplified by PCR (73). Here, specific primers are designed to target regions of the LASV cDNA. When the primers bind to the targeted regions of the cDNA, the enzyme polymerase replicates the cDNA, creating many copies of the targeted LASV regions. The primers used in LASV PCR are designed to specifically bind to specific conserved regions of the LASV genome (74). The forward primer is designed usually to bind to the

region of the viral RNA that codes for the virus nucleoprotein (NP) or polymerase (L) genes while the reverse primer is designed usually to bind the region that codes for the viral glycoprotein (GP) gene (75).

The specificity of the primers ensures that the PCR reaction only amplifies the viral genes of interest and not non-viral or human genes. Different primers targeting different viral genes can be used for specific or broad detection of LASV (6,75). Primers targeting the L-gene would only be specific for LASV, while primers targeting the GP gene could be used to detect other arenaviruses such as Lujo and lymphocytic choriomeningitis virus (76). While primer design is a critical step in the PCR process, and it is crucial to use primers that are specific for LASV to avoid cross-reactivity with other arenaviruses. The design of primers is also influenced by factors such as the genetic variability of the virus and the sensitivity and specificity of the test (77).

The amplified products are analysed by gel electrophoresis or other methods to detect the presence of the LASV. The RT-PCR test is highly specific and sensitive, and it can detect very low levels of the virus in a patient's blood or other body fluids. It is useful not just for diagnosis, but also for monitoring of the disease progression, follow-up of patients and early detection of outbreaks (69,73). RT-PCR is considered the 'gold standard' diagnostic method for Lassa fever, and a positive result is considered confirmatory of an infection (77). However, there are limitations of RT-PCR, such as the need for specialized laboratory settings, reagents and equipment and also the need for specific safety measures.

Several studies have reported successful use of loop-mediated isothermal amplification (LAMP) assay for diagnosis of Lassa fever from various sample types, including blood, urine, and saliva. This technique has been shown to have comparable or higher sensitivity than conventional PCR methods. However, LAMP assay also has some limitations, including the potential for nonspecific amplification and the need for careful primer design to avoid cross-reactivity with closely related viral species. Additionally, LAMP products can be difficult to quantify accurately, which may limit its use in monitoring treatment response (78-80).

Strand displacement assay (SDA) is a sensitive and specific nucleic acid amplification technique that has been used for the diagnosis of Lassa fever (80,81). The assay relies on the use of two target-specific oligonucleotide probes, a capture probe, and a signal probe, both labeled with a fluorophore and a quencher (82). The SDA has several advantages over other nucleic acid amplifica-

tion techniques. It is highly specific, as the hairpin structure ensures that the signal probe only binds to the target sequence (82). Additionally, the isothermal nature of the amplification process eliminates the need for thermocycling equipment, making it easier to use in resource-limited settings. Despite its advantages, the SDA has some limitations, including a lower sensitivity than quantitative RT-PCR and a higher susceptibility to the presence of inhibitors in clinical specimens. However, SDA is a promising diagnostic tool for Lassa fever, and further optimization may improve its diagnostic accuracy and utility in clinical practice.

Quantitative or real-time PCR (qRT-PCR) for the detection and quantification of LASV RNA in clinical samples provides advantages over conventional RT-PCR for the diagnosis of Lassa fever. The qRT-PCR assay provides a quantitative measurement of the amount of viral RNA in the sample, allowing for the determination of viral load and disease severity. This thus allows for earlier diagnosis and can detect disease progression and response to therapy. In general, because of the combined sensitivity and specificity of primers and probes, qRT-PCR is more sensitive and specific than conventional RT-PCR, and can detect low levels of viral RNA in patient samples (77). Unfortunately, the assay requires specialized equipment and trained personnel, which may not be available in all settings. Additionally, qRT-PCR is more expensive than conventional RT-PCR, which may limit its use in resource-limited settings. The qRT-PCR requires careful design and optimization of primers and probes, which may be time-consuming and technically challenging (77).

#### Sequencing for LASV characterization:

Sequencing of LASV is relatively new in the surveillance terrain for Lassa fever. The unbiased sequencing approach was one of the earlier methods used to sequence LASV and it still continues to be used (83). The unbiased sequencing approach was particularly useful when little or no information was available about the genome sequence of LASV because here, there is no bias or preference for specific regions or types of nucleic acids to be sequenced (83). It is an approach that aims to sequence all available DNA or RNA molecules present in a sample, rather than selectively amplifying or sequencing certain regions of the genome. This type of sequencing is particularly useful for identifying unknown or unexpected sequences, such as novel pathogens, and for studying genetic diversity within a population. This method was used to generate a "molecular clock" which showed LASV originated in Nige-

ria 1000 years ago and subsequently spread to other parts of West Africa (83).

Unbiased sequencing methods can be done with whole genome sequencing, metagenomic sequencing and RNA sequencing. Unbiased RNA sequencing (total RNAseq) has been used to characterize the evolution of LASV (83). Unfortunately, RNAseq is prone to contamination from RNA of human source or other viral sources (84). In 2019, the MinION technology (Oxford Nanopore) was used to characterize 19 LASV from patients in the 2019 outbreak with results showing the same lineages as that of the 2018 outbreak (85). Illumina technology is the leading second-generation sequencing technology used for LASV characterization. This method employs a technique called "bridge amplification", which involves amplifying DNA molecules that have adapters ligated to both ends on a solid support and generates high-quality sequence data because of the synthesis of amplified clusters (86). The Illumina unfortunately has <80% of their reference sequences captured at this 100% identity (87).

#### Virus isolation:

Given the challenges with the other diagnostic methods of Lassa fever, viral isolation in cell culture remains the "gold standard" for the diagnosis of Lassa fever (77). This method involves growing the virus in cell culture. The starting sample for viral isolation is patient specimens such as urine or semen, and inoculating it into a cell culture. The most commonly used cell lines for virus isolation are Vero E6 and L929 cells (69,88). These cells are grown in a laboratory and are used to create a monolayer, a single layer of cells, in a tissue culture flask or well. The patient's sample is then added to the monolayer and incubated at 37°C (67). If the LASV is present in the patient's sample, it will infect the cells in the monolayer and begin replicating. After a few days, the virus-infected cells will show typical cytopathic effects such as syncytial (cell to cell fusion) and/or cell death, that are unique to LASV, which can be identified by trained technicians (89).

While virus isolation is considered the "gold standard" for confirming Lassa fever diagnosis, virus isolation is one of the most technically demanding and dangerous methods (76,89). LASV is considered a biosecurity level 4 agent, the highest level of biosecurity, requiring a high level of biosafety measures to handle. Therefore, virus isolation should be carried out only in specialized laboratories that have the necessary facilities and training to safely handle the virus. Virus isolation is not usually carried out as a first-line diagnostic test due to its technical complexity and risk, but usually used as a confirmatory



test for patients who have tested positive for Lassa fever using other methods such as PCR or ELISA.

### Lassa fever vaccines

Currently, there is no licensed vaccine for Lassa fever, and treatment is primarily supportive care. However, there are at least 35 different vaccine candidates in various stages of development (11,90,91). Vaccines that have been shown to be safe and effective in preclinical studies such as the recombinant vesicular stomatitis virus (rVSV) vector-based, virus-like particle (VLP) based, and DNA based vaccines, have also been shown to induce protective immunity in animal models. However, animal models may not translate to humans. Recombinant Lassa vaccines in general aim to stimulate the host immune system to produce protective immunity against the virus. They are generally based on nucleoprotein (NP), glycoprotein (GP), Z protein, L polymerase, and Z matrix proteins of the virus (8,92).

Nucleoprotein-based recombinant Lassa virus vaccines utilize the NP protein of the virus as the main antigenic component (10,93). NP is an abundant protein in the virus and is known to elicit both humoral and cellular immune responses (94). Various recombinant NP-based vaccines have been developed and tested in preclinical studies (92, 95). These vaccines are typically delivered as DNA or protein subunit vaccines, with the NP protein expressed from a bacterial, yeast, or insect cell system. Preclinical studies have shown that NP-based vaccines are able to induce both cellular and humoral immune responses against LASV (95). The cellular immune response is mediated by CD8<sup>+</sup> T cells that recognize NP peptides presented on the surface of infected cells, leading to their elimination. The humoral response involves the production of neutralizing antibodies that recognize the NP protein and prevent its interaction with host cells, thus inhibiting viral replication. However, NP-based vaccines have been found to provide incomplete protection against LASV infection in animal models (95). This is because NP is not the sole antigenic component of LASV, and other viral proteins, such as GP, are also known to elicit strong immune responses. Therefore, current efforts are focused on the development of vaccines that include both NP and GP proteins to provide broad-spectrum protection against LASV.

The GP is the major surface protein of LASV and mediates virus entry into host cells to initiate infection (95,96). Several recombinant Lassa fever vaccines have been developed based on the GP protein (92,95), as protein subunit vaccines, DNA vaccines, viral vector vaccines, and virus-like particle

vaccines. One major approach is use of virus-like particles (VLPs) that present the GP protein on their surface. VLPs are self-assembling structures that mimic the virus but lack the genetic material necessary for replication. They can be produced by expressing the GP protein in a cell line and allowing it to self-assemble into VLPs. The resulting particles can then be purified and used as a vaccine. Another approach is to use DNA vaccines that express the GP protein (96). In this case, the gene encoding the GP protein is inserted into a plasmid vector and delivered to the cells. Once inside the cells, the plasmid is transcribed into RNA and translated into protein, which is then presented to the immune system (95). DNA vaccines have the advantage of being relatively easy to produce and store, but their efficacy in humans has not yet been established. A third approach is to use viral vectors to deliver the GP protein to cells. Viral vectors are modified viruses that can infect cells and express foreign genes, such as the gene encoding the GP protein. The most commonly used viral vectors for Lassa vaccines are adenoviruses and vesicular stomatitis viruses (VSVs) (92,96). Adenoviruses are non-replicating and have been used in several clinical trials for Lassa vaccines. VSVs are also non-replicating but have the advantage of inducing strong cellular immune responses.

The Z protein is another potential target used for LASV vaccine development (92,94). The Z protein is a multifunctional protein that plays a crucial role in the replication cycle of the virus through involvement in assembly and release of viral particles (11), and inhibiting the host immune response. It has been shown that the Z protein can induce a potent immune response and confer protection against LASV infection in animal models. Several studies have investigated the use of Z protein-based vaccines for LASV. One approach has been to use recombinant DNA technology to express the Z protein in a viral vector such as the vaccinia virus or the vesicular stomatitis virus (VSV). One promising specific vaccine candidate is the rVSV vector-based vaccine, which has been shown to be safe and effective in preclinical studies and is currently in phase 1 clinical trials (8,72,91).

The rVSV vector used in this vaccine contains the genetic material of LASV, which is able to induce a protective immune response in animal models and confer protection against LASV infection. Another approach has been to use virus-like particles (VLPs) expressing the Z protein. Studies have shown that VLPs expressing the Z protein can induce a potent immune response and protect against LASV infection in animal models. A third type of vaccine candidates are the DNA based vaccines, which are composed of plasmids con-

taining the genetic material of the virus. These plasmids can be delivered *in vivo* to the patient through different methods, such as electroporation or intramuscular injection (72). When three doses were given intradermally, it was shown to be safe and effective in preclinical studies and currently in ph-

ase 1 clinical trials. DNA/plasmid-based vaccines have been shown to induce a lasting immunity and to cross-protect against other arenaviruses, such as Lujo and lymphocytic choriomeningitis virus (9). Table 2 shows the current vaccine candidates, category and phases of trial.

Table 2: Vaccine types, phase of trial, manufacturer, and category

Vaccine Type	Phase	Manufacturer	Category	Reference
EBS-LASV, a dual attenuated rVSV vectored	Phase 1	Emergent BioSolutions	Viral vector	97
Baculovirus-expressed Lassa virus nucleoprotein vaccine	Phase 2	NewLink Genetics	Protein subunit	98
Baculovirus-expressed Lassa virus nucleoprotein vaccine	Preclinical	CEPI (Coalition for Epidemic Preparedness Innovations)	Protein subunit	99,100
ChAdOx1-vectored Lassa virus vaccine	Phase 1	University of Oxford	Viral vector	101
ML29 L-AttV, rLCMV (IGR/S-S)	Preclinical	The Scripps Research Institute, USA	Live attenuated	102
Viral genome rearrangement for the development of live-attenuated arenavirus vaccines	Preclinical	University of Rochester; The Scripps Research Institute	Live attenuated	103
Single cycle infectious viruses as live attenuated arenavirus vaccines		University of Rochester; The Scripps Research Institute	Live attenuated	103
DNA vaccine expressing Lassa virus nucleoprotein and glycoprotein precursor	Preclinical	University of Texas Medical Branch	DNA vaccine	104
DNA vaccine expressing Lassa virus nucleoprotein and glycoprotein precursor	Preclinical	Thomas Jefferson University	DNA vaccine	105
VSDG/LASVGPC (VSV vector)	Preclinical	International Aids Vaccines Initiative; Public Health Agency of Canada	Viral vector	106
Inactivated Lassa virus vaccine	Phase 2	Themis Bioscience	Inactivated virus	107
Inactivated Lassa virus vaccine	Phase 1	Sinovac Biotech	Inactivated virus	108,109
Influenza-vectored Lassa virus vaccine	Preclinical	University of Geneva	Viral vector	72
Lassa virus replicon particle (VRP) vaccine	Preclinical	University of Texas Medical Branch	RNA vaccine	110
Lassa virus-like particle (VLP) vaccine	Preclinical	GeoVax Labs	VLP vaccine	111
Lassa virus-like particle (VLP) vaccine	Preclinical	Themis Bioscience	VLP vaccine	112
Lassa virus-like particle (VLP) vaccine with codon optimized glycoprotein gene	Preclinical	Thomas Jefferson University	VLP vaccine	113
Alphavirus replicon encoding LASV genes	Preclinical	Medigen, Inc.; University of Louisville	VLP vaccine	114
Measles virus-vectored Lassa virus vaccine	Preclinical	Institute Pasteur & Themis Bioscience	Viral vector	115
Lassa GPCclamp	Preclinical	The University of Queensland; Australian Government – National Health and Medical Research Council (NHMRC)	DNA vaccine	116
Lassa Polyhedrin-encased glycoprotein vaccine	Preclinical	University of Cambridge; Imperial College London	VLP vaccine	117



MVA-vectored Lassa virus vaccine	Preclinical	Oxford University	Viral vector	108
RABV inactivated rabies virus with Lassa Virus coGPC (LASSARAB)	Preclinical	Thomas Jefferson University	Viral vector	8
MV-LASV	Phase 1	Themis Bioscience and CEPI	Viral vector	118
RepliVAX Lassa virus vaccine	Phase 1	SIGA Technologies	Viral vector	119
Self-assembling protein nanoparticles presenting Lassa virus GP epitopes	Preclinical	Medicago	Protein subunit	120
Sendai virus-vectored Lassa virus vaccine	Preclinical	Georgia State University	Viral vector	121
RABV-Lassa virus vaccine candidate GPC	Preclinical	National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH)	Viral vector	8
YF 17D GPC	Preclinical	Texas Biomedical Research Institute; University of Louisville; Leiden University Medical Center	Viral vector	122
ML29 virus – reassortant encodes major immunogenic proteins from LASV and RNA polymerase and Z protein from MOPV	Preclinical	Medigen, Inc.	Viral vector	123
MOPEVAC (Modified Mopeia virus expressing antigens of pathogenic arenaviruses)	Preclinical	Institut Pasteur	Viral vector	124
Live attenuated rLCMV/CD	Preclinical	The Scripps Research Institute	Viral vector	125
GPC441-449 subunit	Preclinical	Emergent BioSolutions, University of Vermont, California and The Scripps Research Institute	DNA vaccine	126
HLA-A02 and 10 HLA-A03-restricted epitopes	Preclinical	The University of Vermont College of Medicine; University of California; Pharmexa-Epimmune	DNA	126
LASV VLP	Preclinical	Tulane University Health Sciences Center; Autoimmune Technologies, LLC	Protein subunit	127

Despite the promise of these vaccine candidates, it should be noted that they are still in early stages of development and it is crucial to perform more studies to evaluate the safety and efficacy of these vaccines in humans. Additionally, some of these vaccines may not prove to be effective for certain population subgroups and may require more than one dose to achieve immunity. In addition, the protective efficacy of Lassa fever vaccines may vary depending on the type of vaccine, the population being vaccinated, and the individual's immune response to the vaccine. Factors such as the genetic variability of the virus, age, and overall health of the person being vaccinated can also affect the efficacy of the vaccine (77,128). It is also worth noting that a single vaccine may not provide enough protection, multiple vaccinations may be needed to reach the desired level of protection, and also the protection could be short-lived and booster shots would be need-

ed. Until these vaccines are fully evaluated and licensed, their protective efficacy remains uncertain.

## Conclusion:

In conclusion, Lassa fever continues to be a significant cause of outbreaks. The evolutionary history of Lassa fever is complex and multifactorial, including host-virus interactions, human population density, and land use changes. Recent molecular epidemiological studies have provided insight into the evolutionary history, origin and dispersal of LASV, revealing a complex evolutionary history with multiple introductions into human populations. The ecological and genetic factors that drive the evolution of LASV are still not fully understood, but it is believed that the virus may have adapted to its human hosts through genetic changes in the viral genome.

Diagnosis of Lassa fever has evolved from basic serological tests to more advanced methods such as qRT-PCR for surveillance and sequencing for more in-depth characterization. Next generation sequencing techniques are increasingly being used to characterize outbreak strains from West Africa. While no vaccines are in clinical use, recombinant vesicular stomatitis virus (rVSV) vector-based, virus-like particle (VLP), and DNA based vaccines have shown to induce protective immunity in animal models and some of these have progressed to phase 2 clinical trials.

The control and prevention of Lassa fever are crucial for the health and well-being of the affected populations. Implementing effective control measures such as rodent control, improved sanitation, and early detection and isolation of infected individuals are crucial for reducing the transmission of LASV. Additionally, ongoing research into the genetic and ecological factors that drive LASV evolution, are essential for reducing the transmission and impact of Lassa fever. Lassa fever is not only a public health problem in West Africa, but also a significant economic burden on the affected communities. Therefore, it is crucial to continue investing in research and control measures to better understand and combat Lassa fever, in order to protect the health and livelihoods of the people in the affected areas.

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NM, BM and KM searched for articles, and jointly wrote the initial draft. AB conceptualised the research, contributed additional information and reviewed the article.

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**Original Article****Open Access****Determinants of COVID-19 vaccine acceptance amongst doctors practising in Cross River State, Nigeria**

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**Abstract:**

**Background:** COVID-19 vaccine is one of the most effective public health intervention approaches for prevention of COVID-19. Despite its well-known efficacy and safety, significant proportion of frontline COVID-19 healthcare workers remain hesitant about accepting the vaccine for whatever reasons. This study aimed to determine acceptance rate and determinants of vaccine refusal among doctors in Cross River State, Nigeria.

**Methodology:** This was a cross-sectional survey of doctors using structured online questionnaire administered via the WhatsApp platform of the medical doctors' association, in order to assess their rate of acceptance of COVID-19 vaccines, and reasons for vaccine refusal. The predictors of vaccine acceptance were analysed by univariate and multivariate logistic regression analyses.

**Results:** Of the 443 medical doctors targeted on the WhatsApp platform, 164 responded to the questionnaire survey, giving a response rate of 37.0% (164/443). The mean age of the respondents is 38 ±6.28 years, 91 (55.5%) are 38 years old and above, 97 (59.1%) are males and 67 (40.9%) are females, giving a male-to-female ratio of 1.4:1. The greater proportion of the respondents are physicians (70/148, 47.3%) and about three-quarter of the participants (127/164, 77.4%) had received COVID-19 vaccine. The proportion of physicians who had received COVID-19 vaccine (57/70, 81.4%) was more than the proportion of general practitioners (31/42, 73.8%) and surgeons (24/35, 68.6%). Low perceived benefit of vaccination was the main reason given for COVID-19 vaccine refusal (45.9%, 17/37). No significant association was found between vaccine refusal and suspected predictors ( $p>0.05$ ).

**Conclusion:** Our study revealed high rate of COVID-19 vaccine acceptance among medical doctors especially among the physicians, with the surgeons showing lowest acceptance rate. A significant proportion would not take vaccine because they perceived it lacks much benefits. To raise vaccine acceptance among doctors, more efforts on vaccine literacy that would target doctors from all sub-specialties especially surgeons and incorporate vaccine benefits should be made.

**Keywords:** COVID-19 vaccine, vaccine acceptance, vaccine hesitance, Calabar, Nigeria

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**Déterminants de l'acceptation du vaccin COVID-19 parmi les médecins exerçant dans l'État de Cross River, au Nigeria**

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## Résumé:

**Contexte:** Le vaccin contre la COVID-19 est l'une des approches d'intervention de santé publique les plus efficaces pour la prévention de la COVID-19. Malgré son efficacité et son innocuité bien connues, une proportion importante de travailleurs de la santé de première ligne contre la COVID-19 hésitent à accepter le vaccin pour quelque raison que ce soit. Cette étude visait à déterminer le taux d'acceptation et les déterminants du refus du vaccin chez les médecins de l'État de Cross River, au Nigeria.

**Méthodologie:** Il s'agissait d'une enquête transversale auprès de médecins utilisant un questionnaire en ligne structuré administré via la plateforme WhatsApp de l'association des médecins, afin d'évaluer leur taux d'acceptation des vaccins COVID-19 et les raisons de leur refus de vaccin. Les prédictors de l'acceptation du vaccin ont été analysés par des analyses de régression logistique univariées et multivariées.

**Résultats:** Sur les 443 médecins ciblés sur la plateforme WhatsApp, 164 ont répondu à l'enquête par questionnaire, soit un taux de réponse de 37,0% (164/443). L'âge moyen des répondants est de 38±6,28 ans, 91 (55,5%) ont 38 ans et plus, 97 (59,1%) sont des hommes et 67 (40,9%) sont des femmes, soit un ratio hommes-femmes de 1,4:1. La plus grande proportion des répondants sont des médecins (70/148, 47,3%) et environ les trois quarts des participants (127/164, 77,4%) ont reçu le vaccin contre la COVID-19. La proportion de médecins ayant reçu le vaccin COVID-19 (57/70, 81,4%) était supérieure à la proportion de médecins généralistes (31/42, 73,8%) et de chirurgiens (24/35, 68,6%). Le faible bénéfice perçu de la vaccination était la principale raison invoquée pour le refus du vaccin COVID-19 (45,9%, 17/37). Aucune association significative n'a été trouvée entre le refus du vaccin et les prédictors suspectés ( $p>0,05$ ).

**Conclusion:** Notre étude a révélé un taux élevé d'acceptation du vaccin COVID-19 parmi les médecins, en particulier parmi les médecins, les chirurgiens affichant le taux d'acceptation le plus bas. Une proportion importante ne prendrait pas le vaccin parce qu'ils estimaient qu'il manque de beaucoup d'avantages. Pour augmenter l'acceptation des vaccins parmi les médecins, davantage d'efforts sur la connaissance des vaccins qui cibleraient les médecins de toutes les sous-spécialités, en particulier les chirurgiens, et intégreraient les avantages des vaccins devraient être faits.

**Mots-clés:** vaccin COVID-19, acceptation du vaccin, hésitation à la vaccination, Calabar, Nigeria

## Introduction:

The world is still grappling with the challenges of the coronavirus disease-2019 (COVID-19), a pandemic of global public health concern (1,2). Globally, as of 18 November 2022, there have been 633,601,048 confirmed cases of the COVID-19, including 6,596,542 deaths and as of 16 November 2022, a total of 12,943,741,540 vaccine doses have been administered (3). As of 21 November, 2022 in Nigeria, a total of 259,640 confirmed cases and 3,155 deaths have been recorded in the 36 States and the Federal Capital Territory (4). There have been reportedly increased poverty and hunger globally occasioned by breakdown in social and economic activities brought about by the COVID-19 pandemic (5).

COVID-19 is an infectious disease that is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Majority of the people infected with the virus will only develop mild to moderate respiratory illness and do usually recover even without any specialised care, however some will develop severe illness that will warrant special treatment (6). There is as yet no known definitive therapy for the treatment of COVID-19, prompting the use of non-pharmacological means for its prevention and control (7). Social distancing, lockdown, use of well fitted face mask, ensuring spaces of stay are well-ventilated, observing of hand and respiratory hygiene are still effective preventive measures against the spread of COVID-19 (6,8,9). However, vaccination still remains the safest and

most efficient means of preventing infectious diseases known to man and it has recorded much successes globally (10).

Currently, there are four main types of COVID-19 vaccines; whole virus, protein sub-unit, nucleic acid, and viral vector-based types. COVID-19 vaccines development is moving at record speed with over 170 different vaccines in trials. They are different from each other but all try to achieve the same result of providing immunity against the virus and stop transmission (11). Other benefits of COVID-19 vaccine includes slowing transmission rate, induction of herd immunity, reduction in risk of COVID-19, including developing severe illness and deaths (12). In as much as COVID-19 vaccines tend to be promising in containing the global pandemic, there are still some concerns surrounding its use. The overall effectiveness of COVID-19 vaccines has been tied to its efficacy, safety, cost-effectiveness and public acceptance (13,14).

Vaccine acceptance has been defined as the extent to which individuals accept, question, or completely refuse vaccination. This determines vaccine uptake rate and ultimately, vaccine distribution success (15). COVID-19 vaccine uptake is an important public health challenge to tackle. There have been fluctuations in COVID-19 vaccine acceptance rates in different studies ranging from 23.6% to 97% (14,16,17,18). The rates among healthcare workers (HCWs) have also fluctuated between 27.7% to 78.1% (19,



20).

COVID-19 vaccine acceptance has also been shown to be influenced by some important demographic factors such as sex, age, education and marital status (14,18,19). Medical doctors are important source of health information to their communities, and their attitudes and perception towards COVID-19 vaccination can positively or negatively affect vaccine acceptance in the community where they work (21,22). Therefore, doctors occupy the position of role models, and even their body languages concerning COVID-19 vaccination have important communication to the patients they treat, family and friends and ultimately extending to the community they practice in.

Doctors amid other HCWs were prioritized during the early days of COVID-19 vaccine paucity among the high-risk-groups and were considered for early vaccination. Currently, there are no data in Cross River State of Nigeria vaccine acceptance rate among doctors and the determinants of their willingness to accept, which can affect their networks. This study was therefore conducted to fill this research gap by determining COVID-19 vaccine acceptance and its determinants among doctors practicing in Cross River State, Nigeria.

## Materials and method:

### Study setting:

The study was conducted in Cross River State, Nigeria, a coastal state named after Cross River. Cross River State is one of the States in the south-south geopolitical zone of Nigeria. The capital city is Calabar. The majority of the medical doctors working in Cross River State reside in Calabar. Other major towns hosting medical doctors and other medical professionals are Akamkpa, Odukpani, Ikon, Obubra, Ogoja, Ugep, Obudu, Akpabuyo, and Obanliku.

Cross River State occupies 23,074 km<sup>2</sup> and shares boundaries with Benue State to the north, Enugu and Abia States to the west, Republic of Cameroon to the east, and to the south, Akwa-Ibom and Atlantic Ocean (23). The State belongs to tropical rainfall belt with humid tropical climate, annual (seasonal) rainfall of about 1300-3000mm and 30°C mean annual temperature. However, Obudu plateau is subtemperate with temperature of 4°-10°C (23).

### Study design:

A cross-sectional online survey was conducted from August 22, 2022 to September 23, 2022 to determine COVID-19 vaccine acceptance and its predictors among medical doctors practicing in Cross River State, Nigeria.

### Study participants and method of sampling:

The convenience sampling method was used to select the study participants from the doctors who were either specialist or non-specialist doctors. The specialists were grouped into; (i) surgeons which comprise of specialist doctors from general surgery, intensive care unit, orthopaedic, urology, ear-nose-throat, and obstetrics and gynaecology; (ii) physicians, comprising of specialist doctors from internal medicine, paediatrics, community medicine, laboratory medicine, and dermatology; and (iii) general practitioners, which consists of both specialists from family medicine plus other non-specialist general practitioners. All the doctors are certificated by the Medical and Dental Council of Nigeria (MDCN) and all belong to one umbrella organization, Nigeria Medical Association (NMA).

### Simple size determination:

The sample size was calculated with an online sample size calculator (25) using a confidence level of 95%, margin of error 5%, population size of 443 (number of doctors on the Cross River State Branch of the Nigerian Medical Association WhatsApp Group at the time of survey), and population proportion of 38.8% based on a similar study (26). A sample size of at least 202 was required for the study, however only 164 doctors responded and submitted their survey.

### Ethical considerations:

The ethical principles of Helsinki Declaration in medical research involving human participants were followed (24). Questionnaires were only self-administered to ensure confidentiality and anonymity, not requiring participants names, emails, or Ip addresses, and only the principal investigator had access to the survey account. The e-consent section followed the introductory section. Participants were asked; "Do you consent to participate in this study?" Answering "No" signified "no consent" and such participant would not proceed to answer further questions on the form while "Yes" signified "consent", which allowed the participants access to proceed to respond to the ensuing study questions.

As the study was based on filling form online with no direct link to the participant, with no plausibility to cause harm or stigma to the participants, and none inclusion of clinical data of the participants, a preliminary evaluation by an Ethical Committee was not applied for.

### Survey instrument and administration:

The questionnaire was designed using the Google forms (Google LLC; Menlo Park, CA,

USA), and consisted of questions to assess the medical doctors' acceptance or refusal of the COVID-19 vaccine as well as COVID-19 vaccine acceptance predictors among practicing doctors in Cross River State. The questionnaire was segmented into; (i) introductory and e-consent, which contained information about the study and its objectives followed by the e-consent for participation; (ii) biodata, which contained information on sociodemographic characteristics of the participants including age, gender and marital status; and (iii) medical sub-specialties, which indicate the different medical sub-specialties (twelve) summarized into three main sub-groups; surgeons, physicians and general practitioners.

The COVID-19 vaccine practice section, has 3-item questions; (i) have you received COVID-19 vaccine?, with the responses of "yes" or "no", which was basically a question used to determine doctors' vaccine acceptance/refusal; (ii) reasons for refusal (not accepting) to receive COVID-19 vaccine, with the participants made to choose the best answer out of the best-of-seven multiple reasons, which included perceived possibility of serious adverse reactions, low perceived benefit of vaccination, low perceived risk of contracting COVID-19, health concerns, lack of information, religious or spiritual reasons, and systemic mistrust; and (iii) have you completed your COVID-19 vaccine dose?, with a "yes" or "no" answer. It took approximately 6-10 minutes to complete the questionnaire.

#### Statistical analysis:

The raw data were entered into Excel sheet and imported into the Statistical Package for Social Sciences (SPSS) software for Windows (version 19.0 SPSS Inc. Chicago, IL. USA) for analysis. Categorical variables were summarized and initially reported as frequencies and percentages, while continuous variables were summarized and reported as mean  $\pm$  standard deviations. Univariate analysis was conducted to evaluate associations between outcome and explanatory variables, while logistic regression was used to predict the determinants of vaccine acc-

eptance, utilizing odds ratio (OR), 95% confidence interval (95% CI), and  $p$ -value. Predicted variable was acceptance of COVID-19 vaccine dichotomized into yes or no. Predictor variables included age, gender, marital status, and sub-specialties. Statistical significance was considered for  $p < 0.05$ .

## Results:

#### Socio-demographic characteristics of the study participants:

Out of 443 medical doctors targeted in the WhatsApp platform in which our questionnaire was posted, 164 responded and submitted the survey, giving a response rate of 37.0% (164/443). The mean age of the participants is  $38 \pm 6.28$  years, 91 (55.5%) are 38 years of age and above, 97 (59.1%) are males and 67 (40.9%) are females, giving a male-to-female ratio of 1.4:1. About three-quarter (74.4%, 122/164) of the participants are married. Concerning the areas of specialization of the participants, majority (47.3%, 70/148) are physicians. Table 1 illustrates the socio-demographic characteristics of the participants.

#### Frequency of acceptance/refusal of COVID-19 vaccine:

One hundred and twenty-seven (77.4%) reported to have received COVID-19 vaccine, while only 37 (22.6%) refused to receive the vaccine (Fig 1). High frequency of doctors aged 38 years and above than below 38 years (56% vs 54%,  $p > 0.05$ ), males than females (81.1% vs 71.6%,  $p > 0.05$ ), married than single (77.7% vs 75.6%,  $p > 0.05$ ) accepted COVID-19 vaccine (Table 2). Furthermore, among the three medical sub-specialties, higher number of physicians than surgeons (81.4% vs 68.6%,  $p > 0.05$ ), and physicians than general practitioners (81.4% vs 73.8%,  $p > 0.05$ ) accepted COVID-19 vaccine.

Fig 2 shows the percentage frequency of acceptance per each sub-specialty. Figs 3-5 illustrate the frequency distribution of acceptance/refusal by age, gender, marital status, and medical sub-specialties.

Table 1. Socio-demographic characteristics of study participants (practising medical doctors) in Cross River State, Nigeria

Socio-demographic characteristics	Frequency	Percentage
<b>Mean age <math>\pm</math> SD (in years)</b>	38.28 $\pm$ 6.28	
<b>Age group (years)</b>		
< 38	73	44.5
$\geq$ 38	91	55.5
<b>Gender</b>		
Female	67	40.9
Male	97	59.1
<b>Marital status</b>		
Single	42	25.6
Married	122	74.4
<b>Medical sub-specialty</b>		
Physicians	70	47.3
General Practitioners	42	28.4
Surgeons	36	24.3

About 46% (17/37) of participants who refused COVID-19 vaccine cited low perceived benefit of vaccination, 19% (7/37) cited perceived possibility of serious adverse reactions, and only 5.4% (2/37) cited religious/spiritual reasons for refusal to accept vaccine. None of the participants cited "lack of information or low

perceived risk of contracting COVID-19" as reasons for not accepting to receive the vaccine (Fig 7). Of the 127 participants who have received COVID-19 vaccine, 110 (89.4%) had completed the required doses of the vaccine while only 13 (10.6%) were yet to complete their required doses (Fig 8).

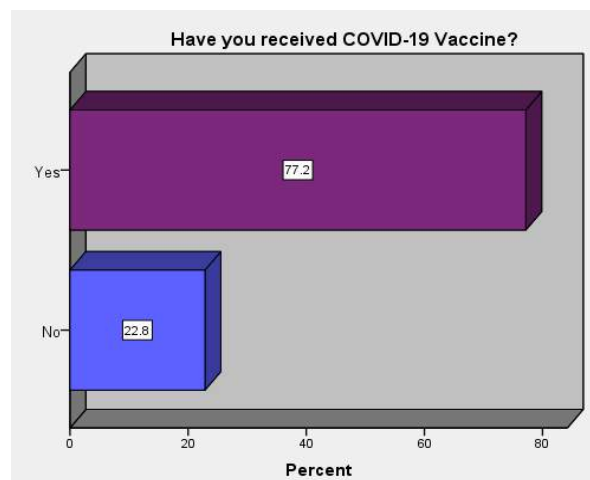


Fig 1: Frequency of COVID-19 vaccine acceptance/refusal among the study participants

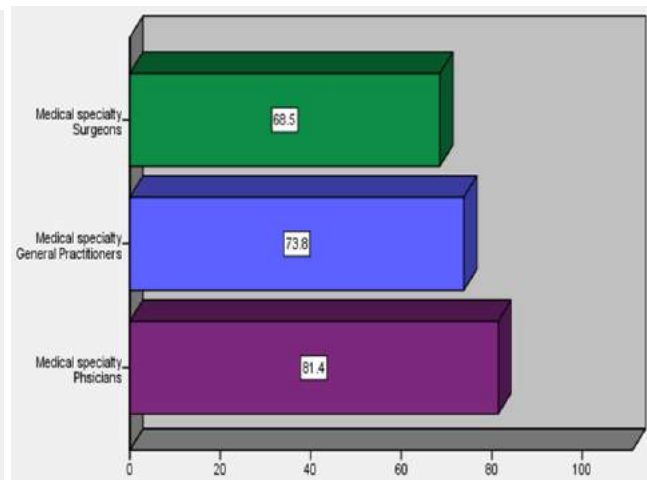


Fig 2: Frequency distribution of doctor participants who received COVID-19 vaccine with respect to sub-specialty

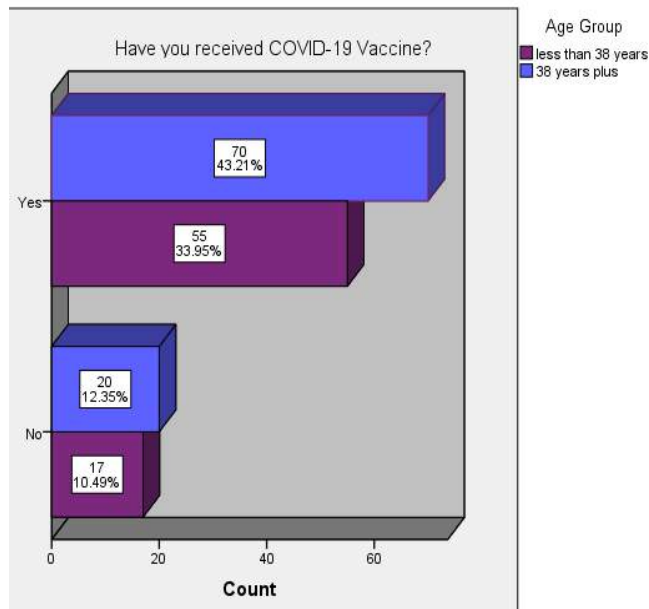


Fig 3: Age group distribution of doctor participants with respect to COVID-19 vaccine acceptance/refusal

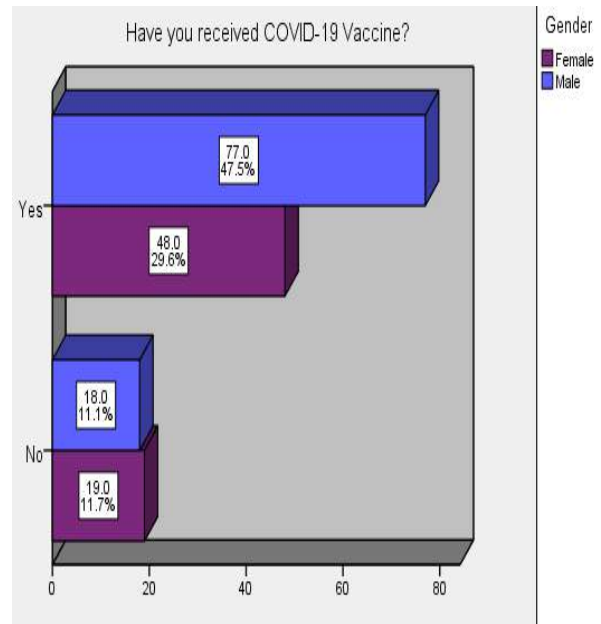


Fig 4: Gender distribution of doctor participants with respect to COVID-19 vaccine acceptance/refusal

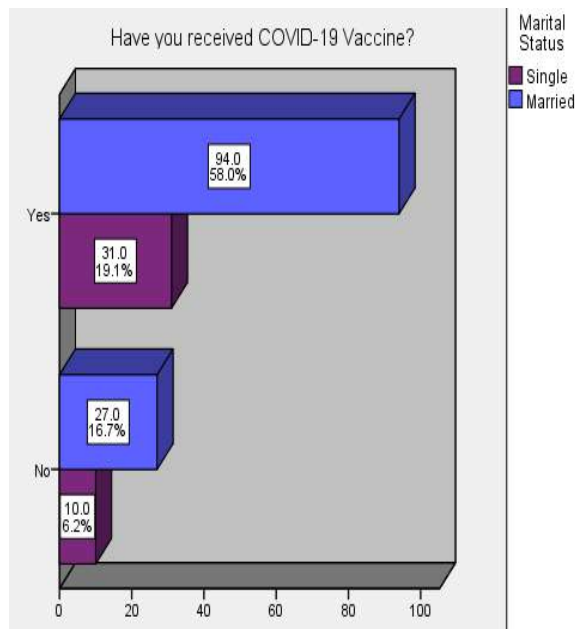


Fig 5: Distribution of doctor participants on COVID-19 vaccine acceptance/refusal by marital status

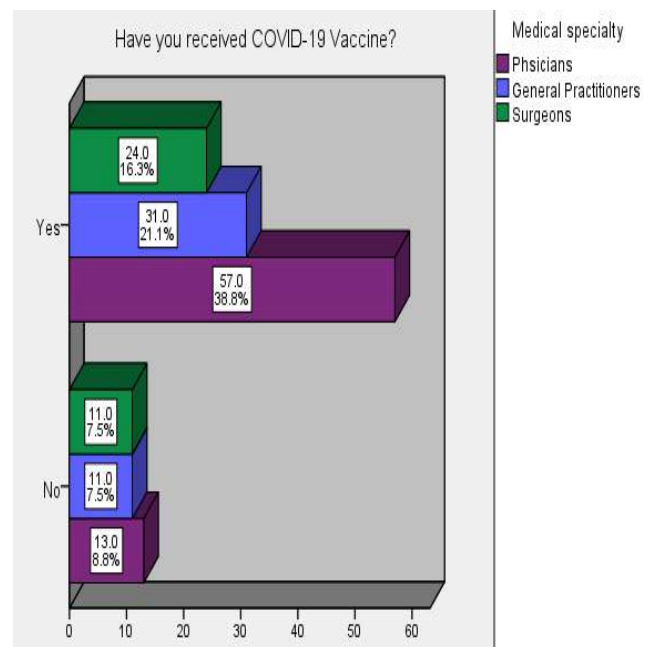


Fig 6: Distribution of doctor participants on COVID-19 vaccine acceptance/refusal by medical sub-specialties

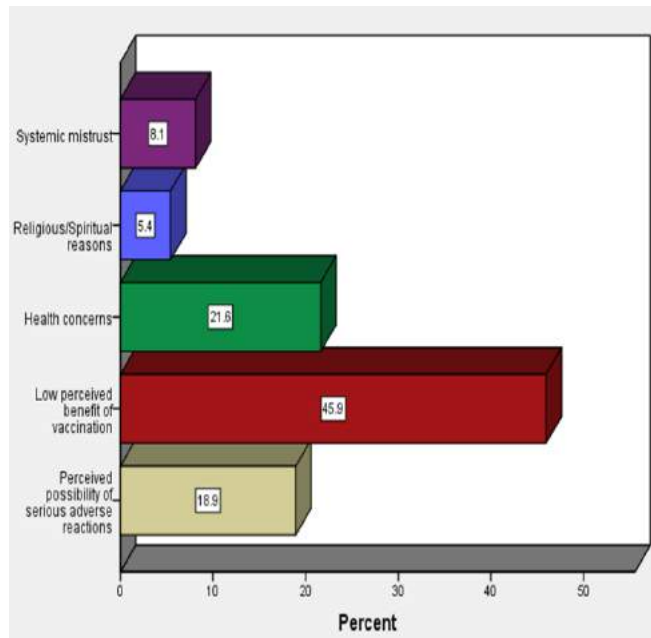


Fig 7: Reasons for COVID-19 vaccine refusals among the doctors

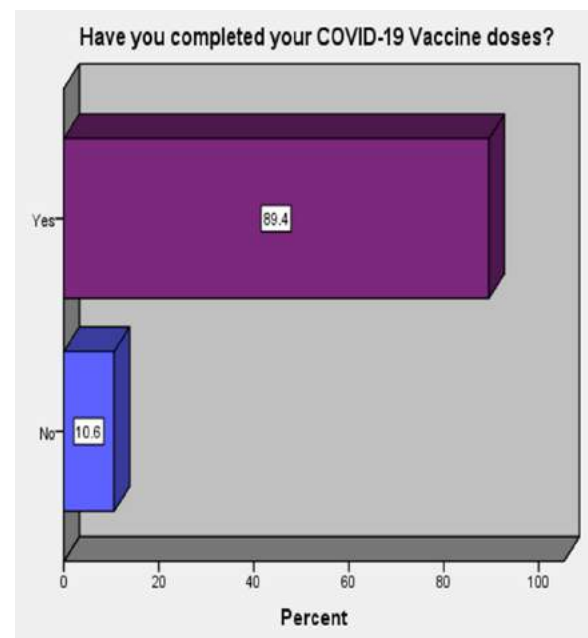


Fig 8: Frequency of COVID-19 vaccine doses completion among the doctors who received COVID-19 vaccine

**Determinants of COVID-19 vaccine acceptance:**

Table 2 illustrates the logistic regression analysis of factors that determine the acceptability of COVID-19 vaccine. Although none of the tested predictors were statistically significant ( $p > 0.05$ ), the odd of receiving COVID-19 vaccines was 1.01 times more among doctors who are 38 years of age and above (OR=1.01, 95% CI=0.44-2.37,  $p=0.97$ ), but the odd of receiving COVID-19 vaccine was less (0.55 times) in females compared to males (OR=0.55, 95%

CI=0.25-1.20,  $p=0.135$ ).

The table also shows that the odd of married doctors receiving COVID vaccine was 1.06 times more than their single (unmarried) counterparts (OR=1.06, 95% CI=0.43-2.63,  $p=0.897$ ) and the odds of physicians receiving the vaccine was 2.04 (OR=2.04, 95% CI=0.79-5.26,  $p=0.141$ ) and 1.37 times (OR=1.37, 95% CI=0.49-3.78,  $p=0.541$ ) more than their surgeon and general practitioner colleagues respectively.

Table 2: Univariate and multivariate logistic regression analyses of COVID-19 vaccine intake and predictors of vaccine acceptance amongst the study participants

Variable	n (%)	Have not received COVID-19 vaccine n (%)	Have received COVID-19 vaccine n (%)	$\chi^2$	p-value	Logistic Regression		
						B	OR (95% CI)	p-value
<b>Age group (years)</b>	162							
<38	(98.8)	17 (23.6)	55 (76.4)	0.00	0.983	0.014	1.01 (0.44-2.37)	0.97
≥ 38		20 (22.2)	70 (77.8)					
<b>Gender</b>	162							
Female	(98.8)	19 (28.4)	48 (71.6)	1.477	0.2243	-0.58	0.55 (0.25-1.20)	0.135
Male		18 (18.9)	77 (81.1)					
<b>Marital status</b>	162							
Single	(98.8)	10 (24.4)	31 (75.6)	0.00342	0.9534	0.06	1.06 (0.43-2.63)	0.897
Married		27 (22.3)	94 (77.7)					
<b>Sub-specialty</b>	147							
Physicians	(89.6)	13 (18.6)	57 (81.4)	2.31	0.315	0.71	2.04 (0.79-5.26)	0.141
General Practitioner		11 (26.2)	31 (73.8)					
Surgeons		11 (31.4)	24 (68.6)					

n=number;  $\chi^2$ =Chi square; OR=Odd ratio; CI=Confidence interval

## Discussion:

Although vaccination is regarded as one of the greatest achievements of science, vaccination hesitancy or outright refusal have persisted (27). Since currently, there is no specific standard treatment for COVID-19, vaccination remains one of the most effective means of preventing the disease (28,29). COVID-19 vaccine acceptance among a large population of susceptible people can positively influence the control of COVID-19 pandemic. As of 22 November 2022, seven vaccines have been approved for use in Nigeria including Moderna (Spikevax), Pfizer/BioNTech (Comirnaty), Gamaleya (Sputnik V), Janssen (Johnson & Johnson) (Jcovden), Oxford/AstraZeneca (Vaxzevria), Serum Institute of India (Covishield), and Sinopharm (Covilo) (30). A successful vaccine campaign in Nigeria might require an investigation about the readiness of medical doctors to take the vaccine as well as the factors that will determine their decisions. This is so because medical doctors are seen as role model everyone look up to in health decision taking. Their unwillingness to accept COVID-19 vaccine will negatively affect the possibility of a successful vaccine campaign. This study therefore aimed to evaluate the acceptance of COVID-19 vaccine among medical doctors as well as the determinants of these decisions to accept or refuse.

According to the findings of this survey, the COVID-19 acceptance rate among doctors in Cross River State, Nigeria is considerably high, about three-quarter of the participants having reportedly received the vaccine. Previous studies have reported that vaccine acceptance is generally higher among HCWs especially medical doctors (15,18,31). This acceptance rate is quite encouraging and shows improvement over the rates obtained in the country previously that ranged between 20.0-58.2% according to data pooled from a systematic review on COVID-19 vaccine acceptance and associated factors in Nigeria (32). The finding has also outperformed the 38.8% rate reported by Nri-Ezedi et al., (26) in a similar study that involved only medical doctors in Nigeria in 2021, although, in their study, the participants were only asked for their intention to accept COVID-19 vaccine, unlike in the current study that involved actual vaccine acceptors. To sum this up, there seems to be significant variations in vaccine acceptance based on the timing of surveys and the type of population involved. The time-trend patterns of vaccine acceptance rates among HCWs in Nigeria had fluctuated from 55.5% in October 2020, 32.5% in January 2021, to 45.6% in March 2021 (32).

Participants knowledge and COVID-19 awareness level among a given population may influence the vaccine acceptance level. Also, availability of the vaccines in-country and the accorded priority to HCWs during the early phase of COVID-19 campaign could be linked to the high acceptance rate (33). Comparable results of our study participants attitude towards COVID-19 vaccination had been reported among HCWs from other countries such as Israel (78.1%) in March 2020, Canada (72.4%) in October 2020 and Thailand (77.0%) (20,34). Strikingly, Colombia (90.7%) in 2021 and Thailand (95.6%) had much higher acceptance rates than in our study (35,36).

Demographic differences such as gender, age, and presence of comorbidity have been observed among vaccine and non-vaccine acceptors (19,20). This current study revealed that greater proportion of doctors 38 years of age and above accepted COVID-19 vaccine than the younger doctors. This finding was expected considering the facts that studies have associated old age with increased risk of morbidity and mortality. This knowledge could have been the driver for the demonstrated higher acceptance among older doctors in this study. There have been reports of lower vaccine acceptance among younger generation of doctors caring for COVID-19 patients (14,19,20,24). The age of medical doctors correlated negatively with their willingness to accept COVID-19 vaccine in a previous study in Nigeria (26). This observation prompted most health facilities in Nigeria then to institute work-from-home concept for elderly doctors to lower the possibility of them contacting a potentially infected patients who came to the health facility (26).

Male doctors were about 2 times more likely than female doctors to accept COVID-19 vaccine in this study. This finding is congruent to an earlier study in Nigeria and in United States of America (26,37). Also, Dror et al., (34) in Israel in 2020 found a similar male preponderance to acceptance of COVID-19 vaccine than female. A likely reason for this observation may stem from the procreation point of view, in that female of child-bearing age, especially expectant mothers may be advised by their HCWs and family members to keep off from drugs other than those prescribed by their doctors. The observation that adverse outcomes from COVID-19 are more with males than females may also be the reason for the male preponderance in COVID vaccine acceptance (38).

Interestingly, we observed in our study that the odd for the physicians to receive COVID-19 vaccine was twice and over one-and-half times more than their colleagues in surgery and

general practice respectively. We could not readily find other studies that have assessed medical sub-specialties association with COVID-19 vaccine acceptance or hesitancy. However, some studies have compared medical doctors and other HCWs. Dzieciolowska et al., (39) observed that physicians were more likely to accept vaccination compared to nurses and other HCWs. According to health belief model, peoples' beliefs about their susceptibility to a disease and their perceptions of benefits of trying to avoid it could have influenced physicians' willingness to accept COVID-19 vaccine (40). Since physicians seem to be more directly involved in the fight against COVID-19, and having witnessed firsthand, the devastating effects of the virus, this could have boosted their willingness to get vaccinated (41). Shehata et al., (42) noted that physicians not taking care of COVID-19 patients relatively had lower acceptance rate than those who indirectly or directly contact COVID-19 patients. Probably, the surgeons and the general practitioners in this study felt they are lower risk for contracting COVID-19 than their physician colleagues. Perhaps, more study will be needed to understand much better factors associated with poor COVID-19 vaccine in other medical sub-specialties when compared with physicians so as to apply tailored health intervention to address it.

In contrast to many other studies that found safety issues as main reasons for vaccine hesitancy/refusal, this study found that low perceived benefit of vaccination followed by safety reasons were the main reasons for vaccine non-acceptance (20,26,43). Based on the theory of diffusion of innovation, there is firm belief that with time, as more positive information emerge about the effectiveness and safety of the vaccines, more vaccine refusers would become vaccine acceptors (44). The use of targeted interventions among HCWs to address safety issues around COVID-19 vaccine should be encouraged. Improving the national safety surveillance and publicly publishing national data to address any concerns the citizens may have with the vaccines other than the use of foreign data should be the way to go (45). There are two sub-groups among those who refuse COVID-19 vaccine; the vaccine hesitant and the firm refusers. Our study would have captured more of the later than the former, considering the prolonged time interval since the first commencement of COVID-19 vaccine in Nigeria.

Our study has some limitations. The first limitation is the study design, in which cause-effect relationship cannot be established. Second, the use of convenience sampling method would produce research findings that cannot be generalized to the population of medical doctors

in Nigeria. Third, being an online survey, only invited doctors who have internet access and are motivated could have participated. Fourth, the sample size used was smaller than the calculated sample size. The calculated sample that would have given a statistical power of at least 80% was 202, however, only 164 doctors responded, therefore, the statistical power to detect differences and associations will be negatively affected.

## Conclusion:

The level of acceptance of COVID-19 vaccine among medical doctors in Cross River State, Nigeria, is considerably high, although there is still room for improvement. There was no significant difference between vaccine accepting and vaccine-refusing doctors with respect to age group, gender, marital status, and medical specialty. Low perceived benefit of the vaccine, health concerns and safety concerns were the main reasons for not accepting vaccine.

To attain optimum COVID-19 vaccine acceptance rate, we recommend increased awareness and health education campaign targeting health institutions to disseminate reliable and accurate information about vaccines. There should also be the need to improve on vaccine safety and efficacy surveillance to counteract the aberrations of fears and mistrust arising from conspiracy theories and social media.

## Contributions of authors:

IAA was involved in the study conceptualization, formal analysis, methodology, supervision, visualization, original manuscript draft writing, review and editing; OGI was involved in methodology, resources provision, supervision, manuscript review and editing; OPA was involved in project administration, original manuscript writing, review and editing; IOI was involved in project administration; UUA was involved in project administration; EDE was involved in project administration, resources provision and visualization; and OAB was involved in resources provision and visualization. All authors approved the final manuscript submitted.

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No conflict of interest is declared

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Copyright AJCEM 2023: <https://dx.doi.org/10.4314/ajcem.v24i2.5>**Original Article****Open Access****Comparative HPV genotype distribution among women with normal and abnormal cervical cytology in Yaoundé, Cameroon**\*<sup>1</sup>Mbimenyuy, C. M., <sup>1</sup>Cho, J. F., <sup>2</sup>Mugyia, A. E., <sup>3</sup>Ikomey, G. M., <sup>4,5</sup>Tebit, D. M., and <sup>6</sup>Nota, D. A.<sup>1</sup>Department of Microbiology and Parasitology, Faculty of Science, P. O. Box 63, Buea,  
University of Buea, South West Region, Cameroon<sup>2</sup>Department of Virology Centre Pasteur Du Cameroun<sup>3</sup>Centre for Studies and Control of Communicable Diseases (CSCCD), FMBS-University of Yaoundé 1,  
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Thohoyandou 0950, South Africa<sup>5</sup>Global Biomed Scientific LLC, P.O. Box 2368, Forest, VA 24551, USA<sup>6</sup>Department of Biological Sciences, University of Bamenda, North West Region, Cameroon\*Correspondence to: [cmbimenyuy@yahoo.com](mailto:cmbimenyuy@yahoo.com)**Abstract:**

**Background:** The epidemiology of human papillomavirus (HPV) infection and the pattern of HPV genotype distribution are parameters needed to assess the risk of cervical cancer. Oncogenic HPV types are well-known pathogen for lower genital tract neoplasias, representing the primary cause of cancer death in Africa and the second in Cameroon. This study was conducted to identify the various genotypes particularly the high-risk HPV types in normal and abnormal cervical cytology from women in Yaoundé, Cameroon.

**Methodology:** This was a hospital-based, analytical cross-sectional study carried out on 226 symptomatic women wherein cervico-vaginal samples were obtained during gynaecological examination for Pap smears, HPV-DNA and genotype detection with linear array HPV strip, conducted from November 2019 to January 2021.

**Results:** From the 226 women whose cervical samples were collected for Pap smears, 71 (31.4%) had abnormal cytology results while 155 (68.6%) had normal results. The overall HPV prevalence in the study population was 34.1% (77/226). The HPV prevalence in women with abnormal Pap smears was 100% (71/71) and are distributed in following descending order; LSIL (21.1%, 15/71), HSIL (21.1%, 15/71), ASC-US (19.7%, 14/71), ICC (19.7%, 14/71) and others (18.4%, 13/71). HPV-DNA was positive in 6 (3.9%) of the 155 women with normal cytology results, 4 (2.6%) of whom were high-risk HPV. There is statistically significant difference in the HPV prevalence between women with abnormal and normal Pap smear results (OR=3289, 95% CI=182.62-59235,  $p<0.0001$ ). The frequently identified oncogenic HPV types were type 16 (31.2%, 24/77), type 45 (14.3%, 11/77) and type 18 (10.4%, 8/77).

**Conclusion:** It is evident from our study that symptomatic women with normal Pap smear can have HR-HPV infection and should therefore be screened for HPV and followed up with periodic Pap smears to detect any abnormal change in cervical cytology results, to prevent cervical cancer development. Women should be encouraged to take up cervical screening, through Pap smears, because it is a non-invasive and cost-effective method for early detection of pre-invasive lesions.

**Keywords:** human papillomavirus; genotypes; low risk; high risk; unclassified risk; cervical cytology

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**Répartition comparative des génotypes du VPH chez les femmes ayant une cytologie cervicale normale et anormale à Yaoundé, Cameroun**\*<sup>1</sup>Mbimenyuy, C. M., <sup>1</sup>Cho, J. F., <sup>2</sup>Mugyia, A. E., <sup>3</sup>Ikomey, G. M., <sup>4,5</sup>Tebit, D. M., et <sup>6</sup>Nota, D. A.

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## Abstrait:

**Contexte:** L'épidémiologie de l'infection par le virus du papillome humain (VPH) et le schéma de distribution des génotypes du VPH sont des paramètres nécessaires pour évaluer le risque de cancer du col de l'utérus. Les types de VPH oncogènes sont des agents pathogènes bien connus des néoplasies des voies génitales inférieures, représentant la première cause de décès par cancer en Afrique et la deuxième au Cameroun. Cette étude a été menée pour identifier les différents génotypes, en particulier les types de VPH à haut risque dans la cytologie cervicale normale et anormale chez les femmes de Yaoundé, au Cameroun.

**Méthodologie:** Il s'agissait d'une étude transversale analytique en milieu hospitalier menée sur 226 femmes symptomatiques dans laquelle des échantillons cervico-vaginaux ont été obtenus lors d'un examen gynécologique pour les frottis Pap, l'ADN du VPH et la détection du génotype avec une bandelette VPH à réseau linéaire, menée à partir de novembre 2019 à janvier 2021.

**Résultats:** Sur les 226 femmes dont les échantillons cervicaux ont été prélevés pour les frottis Pap, 71 (31,4%) avaient des résultats cytologiques anormaux tandis que 155 (68,6%) avaient des résultats normaux. La prévalence globale du VPH dans la population étudiée était de 34,1% (77/226). La prévalence du VPH chez les femmes ayant des frottis de Pap anormaux était de 100 % (71/71) et est répartie dans l'ordre décroissant suivant ; LSIL (21,1 %, 15/71), HSIL (21,1%, 15/71), ASC-US (19,7%, 14/71), ICC (19,7%, 14/71) et autres (18,4%, 13/71). L'ADN du VPH était positif chez 6 (3,9%) des 155 femmes ayant des résultats cytologiques normaux, dont 4 (2,6%) étaient des VPH à haut risque. Il existe une différence statistiquement significative dans la prévalence du VPH entre les femmes ayant des résultats de frottis anormaux et normaux (OR=3289, IC à 95%=182,62-59235,  $p<0,0001$ ). Les types de VPH oncogènes fréquemment identifiés étaient le type 16 (31,2%, 24/77), le type 45 (14,3%, 11/77) et le type 18 (10,4%, 8/77).

**Conclusion:** Il ressort de notre étude que les femmes symptomatiques avec un frottis de Pap normal peuvent avoir une infection HR-HPV et doivent donc être dépistées pour le VPH et suivies de frottis de Pap périodiques pour détecter tout changement anormal dans les résultats de la cytologie cervicale, afin de prévenir le développement du cancer du col de l'utérus. Les femmes devraient être encouragées à entreprendre un dépistage cervical, par le biais de frottis vaginaux, car il s'agit d'une méthode non invasive et rentable pour la détection précoce des lésions pré-invasives.

**Mots clés:** virus du papillome humain; génotypes; faible risque; risque élevé; risque non classé; cytologie cervicale

## Introduction:

Today, it is widely acknowledged that more than 20 human papillomavirus (HPV) genotypes are known to be sexually transmitted pathogen associated with malignancies of the reproductive organ of women, and is one of the main causal factors of cervical cancer (1). Cervical cancer is a gradual and continuous disease that advances from mild to more severe invasive disease caused by persistent infections with oncogenic strains of HPV (2). Among all cancers threatening women's health, cervical cancer remains one of the leading causes of morbidity and mortality worldwide (3), and the leading cause of cancer mortality in Africa (4).

In Cameroon, over 1474 women are diagnosed with cervical cancer each year and approximately 995 women die from the disease annually, rating it as the second most common cause of cancers in Cameroon predominantly affecting women due to poor access to standard health services (5). Cervical cancer is consid-

ered a warning towards women's health and makes it a substantial public health problem.

In developed countries, HPV has been largely controlled by effective screening, prompt diagnosis and vaccination (6). Cervical HPV infections screening is done using Papanicolaou (Pap) smears that detect morphologic changes or by detecting viral DNA presence in cellular scrapes or biopsy tissues (7). The use of Pap smear screening and HPV prophylactic vaccines are effective in preventing cervical cancer (8) and has reduced the incidence of invasive cervical cancer by 70-90% in the developed world (9). In developing countries, merely 5% of women take up cervical screening due to lack of effective and organized cervical cytology screening programs (10).

Cervical cancer remains a major threat to women, especially in the developing countries. Fortunately, it is one of the most preventable cancers worldwide. With emphasis on primary and secondary preventive measures, the disease can be tackled and eradicated in the

ensuing decades with strong government policies since treatment is too expensive and tedious. This will need a collaborative effort of women, healthcare providers, families and the community at large. This study is aimed at determining the prevalence of HPV infections and characterizing the HPV types in cytological grade trends for better follow-up and strategic management.

## Materials and method:

### Study design and setting:

This was an analytical cross-sectional study carried out from November 2019 to January 2021 at the Yaoundé General Hospital and the Yaoundé Gynaeco-Obstetric and Paediatric Hospital, Cameroon.

### Study population and participants:

The study participants consisted of 226 women who came for consultation, and were above 19 years of age, sexually active, with symptoms and signs of cervical pre-cancerous lesions, including women living with HIV (WLWH) who came for their routine gynaecology consultation. Women were excluded if they had had history of hysterectomy, abnormal bleeding, pregnant, had contraindications for Pap smear examination and unwilling to take part in the study.

### Sample size:

A minimum sample size of 226 women was obtained using the statistical formula for proportion;  $N = p(1-p) \times (Z_{\alpha}^2/d^2)$ , where N is the minimum number of participants, p is the prevalence of HPV of 39.0% ( $p=0.39$ ) in Cameroonian women in 2016 (11),  $Z_{\alpha}$  is the 95% confidence interval ( $Z_{\alpha}=1.96$ ), and d is the error rate set at 5% ( $d=0.05$ ).

### Ethical consideration and informed consent:

The study was approved by the Research Ethics Committee of the University of Buea (Reference No. 2017/0491/UB/FS/HOD/MBP). Local ethical clearance was also obtained from the Institutional Ethics Committee for Research of Human Health (CIERSH) at the Yaoundé Gynaeco-Obstetric and Paediatric Hospital (Authorization No. 675/CIERSH/DM/ 2018) and Yaoundé General Hospital (Authorization No. 3616/017/HGY/DG). Informed consent was obtained after explaining to patients either in English, French or Pidgin (a local Lingua Franca) the purpose of the study.

### Data collection:

After signing the consent form, a structure questionnaire was interviewer-administered to collect socio-demographic and other relevant

data from each participant by trained laboratory technicians. Participants without proof of their HIV status were tested for HIV according to the Cameroon national testing guidelines. Those with negative results of more than four months prior to recruitment were re-tested for further confirmation.

### Cervical and vaginal specimen collection:

Each participant underwent a gynaecological evaluation with a non-lubricated, clean and single-use speculum (HybriBio Biochemical Company Limited China) performed by a gynaecologist and two cervical specimens were collected for each participant. One specimen for onco-tic cytological examination was collected using Ayre's spatula which was rolled onto the slide and immediately fixed in 95% ethanol, and allowed to air-dry for subsequent staining following Papanicolaou smear method.

The endo-cervical brush (Cytyc, Mont-rouge, France) was used to collect the second cervical sample and the head of the cytobrush with the specimen was obtained by separation from its handle and then put into commercial aqueous buffered specimen collection and transport media (Roche Diagnostic Systems, Meylan, France), which came in 1.0mL aliquots in capped tubes that accommodated collection devices. The specimens were stored at  $-20^{\circ}\text{C}$  pending DNA extraction and amplification. High vaginal specimens were collected from the participants for bacterial analysis using a sterile cotton swab.

### Cytopathological examination:

The ethanol fixed slides were evaluated in the Cytopathology Laboratory of Yaoundé General Hospital and Yaoundé Gynaeco-Obstetrics and Paediatric Hospital by certified cytopathologists. Detection was done only on smears with adequate number of cells. All smears were reviewed independently by two senior pathologists who were blinded from the clinical or other laboratory findings to avoid bias. In the event of discrepant smear result readings, both pathologists reviewed the slides together and consensus was reached on final diagnosis and grading.

The cytology results were classified according to the 2001 Bethesda classification (12). All cytology results that were identified as abnormal by visual inspection and cytology were referred for colposcopy, and in case of any visible lesions, biopsy was recommended with a definitive diagnosis given to each study participant based on the results of visual inspection, cytology, colposcopy, and biopsy.

### Detection of bacteria in vaginal samples:

Detection of bacterial vaginosis (BV) and

*Trichomonas vaginalis* was carried out at the hospital routine laboratory. High vaginal swabs were used separately for direct wet preparation and then visualized under the light microscope for the presence of clue cells, yeast cells and motile *T. vaginalis*. BV was diagnosed using the Amsel's clinical criteria, which included the presence of any three of the following; homogeneous white vaginal discharge, vaginal pH  $\geq 4.6$ , and release of fishy amine odour (Whiff's test) when 10% (w/v) of KOH was added to a vaginal fluid sample, presence of clue cells on Gram-stained microscopic examination represented about 20% of vaginal epithelial cells (13).

The bacterial types were quantified by calculating the average number of organisms counted in 10 non-consecutive microscopy fields at  $\times 1000$  magnification. Each slide was assigned a score of 0 to 10 using the Nugent score (14) based on the proportion of bacterial flora and morphology. This classification results were evaluated in an overall score in which 0–3 indicates a 'normal flora' lactobacilli-dominated, 7–10 was classified as 'BV-dominated flora', and the score of 4–6 was referred to as an 'intermediate or reduced *Lactobacilli* flora'.

#### Evaluation of cervical inflammation:

The inflamed cervical cells were analyzed by counting the number of neutrophils observed in microscope fields on the Pap slides. Pap-stained smears were evaluated at  $\times 400$  magnification initially to identify cervical mucus. Valid slides were observed at  $\times 1000$  magnification to identify multi-lobed nuclei neutrophils, which were counted in five non-adjacent fields and the average was quantified as 'normal' (0–5 neutrophils/field), 'intermediate' (6–30 neutrophils/field), and 'inflamed' (>30 neutrophils/field).

#### Molecular detection of HPV-DNA:

The cervical specimens for HPV-DNA detection were processed at the Centre for the Study and Control of Communicable Diseases (CSCCD) of the Faculty of Medicine and Biomedical Sciences, Yaoundé 1, Cameroon.

#### DNA extraction:

The DNA of cervico-vaginal samples was extracted using Qiagen DNeasy Kit ((Hamburg, Germany) following the manufacturers instructions. The extracted DNA was quantified using Nanodrop 2000C spectrophotometer (Thermo Scientific, Loughborough, UK). The optical density of 1  $\mu$ L of the DNA sample was measured at 260/280 nm and the DNA concentration was calculated by the Nanodrop 2000 software. The extracted DNA was aliquoted in 50  $\mu$ L aliquots in

sterile Eppendorf tubes and stored at  $-20^{\circ}\text{C}$  until ready for use.

#### Linear Array HPV-DNA amplification:

Polymerase chain reaction assay for amplification of HPV DNA was performed according to the recommendations of the manufacturer. The reaction mixture contained 10  $\mu$ L of sample, 1x PCR buffer, 3.5mM  $\text{MgCl}_2$ , dNTPs mix (200  $\mu$ M of each deoxynucleoside triphosphate), GP5+ primers (5'-TTTGTTACTGTGGTAGATACTAC-3') and 5'-biotinylated GP6+ primers (5'-GA AAAATAAACTGTAAATCA TATTC-3') were used at 1  $\mu$ M each, 1.25U of Taq polymerase (Promega®) and sterile distilled water to a total reaction volume of 50  $\mu$ L. The primers used were synthesized in South Africa by Integrated DNA Technologies (IDT) Inc., and has been described in a previous published study (15).

Amplification was carried out in a thermocycler (MyCycler™ (Biorad) and PCR conditions were as follows; one step of denaturation at  $94^{\circ}\text{C}$  for 4 minutes, followed by 40 amplification cycles each with the following steps; denaturation at  $94^{\circ}\text{C}$  for 1 minute, annealing at  $40^{\circ}\text{C}$  for 2 minutes and elongation at  $72^{\circ}\text{C}$  for 2 minutes. A final elongation step was prolonged by 4 minutes. Amplified PCR products were identified on 2% agarose gel electrophoresis for subsequent genotyping.

#### Linear Array HPV-DNA genotype detection:

HPV-DNA genotype detection on the amplicons was performed using the Linear Array HPV genotyping test (Roche Molecular Systems, Pleasanton, CA, USA). The working hybridization buffer, working ambient wash buffer and working citrate buffers were prepared ahead of time. Briefly, the required number of Linear Array HPV Genotyping strips were removed from the HPV Strip pouch using clean forceps, and placed upward into the appropriate well of the 24-well tray, and 4mL of pre-warmed working hybridization buffer was added. Using a pipette with an aerosol barrier tip, 75  $\mu$ L of denatured amplicon was added into the appropriate wells containing labeled strips, and the 24-well tray with the lid was placed in a  $56^{\circ}\text{C}$  shaking water bath for 30 minutes. The working ambient wash buffer (4 mL) was added to each well containing a strip. The 24-well tray was gently rocked 3 to 4 times to rinse the strips, and the buffer was immediately vacuum aspirated.

Pre-warmed working stringent wash buffer (4mL) was added to each well containing a strip, and the cover was placed on the 24-well tray and then returned to the  $56^{\circ}\text{C}$  shaking water bath for 15 minutes. The tray was removed

from the shaking water bath, and the wash buffer removed from the wells by vacuum aspiration; 4mL of conjugate were added to each well containing a strip, and the tray incubated for an additional 30 minutes. The conjugate was then removed, and the strips were washed by gentle rocking in the presence of the wash buffer. Citrate buffer (4mL) was then added to each well, and the tray was incubated for 5 minutes. After aspiration of this buffer, 4mL of working substrate was added to each well, and the tray was incubated for 5 minutes before aspiration. Deionized water (4mL) was then added to each well, and the strips were removed to a clean surface to dry at room temperature. The HPV genotyping results were interpreted according to the recommendation of the manufacturer using detectable hybridization bands.

#### **Classification of HPV types and cervical lesions:**

HPV genotypes were classified based on the report of Muñoz et al., (16). For the purpose of analysis and oncogenic potential classification analysis used in this present study, eleven HPV genotypes were classified as high risk (types 16, 18, 31, 33, 35, 45, 51, 52, 58, 68, and 82), five as low risk (types 11, 13, 54, 62 and 72) and one as unclassified risk type (type 97). The total HPV and type-specific HPV (total high-risk type, low risk type and unclassified risk type) were scored following the cytology results that were graded as 'normal' and 'abnormal'.

The 'abnormal' cytology results were graded as; atypical squamous cells of undetermined significance (ASCUS); atypical glandular cells (AGC); low-grade squamous intraepithelial lesions (LSILs); atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H); high-grade squamous intraepithelial lesions (HSILs); and intra-epithelial cervical carcinoma (ICC).

#### **Statistical analysis of data:**

Data were entered into a Microsoft Excel spreadsheet and analyzed using SPSS Version 23.0. Categorical explanatory variables were

cross-tabulated against dichotomous outcomes. Continuous variables were summarized using proportions (percentages), means ( $\pm$  standard deviation) and frequency distribution tables. The data were analyzed by Fisher's exact test, and the prevalence rate and descriptive statistics were calculated as required. The distribution and prevalence of HPV type-specific infection among cytological 'normal' and 'abnormal' samples were compared (Odds ratio with 95% confidence interval were computed), and the probability ( $p$ ) type I error was set at 0.05.

## **Results:**

#### **Socio-demographic characteristics of the study participants:**

A total of 226 women, 62 women living with HIV (WLWH) and 164 HIV-negative women, were enrolled in the study over a period of 14 months and screened for cervical abnormality by Pap smears analysis. The age of the participants ranged from 20-79 years with a mean age of  $44 \pm 10.65$  years. Women aged 40-49 years constituted the highest number of the participants (37.2%, 84/226), amongst whom 131 (57.9%) were married and 68 (30.1%) had not attained at least a secondary level education. Most (151, 66.8%) have been exposed to tobacco and 110 (48.7%) have been pregnant 1 to 5 times previously.

The social characteristics showed that majority of the participants (70.4%, 159/226) had sexual exposure on or before 19 years, with exposure in 77.4% (48/62) of the WLWH and 67.7% (111/164) of HIV-negative women, but most (88.1%, 199/226) do not have multiple sexual partners, as 75.8% (47/62) of WLWH and 92.7% (152/164) of HIV-negative women responded not having multiple sexual partners. A high frequency of participants (75.5%, 171/226) had experienced signs and symptoms of HPV infection 1 to 3 times, and more than half (64.2%, 145/226) reported they had never used any form of contraceptives (Table 1).



Table 1: Socio-demographic characteristics of the women participants attending Gynaeco-Obstetrics and Paediatric Hospitals in Yaoundé, Cameroon

Sociodemographic characteristics	HIV negative (%) (n = 164)	WLWH (%) (n = 62)	Total (%) (n = 226)
<b>Age group (years)</b>			
20 – 29	7 (4.3)	4 (6.5)	11 (4.9)
30 – 39	40 (24.4)	16 (25.8)	56 (24.8)
40 – 49	64 (39.0)	20 (32.3)	84 (37.2)
50 – 59	32 (19.5)	15 (24.2)	47 (20.8)
60 – 69	19 (11.6)	5 (8.1)	24 (10.6)
70 – 79	2 (1.2)	2 (3.2)	4 (1.8)
Mean age ± SD (years)	44.93 ± 10.39	46.27 ± 11.34	44 ± 10.65
<b>Marital status</b>			
Married	91 (55.5)	40 (64.5)	131 (57.9)
Single	54 (32.9)	14 (22.6)	68 (30.1)
Divorced	1 (0.6)	1 (1.6)	2 (0.9)
Widow	18 (10.9)	7 (11.3)	25 (11.1)
<b>Educational level</b>			
Primary school or less	48 (29.3)	20 (32.3)	68 (30.1)
Secondary	84 (51.2)	31 (50.0)	115 (50.9)
University	32 (19.5)	11 (17.7)	43 (19.0)
<b>Tobacco use</b>			
No	53 (32.3)	22 (35.5)	75 (33.2)
Yes	111 (67.7)	40 (64.5)	151 (66.8)
<b>Age group of first sexual exposure (years)</b>			
10 – 19	111 (67.7)	48 (77.4)	159 (70.4)
20 – 29	52 (31.7)	14 (22.6)	66 (29.2)
≥ 30	1 (0.6)	0	1 (0.4)
Mean age ± SD (years)	18.28 ± 3.17	17.13 ± 2.84	17.71 ± 3.005
<b>Had multiple sexual partners</b>			
No	152 (92.7)	47 (75.8)	199 (88.1)
Yes	12 (7.3)	15 (24.2)	27 (11.9)
<b>No of times pregnant</b>			
0	12 (7.3)	9 (14.5)	21 (9.3)
1 – 5	84 (51.2)	26 (41.9)	110 (48.7)
6 – 10	57 (34.8)	25 (40.3)	82 (36.3)
> 10	11 (6.7)	2 (3.2)	13 (5.8)
<b>No of times experienced HPV signs and symptoms</b>			
0	8 (4.9)	2 (3.2)	10 (4.4)
1 – 3	127 (77.4)	44 (70.9)	171 (75.7)
> 3	29 (17.7)	16 (25.8)	45 (19.9)
<b>History and types of contraceptives used</b>			
None	110 (67.1)	35 (56.5)	145 (64.2)
IUCD	0	2 (3.2)	2 (0.9)
Natural	2 (1.2)	0	2 (0.9)
Condom	9 (5.5)	6 (9.7)	15 (6.6)
Injection	10 (6.1)	3 (4.8)	13 (5.8)
Norplant	12 (7.3)	3 (4.8)	15 (6.6)
Tablets	21 (12.8)	13 (20.9)	34 (15.0)

WLWH: Women living with HIV; HIV: Human immuno deficiency virus; HPV: Human papilloma virus; IUCD: Intra uterine contraceptive device; SD: Standard deviation

**Vaginal microbial flora of the study participants:**

A total 121 (53.5%) of the 226 women had vaginal infection on microscopic examination with the most frequent infection being bacterial vaginosis caused by *Gardnerella vaginalis* 42 (34.7%) and the least frequent being genital wart 4 (3.3%) (Table 2).

**Cervical inflammatory lesions in the study participants:**

A total 212 (93.8%) of the 226 participants had evidence of cervical inflammation and/or lesion while 14 (6.2%) had no evidence

(Table 3). Fifty-eight (93.5%) of 62 WLWH had cervical inflammation/lesion compared to 154 (93.9%) of 164 HIV-negative women, with no statistically significant difference (OR=1.062, 95% CI=0.3204-3.521,  $p=1.000$ ). Of the 62 WLWH, 21 (33.9%) tested positive for HPV-DNA while 56 (34.1%) of the 164 HIV negative women tested positive for HPV. There was no statistically significant difference in the prevalence of HPV in WLWH and HIV-negative women (OR=0.9878, 95% CI=0.5329-1.831,  $p=1.00$ ). One (25.0%) of 4 WLWH with no cervical inflam-

mation was positive for HPV-DNA while 1 (10%) of 10 HIV-negative women with no cervical inflammation was also positive for HPV-DNA showing no statistically significant difference (OR=3.000, 95% CI=0.1399-64.310,  $p=0.5055$ ).

#### Distribution of HPV genotypes with respect to cervical lesion grades:

Of the 226 cervical samples analyzed, 77 (34.1%) tested positive for any HPV type while 149 (65.9%) tested HPV negative. One hundred and fifty-five (68.6%) samples had 'normal' cytology result, from which 6 (3.9%) tested positive for HPV-DNA, while 71 samples had 'abnormal' cytology result with all (100%) testing positive for HPV-DNA, with a statistically significant difference (OR=3289, 95% CI=182.6-59235,  $p<0.0001$ ). From the 71 samples with 'abnormal' Pap smear results, HR-HPV was detected in 71.8% (51/71), while from 155 samples with 'normal' Pap smear results, HR-HPV was detected in 2.6% (4/155), showing a statistically significant difference (OR=96.263, 95% CI=31.417-294.95,  $p<0.0001$ ) (Table 4).

The most prevalent HPV types in the

study were high risk type 16 (31.2%, 24/77), followed by type 45 (14.3%, 11/77) and type 18 (10.4%, 8/77), although type 16 predominated in all the other cervical cytological abnormalities. In women with observed cytological abnormality, low risk types were frequently identified in women with ASCUS (57.1%, 8/14) and LSIL (53.3%, 8/15), while high risk types were identified in the following ascending order; AGC (66.6%, 4/6), ASC-H (85.7%, 6/7), ICC (92.6%, 13/14) and HSIL (93.3%, 14/15) respectively.

The prevalence of HPV infection in participants with 'abnormal' cervical results is almost the same for ASCUS (19.7%, 14/71), LSIL (21.1%, 15/71), HSIL (21.1%, 15/71) and ICC (19.7%, 14/71). The oncogenic HPV type 16 is the most prevalent types in both normal (33.3%, 2/6) and abnormal Pap smear results (30.9%, 22/71). In general, HPV type 16 predominated in women with ASCUS (21.4%, 3/14), AGC (33.3%, 2/6), LSIL (26.7%, 4/15), ASC-H (42.9%, 3/7) and HSIL (46.7%, 7/15) but for ICC, type 45 was predominant (21.4%, 3/14) (Table 3).

Table 2: Vaginal microbial flora women attending Gynaeco-Obstetrics and Paediatric Hospitals in Yaoundé, Cameroon

Cytology results	Frequency (n)	Percentage (%) at 95% CI
<i>Trichomonas vaginalis</i>	35	28.9
<i>Candida albicans</i>	40	33.1
Genital warts	4	3.3
Bacterial vaginosis ( <i>Gardnerella vaginalis</i> )	42	34.7
<b>Total</b>	<b>121</b>	<b>100</b>

Table 3: Cervical inflammatory lesions of women attending Gynaeco-Obstetrics and Paediatric Hospitals in Yaoundé, Cameroon

Cervical examination	HIV negative (n=164)*			HIV positive/WLWH (n=62)*		
	No HPV -ve (%)	No HPV +ve (%)	Total (%)	No HPV -ve (%)	No HPV +ve (%)	Total (%)
Normal	9 (5.5)	1 (0.6)	10 (6.1)	3 (4.8)	1 (1.6)	4 (6.5)
Inflamed	45 (27.4)	18 (11.0)	63 (38.4)	14 (22.6)	5 (8.1)	19 (30.6)
Lesions	34 (20.7)	20 (12.2)	54 (32.9)	21 (33.9)	3 (4.8)	24 (38.7)
Inflamed + Lesion	20 (12.2)	17 (10.4)	37 (22.6)	3 (4.8)	12 (19.4)	15 (24.2)
<b>Total</b>	<b>108 (65.9)</b>	<b>56 (34.1)</b>	<b>164 (100)</b>	<b>41 (66.1)</b>	<b>21 (33.9)</b>	<b>62 (100)</b>

\*: No significant difference in the prevalence of HPV in HIV positive and HIV negative (WLWH) participants (OR=0.9878, 95% CI=0.5329-1.831,  $p=1.000$ ); WLWH: Women living with HIV; HPV: Human papilloma virus; HIV: Human immunodeficiency virus

Table 4: Prevalence and distribution of HPV genotypes with respect to cervical lesion grade in women attending Gynaeco-Obstetric and Paediatric Hospitals, Yaoundé, Cameroon

HPV type	Total participants (n=226)	Normal cytology (n=155)	Abnormal cytology types (n=71)						
			Total (n=71)	ASC-US (n=14)	AGC (n=6)	LSIL (n=15)	ASC-H (n=7)	HSIL (n=15)	ICC (n=14)
Any HPV	77 (34.1)	6 (3.9)	71 (100)	14 (100)	6 (100)	15 (100)	7 (100)	15 (100)	14 (100)
HR-HPV	55 (24.3)	4 (2.6)	51 (71.8)	6 (42.8)	4 (66.6)	7 (46.7)	6 (85.7)	14 (93.3)	13 (92.6)
LR-HPV	18 (8.0)	2 (1.3)	16 (22.5)	8 (57.1)	1 (16.7)	8 (53.3)	0	0 (0)	0
UR-HPV	4 (1.8)	0	4 (5.6)	0	1 (16.7)	0	1 (12.3)	1 (6.7)	1 (7.1)
<b>High-risk HPV types (n=55)</b>									
16	24 (31.2)	2 (33.3)	22 (31.0)	3 (21.4)	2 (33.3)	4 (26.7)	3 (42.9)	7 (46.7)	2 (14.3)
18	8 (10.2)	0	8 (11.3)	1 (7.1)	1 (16.7)	1 (6.7)	1 (14.3)	3 (20.0)	1 (7.1)
31	1 (1.3)	0	1 (1.4)	0	0	0	0	1 (6.7)	0
35	1 (1.3)	0	1 (1.4)	0	0	0	0	0	1 (7.1)
45	11 (14.3)	1 (16.7)	10 (14.1)	1 (7.1)	1 (16.7)	1 (6.7)	2 (28.6)	3 (20.0)	3 (21.4)
51	1 (1.3)	0	1 (1.4)	0	0	0	0	0	1 (7.1)
52,33,35,58	6 (7.8)	0	6 (8.5)	1 (7.1)	0	1 (6.7)	0	0	3 (21.4)
58	1 (1.3)	1 (16.7)	0	0	0	0	0	0	0
68	1 (1.3)	0	1 (1.4)	0	0	0	0	0	1 (7.1)
82	1 (1.3)	0	1 (1.4)	0	0	0	0	0	1 (7.1)
Total	55 (71.4)	4 (66.6)	51 (71.8)	6 (42.8)	4 (66.6)	7 (46.7)	6 (85.7)	14 (93.3)	13 (92.6)
<b>Low-risk HPV types (n=18)</b>									
11	4 (5.2)	1 (16.7)	3 (4.2)	2 (14.3)	1 (16.7)	1 (6.7)	0	0	0
13	4 (5.2)	0	4 (5.6)	2 (14.3)	0	2 (13.3)	0	0	0
54	6 (7.8)	1 (16.7)	5 (7.0)	2 (14.3)	0	3 (20.0)	0	0	0
62	2 (2.6)	0	2 (2.8)	2 (14.3)	0	0	0	0	0
72	2 (2.6)	0	2 (2.8)	0	0	2 (13.3)	0	0	0
Total	18 (23.4)	2 (33.3)	16 (22.5)	8 (57.1)	1 (16.7)	8 (53.3)	0	0	0
<b>Unclassified risk HPV types (n=4)</b>									
97	4 (5.2)	0	4 (5.6)	0	1 (16.7)	0	1 (14.3)	1 (6.7)	1 (7.1)

ASC-US: Atypical squamous cells of undetermined significance; AGC: Atypical glandular cells; LSILs: Low-grade squamous intraepithelial lesions; ASC-H: Atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; HSILs: High-grade squamous intraepithelial lesions; ICC: Intra-epithelial cervical carcinoma; HPV: Human papilloma virus

## Discussion:

Over the last three decades, the role of HPV infections in the development of cervical cancer has acquired fundamental health significance and HPV testing in addition to Pap smears has become a relevant diagnostic and prognostic tool that has reduced the widespread and incidence of cervical cancer. In view of the proven HPV vaccination efficacy, cervical cytology screening for the detection of pre-invasive cervical lesions, is considered an important public health strategy to assess the impact of HPV, the burden and distribution of the major oncogenic HPV genotypes in different geographical regions of Cameroon.

The result of this study on microbial flora was contrary to the clinical study conducted by Desruisseau (17) on the epidemiology of HPV in fertile women in Cameroon where a significant number of *T. vaginalis*, *Candida albicans* and BV were not detected. Mogtomo et al., (18) in their study on the incidence of cervical diseases associated with HPV in HIV-infected women reported that the main microbial infectious condition was BV as similarly observed in our present study. The high rate of vaginal microbial infections may be attributed to the sexual behavior of the sampled women, poor hygiene, stress, immunosuppression and unhealthy living conditions of the women.

In our study, cases of cervical inflammation and lesions were detected in women who

tested negative for HPV infection. Although this is uncommon, other factors, such as chemical irritants and microbial infections caused by *T. vaginalis*, *C. albicans* or BV may be responsible for cervical lesions (19). Inflammatory process may result in the production of non-specific protective antimicrobial oxidants which may be responsible for the damage to host DNA, leading to neoplasias (20). The association of inflammation with many cancers suggests that inflammation may be considered as a risk factor for carcinogenesis.

From this study conducted in Yaoundé, 226 women were screened for cervical abnormality based on Pap smear analysis out of which 71 (31.4%) had abnormal cervical cytology while 155 (68.6%) had normal cervical cytology results. From the women with normal cytology results, 6 (3.9%) tested positive for HPV, out of which 4 (66.6%) were high-risk types. These results are in accordance with those obtained by Doh et al., (21) in a systemic review of oncogenic HPV prevalence among women with normal cytology results in Cameroon where the researchers observed that the most prevalent HPV types in normal cervical cytology smear were high-risk types. This could be due to the fact that the samples were collected from women referred from other health institutions with complaints of signs and symptoms from suspected HPV infection and cervical lesions. Another plausible reason could be that these tests (PCR based reverse-line strip test and reverse-blot

hybridization to detect HPD-DNA) may vary in their sensitivity and specificity.

Among the 71 participants with abnormal cervical grade results, most reported almost the same trend as follows; LSIL 21.1% (15/71), HSIL 21.1% (15/71), ASCUS 19.7% (14/71), and ICC 19.7% (14/71) but for ASC-H and AGC with almost the score rate of 9.9% (7/71) and 8.5% (6/71) respectively. These results are in accordance with those of Tagne et al., (22) study on the prevalence of precancerous cervical lesions and high-risk HPV types in the same town where this present study was conducted, where the authors reported almost the same trend of prevalence for abnormal cervical cytology results with LSIL (49.6%), HSIL (15.3%), ASC-US (3.7%) and AGC (0.7%) in that order. Desruisseau et al., (17) in a clinical pilot study conducted on the epidemiology of HPV in fertile women in Cameroon reported a contrary result and could not offer any plausible explanation on why participants were not diagnosed with HSIL cervical grade results. In this present study, LSIL precancerous lesions were also common (21.1%, 15/71) possibly due to the fact that it is a mandatory passage for all higher-grade precancerous lesions and cancers. In addition, many high-grade lesions (HSIL, AGC) would regress to LSIL before returning to normal, provided the subject is not exposed to risk factors that would significantly increase the risk of a malignant progression (23).

In our study, all the 71 (100%) participants with 'abnormal' cervical cytology were HPV positive and the most prevalent HPV type among them was the oncogenic with HPV type 16, detected in 22 of 71 (31.0%) samples, followed by HPV type 45 in 10 of 71 (14.1%) samples and HPV type 18 in 8 of 71 (11.3%) women. A high frequency (93.3%, 14/15) of the high-risk or oncogenic HPV types was detected in cervical samples of women with HSIL. Our findings are in concordance with Sarma et al., (24) where HSIL women were mostly infected with high-risk HPV types 16 and 18. A similar trend of results was noted in a study on HPV genotypes in high grade cervical lesions conducted in Cameroon by Sando et al., (25) who reported that majority (89.7%) were the high-risk types in the following descending order; HPV-16 (30.7%), HPV-18 (28.2%) and HPV45 (15.3%).

The distribution of HPV types in our study is also quite similar to a retrospective study conducted by Pirek et al., (26) with the three most common high-risk types noted in descending order as HPV 16 (88%), HPV 45 (32%) and HPV 18 (14.8%). Our study also confirmed the statistically significant association of abnormal cervical cytology and HPV infec-

tions. Compared to women with 'normal' cervical cytology, women with 'abnormal' cervical cytology were 3289 times more likely to have any HPV infections (OR=3289, 95% CI=182.62-59235,  $p<0.0001$ ) and 96 times more likely to have HR-HPV infections (OR=96.263, 95% CI=31.417-294.95,  $p<0.0001$ ). It is well-established that HPV 16 and 18 are the most common HPV types worldwide, accounting for more than 70% of cervical cancer cases, as well as other cancers associated with HPV infections (27,28). The predominance of HPV 16 infections over other HPVs may result partly from the special ability of this HPV type to escape immune surveillance and become more virulent (29). Almost all cervical cancers contain traces of HPV, which infect basal cells within the cervical epithelium or at the squamo-columnar junction causing squamous cell abnormalities and bringing about ASC-US, LGSILs and HGSILs (30,31).

## Conclusion:

Our results agree with the global data that HPV types 16 and 18 are the most common HR-HPV types. In Cameroon, these HR-types including type 45 are the most prevalent in women with normal and abnormal cervical cytology smear, confirming these HPV types as important risk factor for cervical cancer progression. Other high-risk genotypes were also identified in our study, which offers the baseline data for future research with significant implications on the need for raising awareness of women, government authorities and stakeholders about the role of HPV in cervical cancer management and for timely and effective implementation of HPV vaccination campaigns in Cameroon.

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## Contributions of authors:

CMM conceptualized the study, performed the analysis, interpreted the data and wrote the manuscript; DMT drafted, analyzed data and reviewed the manuscript; GMI performed laboratory and data analyses and reviewed the manuscript; DNA contributed to draft manu-

script and analyzed data; AEM analyzed data

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## Conflict of interest:

Authors declare no conflict of interest

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## Original Article

## Open Access

## Awareness of and willingness to use pre-exposure prophylaxis to prevent HIV infection among female sex workers in Anambra State, south-eastern Nigeria

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### Abstract:

**Background:** Pre-exposure prophylaxis (PrEP) is a highly effective preventive measures against HIV infection but its success is strongly based on adherence, which in turn depends on willingness to use. This study is aimed at assessing the level of awareness and willingness to use PrEP to prevent HIV infection among female sex workers (FSWs) in Anambra State, Nigeria, and to identify factors that influence willingness to use PrEP.

**Methodology:** The study was a cross-sectional survey involving 265 brothel-based FSWs recruited through snowballing technique. A structured questionnaire was used to collect relevant information on demographic characteristics, awareness of HIV/AIDS and transmission route, attitudes/behavior related to HIV/AIDS, and awareness of and willingness to use PrEP. Univariate and bivariate analyses with Chi square test (with Odds ratio and 95% confidence interval) was used to determine association of socio-demographic and predictive factors with willingness to use PrEP. Statistical significance was considered when *p* value was less than 0.05.

**Results:** Of 265 FSW respondents, only 81 (31.2%) have heard of PrEP, 10 (3.9%) indicated they have previously used PrEP while 91.0% indicated willingness to use PrEP. Univariate analysis showed that FSWs in Onitsha had a significantly higher odds (OR=28.6, 95% CI=1.718-476.82, *p*=0.0006) while those from Awka had a significantly lower odds (OR=0.184, 95% CI=0.0704-0.1812, *p*=0.0004) of willingness to use PrEP. Also, FSWs with monthly income less than 18,000 Naira had a lower odd of willingness to use PrEP (OR=0.3980, 95% CI=0.1593-0.9945, *p*=0.08). Bivariate analysis shows that FSWs who wish to have more knowledge of HIV/AIDS had higher odd of willingness to use PrEP than those who did not wish to have more knowledge (OR=4.235, 95% CI=1.577-11.374, *p*=0.0066). Similarly, FSWs who are worried of being discriminated against have a lower odd of willingness to use PrEP than those who are not worried of being discriminated against (OR=0.3921, 95% CI=0.1582-0.9718, *p*=0.0439).

**Conclusion:** Our study showed low awareness but high willingness to use PrEP among FSWs in Anambra State, Nigeria. Cost, HIV/AIDS knowledge and fear of discrimination are significant predicting factors of willingness to use HIV PrEP and should be considered when formulating PrEP policy. Adequate enlightenment on PrEP should be emphasized among FSWs.

**Keywords:** HIV; pre-exposure prophylaxis; awareness, willingness to use; female sex workers

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## Sensibilisation et volonté d'utiliser la prophylaxie pré-exposition pour prévenir l'infection à VIH chez les travailleuses du sexe dans l'État d'Anambra, au sud-est du Nigéria

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## Résumé:

**Contexte:** La prophylaxie pré-exposition (PrEP) est une mesure préventive très efficace contre l'infection par le VIH, mais son succès repose fortement sur l'observance, qui à son tour dépend de la volonté de l'utiliser. Cette étude vise à évaluer le niveau de sensibilisation et de volonté d'utiliser la PrEP pour prévenir l'infection par le VIH chez les travailleuses du sexe (FSW) dans l'État d'Anambra, au Nigeria, et à identifier les facteurs qui influencent la volonté d'utiliser la PrEP.

**Méthodologie:** L'étude était une enquête transversale impliquant 265 FSW de bordel recrutés par la technique de la boule de neige. Un questionnaire structuré a été utilisé pour recueillir des informations pertinentes sur les caractéristiques démographiques, la connaissance du VIH/SIDA et de la voie de transmission, les attitudes/comportements liés au VIH/SIDA, et la connaissance et la volonté d'utiliser la PrEP. Des analyses univariées et bivariées avec test du Chi carré (avec rapport de cotes et intervalle de confiance à 95%) ont été utilisées pour déterminer l'association des facteurs sociodémographiques et prédictifs avec la volonté d'utiliser la PrEP. La signification statistique a été considérée lorsque la valeur  $p$  était inférieure à 0,05.

**Résultats:** Sur 265 répondants FSW, seuls 81 (31,2%) ont entendu parler de la PrEP, 10 (3,9%) ont indiqué avoir déjà utilisé la PrEP tandis que 91,0% ont indiqué leur volonté d'utiliser la PrEP. L'analyse univariée a montré que les FSW d'Onitsha avaient une cote significativement plus élevée (OR=28,6, IC à 95%=1,718-476,82,  $p=0,0006$ ) tandis que ceux d'Awka avaient une cote significativement plus faible (OR=0,184, IC à 95%=0,0704-0,1812,  $p=0,0004$ ) de volonté d'utiliser la PrEP. De plus, les FSW dont le revenu mensuel était inférieur à 18000 nairas avaient une probabilité plus faible de vouloir utiliser la PrEP (OR=0,3980, IC à 95%=0,1593-0,9945,  $p=0,08$ ). L'analyse bivariable montre que les FSW qui souhaitent avoir plus de connaissances sur le VIH/SIDA avaient une cote plus élevée de volonté d'utiliser la PrEP que ceux qui ne souhaitaient pas avoir plus de connaissances (OR=4,235, IC 95%=1,577-11,374,  $p=0,0066$ ). De même, les FSW qui craignent d'être discriminés ont une probabilité plus faible de vouloir utiliser la PrEP que ceux qui ne craignent pas d'être discriminés (OR=0,3921, IC 95%=0,1582-0,9718,  $p=0,0439$ ).

**Conclusion:** Notre étude a montré une faible sensibilisation mais une forte volonté d'utiliser la PrEP parmi les FSW de l'État d'Anambra, au Nigeria. Le coût, la connaissance du VIH/SIDA et la peur de la discrimination sont des facteurs prédictifs importants de la volonté d'utiliser la PrEP contre le VIH et doivent être pris en compte lors de la formulation de la politique de PrEP. Une éducation adéquate sur la PrEP devrait être soulignée parmi les FSW.

**Mots clés:** VIH; prophylaxie pré-exposition; sensibilisation; volonté d'utiliser; travailleuses du sexe

## Introduction:

Human immunodeficiency virus (HIV) infection has been a global public health issue for the past thirty years (1). Nigeria has the second largest HIV epidemic in the globe and also has one of the highest rates of new infection in sub-Saharan Africa (2). The West and Central African countries account for 59% of all new HIV infection in 2016 (3), and the Countries population indicates that about 3.1 million persons were living with HIV (PLWH) in 2017 (4). Eighty percent of new infections in Nigeria are on account of unprotected heterosexual intercourse, and the majority of the remaining HIV infections were found to occur in specific affected populations such as sex workers (5). Irrespective of the fact that sex workers, men who sleep with men (MSM) and injection drug users (IDUs) make up only about 3.4% of Nigeria's population, and this group accounts for about 32% of new HIV infections (2). In 2016, estimates of 14.4% of sex workers were living with the disease in Nigeria (6).

Some risk factors contribute to the vulnerability of female sex workers (FSWs) to HIV. This includes the use of injectable substances, large number of clients, unprotected sex with clients, excessive intake of alcohol and having unprotected sex with non-paying clients such as husbands and boyfriends (7, 8). Furthermore, their vulnerability is also increased by some community and societal

factors which include social discrimination, gender inequalities, gender-based violence, and limited access to social opportunities and health services (9). On the other hand, biologically, there is a high prevalence of sexually transmitted infections (STIs) in sex workers (10). These infections have a synergistic relationship with HIV (11). Studies have shown that the prevalence of HIV among FSWs is higher at the rate of 24.5% compared to their male counterparts which is at 18.6% in Nigeria (14). Similarly, FSWs who are brothel-based face greater HIV risks in the country, with a prevalence of 27.4% (15).

The Constitution of Nigerian recognizes sex work as illegal in the country (16). The criminalizing law makes it difficult for individuals to disclose that they are sex workers to healthcare personnel and therefore have less access to quality healthcare. This increases the vulnerability FSWs to HIV and AIDS, the more reason why pre-exposure prophylaxis (PrEP) among FSWs in Nigeria is very important. The brothel-based FSWs are known to have more clients than non-brothel based FSWs, thus, at higher risk of contracting HIV (17). Anambra is one of the States in south-eastern zone of Nigeria, one of the six geo-political regions in the country, and ranks third in HIV prevalence with 1.9% (18).

In Nigeria, PrEP is not available to the general public, however, some sero-discordant couples have been able to access the drug through demonstration projects. As at



1<sup>st</sup> May 2019, the estimated number of current PrEP users are 400-600 persons in Nigeria (19) and the recipients are sero-discordant couples. The new national guidelines for HIV prevention and care states that the future expansion of PrEP will include commercial sex workers, sero-discordant couples, people who inject drugs, and individuals who engage in anal sex on a prolonged and regular basis (21). The study aims at assessing level of awareness and willingness to use of PrEP against HIV among female sex workers in Anambra State, Nigeria and identify factors that influence willingness to use PrEP. It is hoped that the results of the study will be used to inform a nation-wide scale-up of PrEP as part of a comprehensive HIV-prevention package (20).

## Materials and method:

### Study setting, design and participant recruitment:

The study was a cross-sectional survey which was conducted between October and November 2019 in three major cities in Anambra state, Nigeria. These cities include; Awka, Nnewi and Onitsha. The study group involved Brothel-Based FSWs. Inclusion criteria were age 18 years and above, being sexually active, having HIV-negative status, having multiple sexual partners, ability to read and understand the questionnaire and willingness to participate in the study.

The study used questionnaire survey. Three social workers were recruited and given a one-day training. The study adopted the snowballing technique whereby 'lead' participants were first recruited and trust was built, and also with monetary incentives. These lead participants then helped in the recruitment of other participants until the required number of participants was recruited.

### Ethical considerations:

Ethical approval was obtained from the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria (NAUTH/CS/66/VOL.12/107/2018/048). Participation in the survey was strictly voluntary. Assurances of confidentiality were given to the participants. To guarantee anonymity, names of participants were not required. Consent forms were signed by participants.

### Data collection:

Pretested and validated structured questionnaire was used to collect data. Validation was done using face and construct validity. Afterwards, pilot study was conducted for feasibility. In the pilot study, about 10.0 % of the total sample size not included in the study was pre-tested with the questionnaires. Some questions were further modified accordingly after the pre-test to enhance comprehension by the participants

Before the questionnaire was self-administered, FSWs were given brief explanation on PrEP and its use. They were encouraged to ask questions on terms not understood. After the explanation had been done, questionnaire was given to the female sex workers to fill. Majority of the participants filled the questionnaire in 15-30 minutes. Items in the questionnaire were questions related to Demographics, awareness of HIV/AIDS and routes of transmission, and attitudes towards HIV/AIDS. Participants were also asked on the awareness of and use of PrEP, source of their information, attitudes towards the use of PrEP in terms of cost and discrimination, and likely behavioral changes as a result of PrEP use. Willingness to use PrEP was measured with questions such as "how likely would you use PrEP if it were free?", "how likely would you use PrEP if you can only get it from the hospital?" etc. These intentions were reported on a 5-point scale; [definitely (D), probably (P), not sure (N), probably not (PN), and definitely not (DN)].

### Statistical analysis of data:

Data were entered into pre-designed excel worksheets to capture the important information, and questionnaire responses were coded into the worksheet. Data were then entered and analyzed using Statistical Package for Social Sciences (SPSS) version 23 computer software. Descriptive statistics was used to assess factors such as demographic characteristics, awareness of HIV/AIDS transmission and prevention, attitudes/behavior related to HIV/AIDS, awareness of PrEP, and willingness to use PrEP. Univariate and bivariate analyses (with Odds ratios and their 95% CI) were used to determine significant association of predictors with willingness to use PrEP using Chi Square Test of independence with  $p$  value less than 0.05 taken as statistical significance.

## Results:

### Demographic characteristics:

Of the 329 respondents, 64 indicated being HIV positive and were excluded from the study, leaving 265 respondents. Seventy-four (27.9%) of the 265 female sex workers (FSWs) in the survey were from Nnewi, 88 (33.2%) from Onitsha and 103 (38.9%) from Awka metropolitan. The age group 25-29 yrs constitute the largest number of respondents (34.8%) compared to other groups. Most of the respondents were Christians 236 (90.4%), single 184 (69.7%) and belonged to the Igbo ethnicity 179 (68.3%). One hundred & forty-five (62.5%) had secondary school education and only 87 (37.0%) reported that they have

children. The demographic characteristics of the respondents with respect to willingness to use PrEP is shown in Table 1.

#### Attitudes towards HIV/AIDS:

Among the respondents, 68.8% indicated worry when they hear about a family member infected with HIV/AIDS while 67.8% indicated their concerns of being infected themselves. Majority of the respondents (82.9%) have gone for trainings where HIV/AIDS related issues were taught and 89.4% indicated interest in getting more knowledge on HIV/AIDS.

Of the 260 participants who responded to the question of ever being diagnosed of STI, 50% responded in the affirmative, 71.5% correctly believed that STIs are risk factors for HIV/AIDS while 43.1% responded that they use medications they believed will prevent them from contracting STIs. Nineteen percent (49/260) of the FSWs affirmed that taking drugs or concoctions would prevent them from contracting HIV/AIDS. The relationship between attitude towards HIV/AIDS and willingness to use PrEP is shown in Table 2.

Table 1: Univariate analysis of the relationship between demographic characteristics and willingness to use HIV pre-exposure prophylaxis among female sex workers in Anambra State, south-eastern Nigeria

Variables		Willingness to use PrEP		OR (95% CI)	p value
		Yes (%)	No (%)		
Location (n=263)	Nnewi	66 (91.7)	6 (8.3)	1.145 (0.435 - 3.009)	0.973
	Onitsha	88 (100)	0	28.624 (1.718 - 476.82))	0.0006*
	Awka	85 (82.5)	18 (17.5)	0.184 (0.0704 - 0.1812)	0.0004*
Age group (yrs) (n=262)	18-24	81 (90)	9 (10)	0.8599 (0.3606 - 2.050)	0.9082
	25-29	82 (90.1)	9 (9.9)	0.8761 (0.3675 - 2.089)	0.9411
	30-34	44 (93.6)	3 (6.4)	1.588 (0.4533 - 5.561)	0.6530
	35-39	12 (92.3)	1 (7.7)	1.221 (0.1518 - 9.826)	0.8507
	40-44	9 (90)	1 (10)	0.9038 (0.1095 - 7.640)	0.9252
	45-49	3 (75)	1 (25)	0.2936 (0.0293 - 2.940)	0.8155
	50-54	3 (100)	0	0.7282 (0.0365 - 14.525)	0.5801
	55-59	2 (100)	0	0.5180 (0.02415 - 1.108)	0.6521
	60-64	1 (100)	0	0.3095 (0.0123 - 7.81)	0.7504
	65>	1 (100)	0	0.3095 (0.0123 - 7.81)	0.7504
Marital status (n=262)	Single	162 (88.5)	21 (11.5)	0.3045 (0.0881 - 1.053)	0.0812
	Married	26 (96.3)	1 (3.7)	2.821 (0.3655-21.772)	0.4930
	Divorced	19 (95.0)	1 (5.0)	1.995 (0.2551-15.606)	1.000
	Separated	22 (95.7)	1 (4.3)	2.343 (0.3015-18.199)	0.7888
	Widow	9 (100)	0	2.028(0.1144-35.948)	0.7028
	Others	0	0		
Ethnicity (n=260)	Igbo	161 (91.0)	16 (9.0)	0.9268 (0.3659-2.347)	0.8726
	Yoruba	35 (92.1)	3 (7.9)	1.155 (0.3258-4.096)	0.8231
	Hausa	17 (100)	0	3.730 (0.2171-64.089)	0.3751
	Others	24 (85.7)	4 (14.3)	0.5352 (0.1681-1.704)	0.4710
Religion (n=259)	Christianity	210 (89.7)	24 (10.3)	0.1685 (0.00993-2.857)	0.1874
	Islam	19 (100)	0	4.413 (0.2582-75.443)	0.3001
	Others	6 (100)	0	1.388 (0.0758-25.404)	0.9364
Educational status (n=230)	None	11 (91.7)	1 (8.3)	1.050 (0.1285-8.585)	0.9635
	Primary	26 (86.7)	4 (13.3)	0.5652 (0.1754-1.822)	0.5357
	Secondary	133 (91.7)	12 (8.3)	1.152 (0.4508-2.941)	0.9580
	Tertiary	40 (93.0)	3 (7.0)	1.333- (0.3725-4.772)	0.8859
Monthly income (x ₦ 1000) (n=259)	<18	39 (83.0)	8 (17.0)	0.3980 (0.1593-0.9945)+	0.0804
	18 - 29	65 (86.7)	10 (13.3)	0.5353 (0.2264-1.266)	0.2282
	30 - 49	58 (90.6)	6 (9.4)	0.9831 (0.3724-2.595)	0.9725
	50 -90	34 (100)	0	8.390 (0.4982-141.29)	0.0926
	100 - 200	27 (100)	0	6.463 (0.3819-109.38)	0.1604
	>200	12 (100)	0	2.740 (0.1573-47.75)	0.532
Have children (n=240)	Yes	78 (91.8)	7 (8.2)	0.4903 (0.2291-1.050)	0.0960
	No	131 (90.3)	14 (9.7)		

n= number of respondents; \*= statistically significant; + = although not statistically significant, ( $p=0.0804$ ), the odd ratio ( $<1.0$ ) and 95% CI values of ( $<1.0$ ) indicate that the odd of PrEP was lower when monthly income was less than ₦18,000 (i. e. FSWs with low income are less willing to use PrEP, likely due to cost)

Table 2: Bivariate analysis of attitude to HIV/AIDS and PrEP with willingness to use HIV pre-exposure prophylaxis among female sex workers in Anambra State, south-eastern Nigeria

Predictors	Response	Willingness to use PrEP		OR (95% CI)	p value
		Yes (%)	No (%)		
Worried of getting infected with HIV/AIDS (n=259)	Yes	156 (89.1)	19 (10.9)	0.410 (0.1351-1.248)	0.1673
	No	80 (95.2)	4 (4.8)		
Listened to or received training on HIV/AIDS (n=256)	Yes	192 (90.6)	20 (9.4)	0.9600 (0.3112-2.962)	0.9434
	No	40 (90.9)	4 (9.1)		
Wish to get more knowledge about HIV/AIDS (n=261)	Yes	216 (92.7)	17 (7.3)	4.235 (1.577-11.374)	0.0066*
	No	21 (75.0)	7 (25.0)		
Noticed or ever been diagnosed of STI (n=259)	Yes	122 (94.6)	7 (5.4)	2.446 (0.9706-6.165)	0.00840
	No	114 (87.7)	16 (12.3)		
Take drugs to prevent HIV (n=257)	Yes	43 (87.8)	6 (12.2)	0.6379 (0.2375-1.713)	0.5352
	No	191 (91.8)	17 (8.2)		
Heard of PrEP (n=259)	Yes	77 (95.1)	4 (4.9)	2.437 (0.8048-7.378)	0.1647
	No	158 (88.8)	20 (11.2)		
Highest amount willing to spend on PrEP (n=242)	<500	110 (94.8)	6 (86.7)	2.477 (0.9270-6.621)	0.1031
	500 – <1000	26 (86.7)	4 (13.3)	0.5667 (0.1770-1.815)	0.5344
	1000 – <3000	50 (86.2)	8 (13.8)	0.4751 (0.1864-1.211)	0.1876
	3000 – <5000	32 (91.4)	3 (8.6)	1.016 (0.2828-3.649)	0.9807
	>5000	3 (100)	0	0.6888 (0.0344-13.789)	0.5911
Worried of discrimination (n=253)	Yes	94 (87.0)	14 (13.0)	0.3921 (0.1582-0.9718)	0.0439*
	No	137 (94.5)	8 (5.5)		

n=number of respondents; \*=statistically significant

**Awareness of and willingness to use HIV PrEP:**

Among the 260 FSWs who responded to this question, 179 (68.8%) stated that they have never heard of PrEP prior to the study, and only 10 (3.9%) participants indicated that they have previously used PrEP. The willingness to use PrEP was qualitatively measured using the parameters; “definitely” and “probably” as willingness, while “not sure”, “probably not” and “definitely not” as unwillingness (as shown in Table 3a and summarized in Table 3b). Overall, majority of the respondents (91.0%) are willing to use PrEP from the bivariate analysis.

**Attitudes towards PrEP use in terms of cost and discrimination:**

Among 242 participants who responded, high number of FSWs 116 (47.9%) were

willing to spend only less than 500 Naira on PrEP in a month, 30 (12.4%) were willing to spend 500-<1,000 Naira, 58 (24.0%) were willing to spend 1,000-3,000 Naira, 35 (14.5%) were willing to spend 3,000-<5,000 Naira, while only 3 (1.2%) were willing to spend >5,000 Naira on PrEP in a month. On the issue of worry about being discriminated by others for using PrEP, 108 (42.7%) of the respondents indicated their concerns in the affirmative.

**Perceived changes in behavior as a result of PrEP use:**

Among 250 participants who responded to willingness to use PrEP, 87 (34.8%) reported willingness to reduce condom use while 163 (65.2%) of the respondents were willing to be screened for HIV every 3 months as a prerequisite for PrEP use.

Table 3a: Ranking of the predicting factors of willingness to use HIV pre-exposure prophylaxis among female sex workers in Anambra State, south-eastern Nigeria

Predicting factors	D = 5	P = 4	N = 3	PN = 2	DN = 1	Mean	Decision
	n (%)	n (%)	n (%)	n (%)	n (%)		
Willingness to use PrEP if safe and effective in preventing HIV	217 (83.1)	24 (9.2)	13 (5.0)	5 (1.9)	2 (0.8)	4.72	Accept
Willingness to use PrEP if available for free	194 (74.0)	47 (17.9)	15 (5.7)	4 (1.5)	2 (0.8)	4.63	Accept
Willingness to use PrEP if available for free and used by few people	128 (49.0)	73 (28.0)	50 (19.2)	8 (3.1)	2 (0.8)	4.21	Accept
Willingness to use PrEP if available for free and used by fairly large people	161 (62.6)	61 (23.7)	24 (9.3)	8 (3.1)	3 (1.2)	4.44	Accept
Willingness to buy PrEP if readily available but not free	54 (20.8)	103 (39.8)	74 (28.6)	7 (2.7)	21 (8.1)	3.63	Accept
Willingness to use PrEP if only available in Hospital	62 (23.7)	122 (46.6)	70 (26.7)	4 (1.5)	4 (1.5)	3.89	Accept
Willingness to use PrEP if available in Pharmacy shops	99 (39.6)	48 (19.2)	84 (33.6)	17 (6.8)	2 (0.8)	3.9	Accept
Willingness to use PrEP if one must take it once daily for effectiveness	183 (69.6)	52 (19.8)	17 (6.5)	6 (6.3)	5 (1.9)	4.53	Accept
<b>Total mean</b>						<b>4.24</b>	<b>Accept</b>

n=number of respondents; D = definitely; P = probably; N = not sur; PN = probably not; DN = definitely not

Table 3b: Determining factors of willingness to use HIV pre-exposure prophylaxis among female sex workers in Anambra State, south-eastern Nigeria

Determining factors	Willingness n (%)	Unwillingness n (%)
Safe and effective	241 (92.3)	20 (7.7)
Available for free	241 (91.9)	21 (15.2)
Free and few are using	201 (77.0)	60 (23.1)
Free and many are using	222 (86.3)	35 (13.6)
Not free	157 (60.6)	102 (39.4)
Available only in hospitals	184 (70.3)	78 (29.7)
Available only in pharmacy shops	147 (58.8)	103 (41.2)
If taken once daily	235 (89.4)	28 (14.7)

n=number of respondents

**Predictors of willingness to PrEP use:**

As shown in Table 1, willingness to use PrEP was observed in majority of the respondents. Univariate analysis showed that FSWs from Onitsha were more willing to use PrEP (100%, OR = 28.624, 95% CI = 1.718–476.82,  $p = 0.0006$ ) than others from Awka, who were less willing (82.5%, OR = 0.184, 95% CI = 0.0704 – 0.1812,  $p = 0.0004$ ) and Nnewi, who were indifferent (91.7%, OR = 1.145, 95% CI = 0.435 – 3.009,  $p = 0.973$ ). In relation to educational level, there was no significant difference in FSWs to use PrEP ( $p > 0.05$ ). Although not statistically significant ( $p = 0.0804$ ), FSWs who earned less than

18,000 Naira were less willing to use PrEP (OR = 0.3980, 95% CI = 0.1593 – 0.9945), which may indicate that FSWs with low income are less willing to use PrEP, likely due to cost.

Table 2 shows bivariate analysis of attitude to HIV and PrEP of the FSWs as predictors of willingness to use PrEP. There are two statistically significant predictors of willingness to use PrEP in the Table. Firstly, among FSWs who wish to have more knowledge of HIV/AIDS, 92.7% (216/233) were willing to use PrEP with only 6.3% (17/233) not willing, while among those who do not

wish to have more knowledge of HIV/AIDS, only 75% (21/28) were willing to use PrEP with 25% (7/28) not willing to use PrEP (OR = 4.325, 95% CI=1.577-11.374,  $p=0.0066$ ). This indicates a higher odd ( $\sim 4$  times) of willingness to use PrEP among FSWs who wish to have more knowledge of HIV/AIDS than those who did not wish to have more knowledge of HIV/AIDS. Secondly, with respect to discrimination, 13% (14/108) of the FSWs who worried about being discriminated against are not willing to use PrEP compared to 5.5% (8/145) of those not worried about being discriminated against (OR = 0.3930, 95% CI = 0.1593 - 0.9945,  $p=0.0439$ ). This indicates a lower odd ( $\sim 1/3$ ) of willingness to use PrEP among FSWs who worried about being discriminated against.

## Discussion

There are sparse data on awareness and willingness to use PrEP among FSWs especially in Nigeria, and this formed the basis for our study. The findings showed that awareness of PrEP among FSWs in our study was 31.2% (81/260), indicating a low awareness of PrEP as reported in other studies where awareness rates were 10.2% (22), 15.1% (23), and 11.55% (24). A systematic review showed that FSWs had low awareness of PrEP ranging from 12 to 17% (25). However, the findings in these studies contrast that of a study carried out among men who sleep with men (MSM) and FSWs in Nigeria where 95.2% online respondents indicated awareness of PrEP (26). We reported a high rate of willingness to use PrEP (91%), similar to a study that reported 85.9% of FSWs were willing to use PrEP if it was deemed to be effective, safe and provided for free (23). About 69.29% were willing to take PrEP in yet another study (24), which contrasted a more recent finding where only 35.5% were willing to use PrEP (22).

Majority of the participants who indicated having heard of PrEP in our study stated their source of information to be through health training (42.5%) which was as a result of health trainings carried out by community-based Non-Governmental Organizations (NGOs), in partnership with Society for Family Health (SFH) among at-risk groups in their quest for eradication and prevention of the spread of HIV/AIDS. These trainings are organized for the purpose of enlightenment and empowerment of key populations. On PrEP use, only 3.9% of the participants indicated its usage which is consistent with a similar study where only 1.4% of the participants had used PrEP (27), which is the consequence of low awareness of PrEP among FSWs. Although, our result showed that FSWs who have heard of PrEP prior to the study

were more willing to use PrEP (95.1%) than those who have not heard (88.8%), the difference did not reach statistical significance (OR=2.437, 95% CI=0.81-7.38,  $p=0.164$ ).

Willingness to use PrEP varied with predicting factors. Although, rate of willingness to use PrEP was generally high, it was reduced when PrEP was available only at the hospital (70.3%) and less so, only at the pharmacy (58.8%). This is because of the illegality and discrimination associated with sex works which make FSWs in Nigeria less willing to identify with health workers and the general public. A systematic review of studies carried out among at-risk population across several countries on their values and preferences to PrEP showed that dispensing site was an important factor of willingness especially for African countries (25). Moreso, perceived high cost of these drugs at pharmacy, and lower cost at the hospital or health facility (28), could be the reason while FSWs, even though were less willing to use the drug if it were available only at the hospitals, were even more unwilling to get them from the pharmacy. This is also in line with the study where PrEP drugs were preferred to be prescribed and given by healthcare workers in health facilities (29). On willingness to use PrEP if it were to be taken once daily, a high number of participants (89.4%) indicated willingness which is similar to a previous study where sex workers were willing to take PrEP on a daily as it aligns with the nature of their work (28).

From the result of our study, cost seems to be an important predicting factor to PrEP use. Sex workers who earned less than 18,000 Naira per month were less willing to use PrEP. Also, most of the sex workers (47.9%) were willing to spend only less than 500 Naira on PrEP in a month, and as the cost increased, fewer number of sex workers showed interest in using it. This is an important consideration factor to PrEP implementation program in achieving effectiveness of PrEP use. In a similar study carried out in Nigeria, participants suggested that PrEP should be provided for free, one of the reasons for this recommendation was to reduce the incidence of fake drugs which will make the purpose of the medication ineffective, however, if it were to be sold, the drug should be sold not more than 2,000 Naira per prescription (29). Another study carried out among Peruvian at-risk population, cost was the greatest determinant in PrEP acceptability and use (28).

A high number of the FSWs (93.3%) were willing to undergo HIV screening every 3 months as a prerequisite for PrEP use. This screening is presumed to be free as always been done for at-risk groups including FSWs, thus the high willingness to be tested, ano-

ther strong indicator that cost is an important factor in PrEP implementation in the country. HIV commodities (medications and tests) in Nigeria are hugely funded by foreign agencies (30). These commodities and services have been provided for free for some years in the country. The aids are beginning to decline gradually as the country is expected to keep on with provision of the commodities (30). Presently, the availability of these services is also being affected. PrEP drugs, which are part of HIV commodities, may pose additional burden to the already burdened cost and expectations, thus, implementation of PrEP in the country may seem a long way coming.

Some limitations were encountered in this study. Firstly, we adopted a non-probability sampling method, snowballing technique, because the study participants (sex workers) are a difficult group to find, but our method could result in potential selection bias. Secondly, the cross-sectional design used may be associated with potential recall bias as participants are expected to give information about past events. Thirdly, the questionnaire administered by social workers could lead to potential bias as the participants may want to give socially acceptable answers in line with provision of health commodities for their business. Lastly, willingness to use PrEP does not necessarily translate to its actual practice of PrEP.

## Conclusion:

Our study showed low awareness but high willingness to use PrEP among FSWs in Anambra State, Nigeria. Adherence, which is very important in achieving the goal of PrEP use could be affected by some factors such as cost, awareness, and stigmatization among others. Factors which may mitigate PrEP effectiveness include discrimination by others over PrEP use, reduced condom use and most importantly, cost. PrEP and its benefits require good information dissemination among FSWs. The place of adequate knowledge and enlightenment about HIV/AIDS and PrEP cannot be over emphasized. Even though PrEP is very effective in HIV prevention, FSWs must be enlightened on the need to sustain the use of condom as PrEP use is just one of the preventive measures for HIV/AIDS.

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## Contributions of authors:

ECN OIE and ACM were involved in the conceptualization, design and execution of the study; OIE and OCK were involved in the supervision, data analysis and writing up the manuscript

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No conflict of interest is declared

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## Original Article

## Open Access

## Comparative gender analysis of the seroprevalence of varicella zoster virus among HIV-infected individuals receiving care at Offa, north-central Nigeria

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### Abstract:

**Background:** Varicella zoster virus (VZV) infections are common and contribute substantially to morbidity and mortality among HIV-infected patients. This study was conducted to determine the level of exposure, compare the gender distribution pattern and correlate with CD4 count, history of chicken pox and demographics among HIV patients.

**Methodology:** Blood samples were collected from 273 randomly selected HIV-positive patients (93 males and 180 females) receiving care and management at the General Hospital Offa, Kwara State, Nigeria, between September 2019 and March 2020, after obtaining informed consent. Sera were separated from the blood samples and tested for the presence of VZV-specific IgG antibodies using Enzyme Linked Immunosorbent Assay (ELISA).

**Results:** The seroprevalence rate of VZV in the selected HIV patients was 76.9% (210/273), which was similar in both male (83.9%, 78/93) and female (73.3%, 132/180) patients ( $\chi^2=3.265$ ,  $p=0.071$ ). The seroprevalence rates of VZV in both male and female patients were significantly associated with marital status, occupational status, and CD4<sup>+</sup> cell count ( $p<0.05$ ), however, age group was not significantly associated with VZV seroprevalence in both male ( $\chi^2=8.014$ ,  $p=0.155$ ) and female ( $\chi^2=4.689$ ,  $p=0.455$ ) patients. The seroprevalence of VZV in males (32%) who reported history of chicken pox was about twice that of females (17.4%) (OR=2.235, 95% CI=1.162-4.302,  $p=0.023$ ).

**Conclusion:** The level of exposure of HIV-infected individuals to VZV in Offa, Nigeria is high and is similarly distributed in both male and female genders. However, more males with VZV exposure reported history of chicken pox (acute infection) than their female counterparts.

**Keywords:** Seroprevalence; VZV; HIV; gender; Nigeria

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## Analyse comparée selon le sexe de la séroprévalence du virus varicelle-zona chez les personnes infectées par le VIH recevant des soins à Offa, dans le centre-nord du Nigeria

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### Résumé:

**Contexte:** Les infections par le virus varicelle-zona (VZV) sont courantes et contribuent considérablement à la morbidité et à la mortalité chez les patients infectés par le VIH. Cette étude a été menée pour déterminer le

niveau d'exposition, comparer le schéma de répartition entre les sexes et établir une corrélation avec le nombre de CD4, les antécédents de varicelle et les données démographiques chez les patients atteints VIH.

**Méthodologie:** Des échantillons de sang ont été prélevés sur 273 patients séropositifs sélectionnés au hasard (93 hommes et 180 femmes) recevant des soins et une prise en charge à l'hôpital général d'Offa, dans l'État de Kwara, au Nigéria, entre septembre 2019 et mars 2020, après avoir obtenu leur consentement éclairé. Les sérums ont été séparés des échantillons de sang et testés pour la présence d'anticorps IgG spécifiques au VZV à l'aide d'un dosage immuno-enzymatique (ELISA).

**Résultats:** Le taux de séroprévalence du VZV chez les patients VIH sélectionnés était de 76,9% (210/273), ce qui était similaire chez les patients masculins (83,9%, 78/93) et féminins (73,3%, 132/180) ( $\chi^2=3,265$ ,  $p=0,071$ ). Les taux de séroprévalence du VZV chez les hommes et les femmes étaient significativement associés à l'état matrimonial, au statut professionnel et au nombre de cellules CD4<sup>+</sup> ( $p<0,05$ ), cependant, le groupe d'âge n'était pas significativement associé à la séroprévalence du VZV chez les hommes ( $\chi^2=8,014$ ,  $p=0,155$ ) et de sexe féminin ( $\chi^2=4,689$ ,  $p=0,455$ ). La séroprévalence du VZV chez les hommes (32,0%) ayant déclaré des antécédents de varicelle était environ le double de celle des femmes (17,4%) (OR=2,235, IC à 95%=1,162-4,302,  $p=0,023$ ).

**Conclusion:** Le niveau d'exposition des personnes infectées par le VIH au VZV à Offa, au Nigeria, est élevé et est réparti de manière similaire chez les hommes et les femmes. Cependant, plus d'hommes exposés au VZV ont signalé des antécédents de varicelle (infection aiguë) que leurs homologues féminins.

**Mots clés:** Séroprévalence; VZV; VIH; genre; Nigeria

## Introduction:

Herpes group of viruses constitute the major viral opportunistic infections (OIs) among HIV-1 infected individuals. Opportunistic infections occur as a result of immune deficiency and have been recognised as the main reason for hospitalization and substantial morbidity in HIV-infected patients (1). Currently eight human herpes viruses belonging to herpesviridae family are known; herpes simplex virus 1, herpes simplex virus 2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6, human herpesvirus 7, and human herpesvirus 8 (HHV-8). These viruses share a characteristic ability to remain latent within the body over a long period of time.

Varicella zoster virus, a member of the  $\alpha$ -herpesvirinae subfamily is exclusively human pathogen. It is highly infectious virus and is endemic worldwide. Primary infection with VZV leads to acute varicella or "chicken pox". Infection is usually through direct contact with skin lesion or through airborne spread from respiratory droplets (2). Initial infection is followed by establishment of lifelong latency in cranial nerves and dorsal root ganglia from where reactivation can occur years to decades later as herpes zoster or "shingles" which is characterized by painful, localized, vesicular rash in one or adjacent dermatomes.

In HIV-infected individuals, reactivation of VZV causes prolonged and severe manifestation of herpes zoster (3). Although zoster is not viewed as AIDS-defining illness, it can indicate immunodeficiency and tends to occur more often in patients with HIV (4). In fact, herpes zoster occurs at all stages of the HIV infection (5), with reactivation occurring as a result of HIV-induced immunodepression. Other risk factors of herpes zoster include cancers and chronic medical conditions (6). Zoster is said to afflict about 20% of the gene-

ral population during their lifetime, especially the elderly, and about 8% to 11% of patients with AIDS (7). While recurrent episodes of zoster in non-immunocompromised patients are rare, occurring in about 1%-4% of cases (8), recurrence increases in AIDS patients to about 10%-27% of zoster cases (9).

Reactivation in the trigeminal ganglion also results in herpes zoster ophthalmicus (HZO) which may likely be the initial manifestation of HIV infection. The rate of reactivation is higher in immunocompromised patients and older people (10). Incidence of HZO is believed to be six times greater in HIV/AIDS patients than in healthy people and occurs in 5%-15% of HIV-positive patients (11). Studies have increasingly shown gender bias in the incidence of zoster with females more likely to acquire zoster than males. This has been highlighted by review of several epidemiological studies (12). Hitherto attributed to the greater longevity of females than males, research by Fleming et al., (13) however showed greater female incidence in almost all age groups, indicating involvement of factors other than age which are yet to be fully understood.

Although reports have it that over 60% of patients with HZO in Nigeria are HIV-positive (14,15), there is paucity of data showing the true burden of the infection among HIV infected persons in the country. In this study, we determined the seroprevalence of VZV in a cross section of HIV-1 infected patients who are receiving treatment at the General Hospital Offa, Kwara State, north-central Nigeria. We also compared male cum female distribution patterns as well as other associated risk factors.

## Materials and method:

### Study setting, design and population:

This comparative cross-sectional hospital-based study was conducted among HIV-

infected individuals accessing antiretroviral treatment at the General hospital Offa, Kwara State, northcentral Nigeria.

#### **Sample size and method of sampling:**

A total of 273 HIV-infected patients were selected by simple random sampling technique, comprising 93 (34.1%) males and 180 (65.9%) females. The sample size of 273 was determined using the Fischer's formula (16).

#### **Data and sample collection:**

Relevant socio-demographic and clinical information including age, marital status, occupation, CD4<sup>+</sup> cell count, history of chicken pox and history of vaccination against VZV were recorded using well-structured questionnaire forms. These information were obtained both directly from the patients and their clinical record books.

About 5ml of venous blood was obtained from each patient into a sterile bottle and allowed to clot at room temperature. The serum was aspirated into a new Eppendorf tube, appropriately labelled and stored at -20°C until tested.

#### **Serological assay:**

Serum samples were tested for the presence of IgG antibodies using commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Diagnostic Automation, Inc., Calabasas, CA, USA) for the detection of VZV specific IgG antibodies. The tests were performed and interpreted according to the manufacturer's instructions.

#### **Ethical clearance:**

Ethical clearance for the study was obtained from the Ethical Review Committee (ERC) of Kwara State Ministry of Health, Nigeria (MOH/KS/EU/777/174). The ethical standards of the committee and the Helsinki Declaration of 1975 (revised 2000) were strictly adhered to in carrying out the study. Prior to the study, informed consent was obtained from each patient participant and from the parents where the participants were below 18 years of age.

#### **Statistical analysis:**

Data were analysed using Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, USA). Pearson's Chi-square or Fisher's exact test was used where appropriate to test association at 95% confidence interval. P value <0.05 was considered statistical significance.

## **Results:**

#### **Comparative seroprevalence of VZV in male & female patients with respect to other socio-demographic characteristics:**

Over the period of September 2019 to

March 2020, a total of 273 HIV-infected patients were randomly selected for the study with 93 males (mean age 47.4 years) and 180 females (mean age 43.3 years). Of these, 210 (76.9%) were seropositive for VZV infection, 78 (83.9%) of 93 and 132 (73.3%) of 180 male and female patients tested positive to anti-VZV IgG antibodies ( $\chi^2=3.265$ ,  $p=0.071$ ).

The seroprevalence of VZV was not significantly associated with the age group in both male ( $\chi^2=8.014$ ,  $p=0.155$ ) and female ( $\chi^2=4.689$ ,  $p=0.455$ ) patients. However, the highest seroprevalence (100%) was recorded among patients in age group  $\leq 20$  years in both the male and female patients, while the lowest seroprevalence (70.0%) among the males was recorded among patients in both age groups 21-30 and >60 years. Among the females, the lowest seroprevalence (67.7%) was recorded among patients in age group 31-40 years (Table 1).

The VZV seroprevalence was strongly associated with marital status in both male ( $\chi^2=12.46$ ,  $p=0.006$ ) and female ( $\chi^2=12.139$ ,  $p=0.007$ ) patients. While the highest seroprevalence (89.8%) was seen among married male participants, the highest seroprevalence (100%) was recorded among widows and single (unmarried) female patients. The lowest seroprevalence (40%) in the males was recorded among the widowers while in the females, the lowest seroprevalence (50%) was recorded among the divorced women (Table 1).

Analysis also showed strong association of VZV seroprevalence with occupation in both male ( $\chi^2=21.515$ ,  $p=0.0007$ ) and female ( $\chi^2=11.173$ ,  $p=0.025$ ) patients. While artisans, civil servants and participants whose occupation were not determined all had 100% VZV seroprevalence, the lowest seroprevalence was recorded among the students in the male patients. In the female patients, the highest seroprevalence (86.4%) was recorded among patients with undetermined occupation while the lowest seroprevalence (57.1%) was recorded among the artisans (Table 1).

#### **Comparative seroprevalence of VZV in male & female patients with respect to CD4<sup>+</sup> count:**

The seroprevalence of VZV infection was statistically associated with the CD4<sup>+</sup> cell count in both male ( $\chi^2=5.648$ ,  $p=0.017$ ) and female ( $\chi^2=6.448$ ,  $p=0.011$ ) patients. The highest seroprevalence (88.2% for males and 78.7% for females) were recorded among patients with CD4<sup>+</sup> cell count  $\leq 350/\mu\text{L}$  while the lowest seroprevalence rates (64.7% for males and 60.4% for females) were recorded among patients with CD4<sup>+</sup> count  $>350/\mu\text{L}$  (Table 2).

#### **Comparative seroprevalence of VZV in male & female patients with respect to past history of chicken pox:**

Records showed that males seroposi-

tive for VZV were about twice more likely to have suffered from chicken pox than their female counterparts. Out of 78 males who were seropositive for VZV, 25 (32%) had history of chicken pox either as a child or adult,

while out of 132 females who were seropositive for VZV, 23 (17.4%) had previous history of chicken pox ( $\chi^2=5.148$ , OR=2.235, 95% CI=1.162-4.302,  $p=0.023$ ) (Fig 1).

Table 1: Comparative seroprevalence of VZV among male and female HIV patients in relation to demographic variables

Variable	Males		$\chi^2$ (p-value)	Females		$\chi^2$ (p-value)
	Total	Positive (%)		Total	Positive (%)	
<b>Age group (years)</b>						
≤20	8	8 (100)	8.014 (0.155)	3	3 (100)	4.689 (0.455)
21-30	10	7 (70.0)		19	13 (68.4)	
31-40	14	11 (78.6)		65	44 (67.7)	
41-50	37	34 (91.9)		34	28 (82.4)	
51-60	14	11 (78.6)		34	25 (73.5)	
>60	10	7 (70.0)		25	19 (76.0)	
<b>Total</b>	<b>93</b>	<b>78 (83.9)</b>		<b>180</b>	<b>132 (73.3)</b>	
<b>Marital status</b>						
Single	22	19 (86.4)	12.46 (0.006)	6	6 (100)	12.139 (0.007)
Married	59	53 (89.8)		148	112 (75.7)	
Widow/widower	5	2 (40.0)		2	2 (100)	
Divorced	7	4 (57.1)		24	12 (50.0)	
<b>Total</b>	<b>93</b>	<b>78 (83.9)</b>		<b>180</b>	<b>132 (73.3)</b>	
<b>Occupation</b>						
Trading	49	43 (87.8)	21.52 (0.0007)	103	79 (76.7)	11.173 (0.025)
Artisan	6	6 (100)		35	20 (57.1)	
Civil servant	12	12 (100)		20	14 (70.0)	
Student	6	3 (50.0)		-	-	
Professionals	14	8 (57.1)		-	-	
Undetermined	6	6 (100)		22	19 (86.4)	
<b>Total</b>	<b>93</b>	<b>78 (83.9)</b>		<b>180</b>	<b>132 (73.3)</b>	

Table 2: Comparative seroprevalence of VZV infection among male and female HIV-infected patients in relation to CD4<sup>+</sup> count

Variable	Males		$\chi^2$ (p-value)	Females		$\chi^2$ (p-value)
	Total	Positive (%)		Total	Positive (%)	
CD4 <sup>+</sup> count						
≤350	76	67 (88.2)	5.648 (0.017)	127	100 (78.7)	6.448 (0.011)
>350	17	11 (64.7)		53	32 (60.4)	
Total	93	78 (83.9)		180	132 (73.3)	

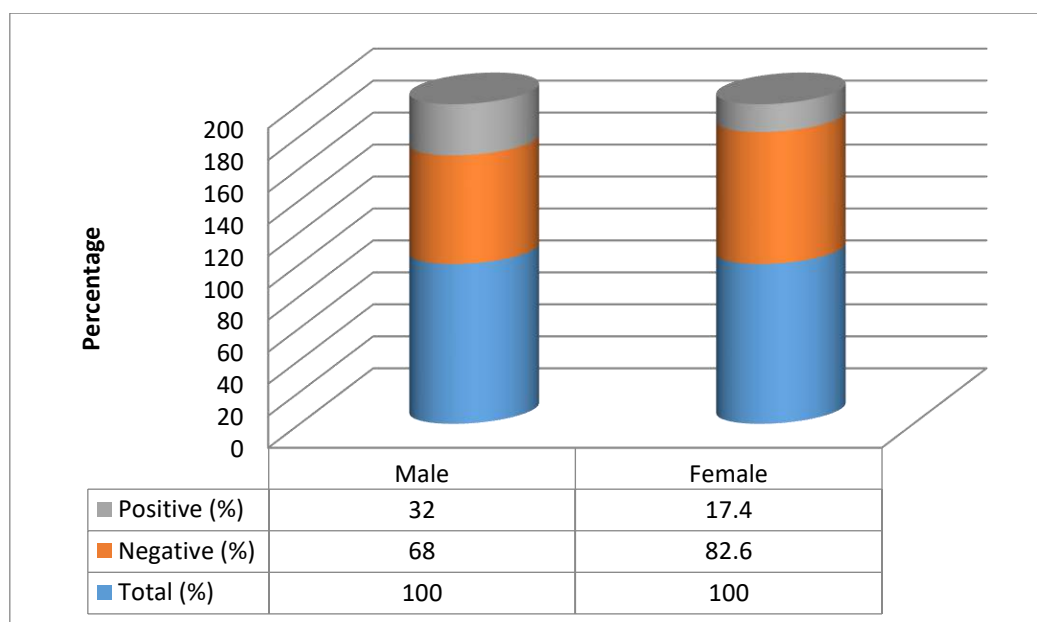


Fig 1: Gender-specific seroprevalence of varicella zoster virus infection with respect to history of chicken pox

## Discussion:

Varicella zoster virus infection is common and contributes significantly to morbidity and mortality especially among HIV-infected individuals. Despite this, it has not been given adequate recognition especially in Nigeria. In this study, prevalence of 83.9% and 73.3% were recorded for male and female patients respectively. As far as it can be ascertained, there has not been any previous epidemiological study on VZV infection among HIV-positive patients in Nigeria thus comparing our result with any local finding is difficult. This reflects how neglected the infection is in Nigeria. Since vaccination against VZV is not yet routine in Nigeria and since none of the study participants reported having received VZV vaccination, the seroprevalence obtained in this study indicates the level of previous natural exposure to the virus.

The 83.9% and 73.3% VZV seroprevalence rates recorded for male and female participants in this study indicates similar rates of exposure of both genders to the infection and is comparable to report from similar study in Italy (17). Although a lower prevalence has been reported in India (18), the rates obtained in this study are appreciably lower compared to the reported 90% -100% seroprevalence in more developed regions of the world such as North America, Western Europe, New Zealand and Japan (19,20). It has earlier been noted that about 50% of young adults in tropical regions have not been exposed to primary VZV infection (20). High ambient temperature and humidity (22) as well as high ultraviolet radiation (23) such as seen in tropical regions like Nigeria have all

been reported to have deleterious effect on the virus. This may partly explain the relatively lower seroprevalence in this study compared to more developed regions of the world.

The age group of the patients was not significantly associated with the seroprevalence of VZV infection, and this was observed for both male and female participants in this study. Other similar studies did not find any association between age and prevalence of VZV antibodies (24,25). High seroprevalence in the younger age group in this study is not out of place since the virus is known to be acquired at very young age. In the temperate countries, most of the infections are known to occur before adolescence (26), while in the tropical regions, primary infection is usually delayed till adolescence (27). Seroprevalence of 66.3% has been previously reported in Kaduna, Northern Nigeria among children  $\leq 15$  years (28).

On the other hand, marital and occupational status were significantly associated with the seroprevalence of VZV infection in both male and female patients in our study. While being students and professionals correlated with low VZV seroprevalence among the male patients, this was not so among the female patients. Few studies have looked into the association between occupation and acquisition of VZV. In one of such studies conducted in a healthcare setting, positive association was observed between VZV sero-negativity and job of the patients (29). Although no immediate reason can be advanced for the observed association of prevalence with marital and occupational status of the patients in our study, it may not be unconnected with increased risk of contact with infected individuals in

certain group of individuals than the others. Some other studies however, did not find any association with these variables (25).

Immunologically, significant association was observed between VZV seroprevalence and CD4<sup>+</sup> count in both the male ( $p=0.017$ ) and female ( $p=0.011$ ) patients in our study. Cell-mediated immunity is believed to play significant role in maintaining the latent state of VZV infection (30), the lower seroprevalence observed among patients with higher CD4<sup>+</sup> count may therefore be as a result of their relatively strong cellular immunity. On the other hand, individuals with more advanced HIV with decreased CD4<sup>+</sup> cell count ( $\leq 350$  cells/ $\mu$ L) had higher seroprevalence of VZV due to their low cellular immunity and therefore stand higher chances of developing zoster in the near future (31).

We also looked into the medical history of the participants and discovered that males are twice as likely to suffer from chicken pox following VZV infection than their female counterparts (ratio 1.8:1). Thirty-two percent of the males seropositive for the virus had history of chicken pox while 17.4% of the females had history of chicken pox indicating that more females had subclinical infection (without chicken pox) than their male counterparts following exposure. Whether ability to suffer from more acute infection (leading to chicken pox) as observed among males in this study translates to reduced chances of suffering from zoster in the future remains to be investigated.

## Conclusion:

The seroprevalence rate of VZV in HIV-infected individuals in Offa, Kwara State, Nigeria, is high and the rate is similar in both male and female patients in our study. However, greater percentage of seropositive males than females reported history of chicken pox.

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## Contributions of authors:

AU conceived, designed and supervised the study. AU and MO were involved in the recruitment of participants, sample and data collection and analysis. AU drafted the manuscript. AU and MO read and approved the final manuscript.

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## Conflict of interest:

Authors declare no conflict of interest

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## Original Article

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## Phylogenetic variants of *Mycoplasma hominis* from pregnant women and women presenting with infertility in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria

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### Abstract:

**Background:** Much controversies have been associated with the pathogenicity of *Mycoplasma hominis* but little has been done to unravel the mystery behind the different views. This study aimed at investigating the genetic variants abounding within *M. hominis* and the distribution of the virulent genes among the variants.**Methodology:** Twenty (20) *M. hominis* isolates from high vaginal swabs of women (11 from pregnant women and 9 from women presenting with infertility) attending the Obstetrics and Gynaecology clinics of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Nigeria, were sequenced using 16S rRNA universal gene target for the purpose of phylogenetic analysis and epidemiological typing. The isolates were also screened for the presence of *M. hominis* variable adherence antigen (*vaa*) and p120 virulent genes using primer constructs from the respective genes in a conventional PCR protocol.**Results:** Of the 20 *M. hominis* vaginal isolates, 4 phylogenetic strains were detected; strain MHS43 constituted 10/20 (50.0%) [2/9 (22.2%) from infertile women and 8/11 (72.7%) from pregnant women]; strain MHBS constituted 3/20 (15%) [3/9 (33.3%) from infertile women and 0/11 (0%) from pregnant women]; strain MHSWP2 constituted 4/20 (20.0%) [3/9 (33.3%) from infertile women and 1/11 (9.1%) from pregnant women]; while strain MHKC87 constituted 3/20 (15%) [1/9 (11.1%) from infertile women and 2/11 (18.2%) from pregnant women]. Each of *vaa* and p120 genes was detected in 14 of 20 isolates, while 6 isolates did not carry the genes. A 2-way ANOVA test showed that none of the genes was significantly associated with a particular strain ( $p=0.8641$ ).**Conclusions:** The different views regarding the pathogenicity of *M. hominis* may be linked to the heterogeneity within the species and lack of homogeneity in the virulent genes as witnessed both in the intra species and intra strain levels.**Keywords:** *Mycoplasma hominis*, pathogenicity, virulence, strains, heterogeneity

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## Variantes phylogénétiques de *Mycoplasma hominis* chez les femmes enceintes et les femmes présentant une infertilité au Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria

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## Résumé:

**Contexte:** De nombreuses controverses ont été associées à la pathogénicité de *Mycoplasma hominis*, mais peu a été fait pour percer le mystère derrière les différents points de vue. Cette étude visait à étudier les variantes génétiques abondantes au sein de *M. hominis* et la distribution des gènes virulents parmi les variantes.

**Méthodologie:** Vingt (20) isolats de *M. hominis* provenant d'écouvillonnages vaginaux élevés de femmes (11 de femmes enceintes et 9 de femmes présentant une infertilité) fréquentant les cliniques d'Obstétrique et de Gynécologie du Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Nigeria, ont été séquencé à l'aide du gène cible universel de l'ARNr 16S à des fins d'analyse phylogénétique et de typage épidémiologique. Les isolats ont également été criblés pour la présence de l'antigène d'adhérence variable de *M. hominis* (*vaa*) et des gènes virulents p120 en utilisant des constructions d'amorces à partir des gènes respectifs dans un protocole de PCR conventionnel.

**Résultats:** Sur les 20 isolats vaginaux de *M. hominis*, 4 souches phylogénétiques ont été détectées; la souche MHS43 constituait 10/20 (50,0%) [2/9 (22,2%) de femmes infertiles et 8/11 (72,7%) de femmes enceintes]; la souche MHBS constituait 3/20 (15,0%) [3/9 (33,3%) de femmes infertiles et 0/11 (0%) de femmes enceintes]; la souche MHSWP2 constituait 4/20 (20,0%) [3/9 (33,3%) de femmes infertiles et 1/11 (9,1%) de femmes enceintes]; tandis que la souche MHKC87 constituait 3/20 (15,0%) [1/9 (11,1%) de femmes infertiles et 2/11 (18,2%) de femmes enceintes]. Chacun des gènes *vaa* et p120 a été détecté dans 14 des 20 isolats, tandis que 6 isolats ne portaient pas les gènes. Un test ANOVA à 2 voies a montré qu'aucun des gènes n'était significativement associé à une souche particulière ( $p=0,8641$ ).

**Conclusions:** Les différents points de vue concernant la pathogénicité de *M. hominis* peuvent être liés à l'hétérogénéité au sein de l'espèce et au manque d'homogénéité des gènes virulents, comme en témoignent les niveaux intra-espèce et intra-souche.

**Mots clés:** *Mycoplasma hominis*, pathogénicité, virulence, souches, hétérogénéité

## Introduction:

*Mycoplasma hominis* is an opportunist human pathogen capable of colonizing epithelial cells and causing urogenital and extragenital pathologies (1,2). At present, it remains unresolved which factors lead to virulence; however, as a first step of invasion, adherence to the epithelium must occur. As the cell wall is absent, this is most likely mediated by structures in the mycoplasma cell membrane. A characteristic feature of the mycoplasmas is the presence of variable surface proteins which may play an important role in the adaptation of the cell-wall less organisms to their host environments (3,4).

The variable adherence antigen (*vaa*) has been shown to be a major adhesin of *M. hominis* and displays a prominent mutational variation in size as well as sequence and antigenic variations (5,6). Variability of *vaa* cytoadhesin has been postulated to be responsible for *Mycoplasma* persistence in various individuals. These play important roles in the pathogenesis of *M. hominis* infections by providing altered structures for escape from immune responses and protein structures that enhance cell and tissue colonization and penetration of the mucosal barrier (7).

Much controversies have been associated with the virulence of *M. hominis* but less has been done to unravel the mystery behind the differences in opinions. *Mycoplasma hominis* have long resisted detailed analysis due to high levels of heterogeneity within the specie (3,8) and although some studies have been conducted in Nigeria to isolate these pathogens, the isolates have not been molecularly typed (1,9,10). The aim of this study is to determine the variability within *M. hominis* isolates from pregnant women and women presenting with infertility using primers targeted at the universal 16SrRNA, *vaa* and p120 genes, in order to detect genetic polymorphisms within the species.

## Materials and method:

### Study design, setting and participants:

The design was quantitative cross-sectional observational study of pregnant women and women with infertility attending the Obstetrics and Gynaecology clinics of the Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, southeast Nigeria from January 2021 to August 2022.

### Ethical approval and consent to participate:

This study was performed in line with

the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi with approval number NAUTH/CS/66/VOL/9/31/2017/026. Informed consent was obtained from all individual participants included in the study.

#### **Isolation *M. hominis* from participants**

*Mycoplasma hominis* were isolated as described in previous studies (9,10), from high vaginal swab samples collected by a consultant gynaecologist using a plastic sterile disposable speculum and a commercial sterile cotton tipped swabs. All samples were collected before initiation of any antimicrobial therapy. Mycoplasma agar base (CM0401), Mycoplasma broth base (CM 0403), and Mycoplasma supplement G (SR 0059) from Oxoid Ltd, UK, were reconstituted and used according to the manufacturer's instruction.

All inoculated broths were incubated aerobically for up to 7 days and examined periodically for color change. Bottles showing color change were sub-cultured onto agar plates (55 mm) prepared with Mycoplasma agar base CM0401, Mycoplasma supplement-G (SR0059) and L-arginine, for further examination. Plates were incubated in moist chambers aerobically, anaerobically and in 10% CO<sub>2</sub> atmosphere. The agar surfaces were examined for characteristic colonial appearances after 3 to 7 days' incubation with a dissecting microscope at 60x magnification using obliquely transmitted light. A characteristic colony with the center of the colony embedded beneath the surface, giving a 'fried-egg' appearance indicated presumptive isolation of *M. hominis*.

Purification of the suspected *M. hominis* isolates was achieved by subculturing a single colony three times to achieve a pure colony before subjecting the isolates to molecular identification. This was carried out by removing a plug of agar containing a colony from the plate and using it to inoculate further plates of medium and finally-sub cultured into Mycoplasma broth medium and stored at -80°C until needed for molecular identification.

#### **Extraction of DNA from *M. hominis* isolates:**

The molecular analysis of the isolates was carried out at Iykenson Medical and Diagnostics Co. Ltd; Road 2 Mofor Estate Unizik Temporary site, Awka, and Inqaba Biotec West Africa Ltd PMB 5320, Oyo Road, Ibadan 200001 Oyo State, Nigeria. Pure isolates of *M. hominis* in the Mycoplasma broth kept frozen at -80°C were recovered by thawing. The isolates were heat-inactivated by incubating at 95°C for 30

minutes. Total genomic DNA was extracted from the bacterial colonies using the Quick DNATM Miniprep Plus Kit (Catalog nos. D4068 & D4069) which was set up according to manufacturer's instructions.

To release the bound DNA on the silica membrane spin column, 50 µl of DNA Elution Buffer (10 mM Tris, pH 8.5, 0.1 mM EDTA) were added to the extracted DNA, incubated for 5 minutes, and then centrifuged for 1 min at full speed and the DNA extracts were collected into a clean microcentrifuge tube. The DNA concentration was determined by measuring the absorbance of the sample at 260 nm.

#### **PCR amplification of *M. hominis* isolates:**

Conventional polymerase chain reaction was performed on the DNA extract of the *M. hominis* isolates using primers as listed in Table 1 and in accordance with previous studies (11,12,13,14). All primers were synthesized by Inqaba Biotec West Africa Ltd. PCR amplifications were performed in a thermal cycler (Tecne Prime; Stone, UK), using 50µl reaction mix containing Quick load One Taq One Step PCR Master Mix (2x) 25µl, forward primer (10 µM) 5µl, reverse primer (10µM) 5µl, template DNA 1-10µl and making up to 50µl with nuclease free water. PCR reactions consisted of an initial denaturation step of 3 minutes at 94°C, followed by 35 cycles of 60 seconds at 94°C. A final extension step was maintained for 10 minutes at 72°C. Water blank was included in every PCR run.

#### **Agarose gel electrophoresis of PCR products:**

Ten microliters of the PCR products were analyzed on 1% agarose gel electrophoresis, stained with ethidium bromide for visualization. Ten microliters of ready to use DNA ladder (100-1500bp) mixed with loading dye was loaded in the first well of every run. It was set at 120volts for 20 mins for genomic DNA and was viewed under gel documentation system with UV transilluminator.

#### **PCR amplification of *M. hominis* isolates with bacterial universal 16SrRNA gene target:**

The 16SrRNA gene target-typing of *M. hominis* included a nested step, where the PCR product from first amplification round was used as the template DNA in the second round. PCR amplifications were performed as above on the DNA extracts using 16SrRNA gene target universal to all bacteria. Agarose gel electrophoresis and visualization were also performed as above. Bacterial amplicons of approximately 750bp in size were expected

Table 1: Forward and Reverse Oligonucleotide Primers Used

Sequence target	Used for	Primer information	Reference
16SrRNA Universal bacterial gene	Sequencing	F-5'-GTGCCAGCAGCCGCGTAA-3' Barcode: S3B 1E; Length: 19 bases, 886bp. For 100uM stock solution, add 285.97ul buffer  R-5'-AGACCCGGGAACGTATTCAC -3' Barcode: S3B 1F; Length: 20 bases, 886bp. For 100uM stock solution, add 490.85ul buffer	11
Open-reading frame encoded by the <i>vaa</i> gene	Amplification of <i>vaa</i> gene	F-5'-CCCCGGAGATTATTAAGTCTC-3' R-5'-GTGCCCATAGTAGCACTATTTTGTG-3'	12
Hypervariable domain of p120 gene	Amplification of p120 gene	F-5'-GCTATTGTTGAAATTCC-3' R-5'-CTTTGATTGTTCCAGCAGTAGG-3'	13
16SrRNA gene of <i>Mycoplasma hominis</i>	Amplification of 16SrRNA gene	F-5'-CAATGGCTAATGCCGGATACGC-3' R-5'-GGTACCGTCAGTCTGCAAT-3'	14

### PCR products cleaning

The PCR products were cleaned using Exo-SAP IT kit. The Exo-SAP IT Master mix was prepared by adding 50.0 µl Exonuclease I (NEB M0293) 20 U/ul and 200.0 µl Shrimp Alkaline Phosphatase (NEB M0371) 1U/ul in a 0.6 ml microcentrifuge tube. Exo-SAP IT Mix of 2.5 µl was then added to 10.0 µl of PCR mixture, mixed properly and incubated at 37°C for 30 minutes after which the reaction was stopped by heating at 95°C for 5 minutes.

### Phylogenetic analysis of *M. hominis* with bacterial universal 16SrRNA sequencing:

The ultra-pure DNA was sequenced with ABI3500XL analyzer with a 50cm array, using POP7. Sequence data generated were analyzed with Geneious version 9.0.5 and phylogenetic tree were constructed using neighbor joining method based on the nucleotide sequences of the 16SrRNA gene target. Neighbor-joining trees were constructed for each individual locus. Genes of interest were identified in scaffolds using NCBI Blast (<http://blast.ncbi.nlm.nih.gov/>) with the setting; 'align two sequences'. The gene sequence from the reference genome was used to identify the same/similar sequence in the scaffolds.

### Statistical analysis of data:

Data were entered and analyzed using Statistical Package for Social Sciences (SPSS) version 20 and Graph Pad Prism version 6.0. Descriptive statistics were used to describe the study participants in relation to relevant variables. Chi-square and Fisher's exact test were employed to compare variables. P value < 0.05 was considered statistically significant.

## Results:

The 16SrRNA universal gene sequencing revealed four strains (clonal or phylogenetic) of *M. hominis* (Table 2). The Fisher's exact test showed no significant difference in occurrence of the strain (clonal) types between pregnant and infertile women ( $p>0.05$ ). Although strain MHS43 (72.7% v 22.0%) and MHKC87 (18.2% v 11.1%) appeared to be more frequently associated with pregnant than infertile women, strain MHBS (33.3% v 0%) and MHSWP2 (33.3% v 9.1%) appeared to be more frequently associated with infertile compared to pregnant women. However, the differences in frequency of occurrence were not statistically significant for all strains between both groups ( $p>0.05$ ).

Table 3 showed distribution of virulent genes among the four phylogenetic types of *M. hominis* in the pregnant and infertile women, and with the Fisher's exact test, neither *vaa* nor p120 gene was significantly associated with frequency of occurrence of any strain type with respect to source ( $p>0.05$ ). The 3 MHBS strains were all exclusive to infertile women and all possessed virulent genes.

Table 4 showed the strain types and how the virulent genes (*vaa* and p120) were distributed among them. A two-way ANOVA analysis showed none of the gene was significantly associated with a particular strain ( $p=0.8641$ ). All strains appear to possess different genes in different capacity with no particular pattern apart from MHBS which showed strain homology in pattern.

Plates 1 and 2 showed the gel electrophoresis picture of *vaa* and p120 genes respectively while Figs 3.1 to 3.4 represent the phylogenetic variants (strain types) of *M. hominis*.

Table 2: Phylogenetic variants (strain) of *Mycoplasma hominis* and distribution among study participants

Clonal type	Number (%)	Infertile women (%)	Pregnant women (%)	p-value	OR (95% CI)
MHS43	10 (50.0)	2 (22.2)	8 (72.7)	0.06	0.1071 (0.014 - 0.838)
MHBS	3 (15.0)	3 (33.3)	0	0.07	12.385 (0.549 - 279.4)
MHSWP2	4 (20.0)	3 (33.3)	1 (9.1)	0.28	5.00 (0.419 - 59.7)
MHKC87	3 (15.0)	1 (11.1)	2 (18.2)	1.00	0.5625 (0.043 - 7.45)
<b>Total</b>	<b>20</b>	<b>9</b>	<b>11</b>		

OR: Odds ratio; CI: Confidence interval

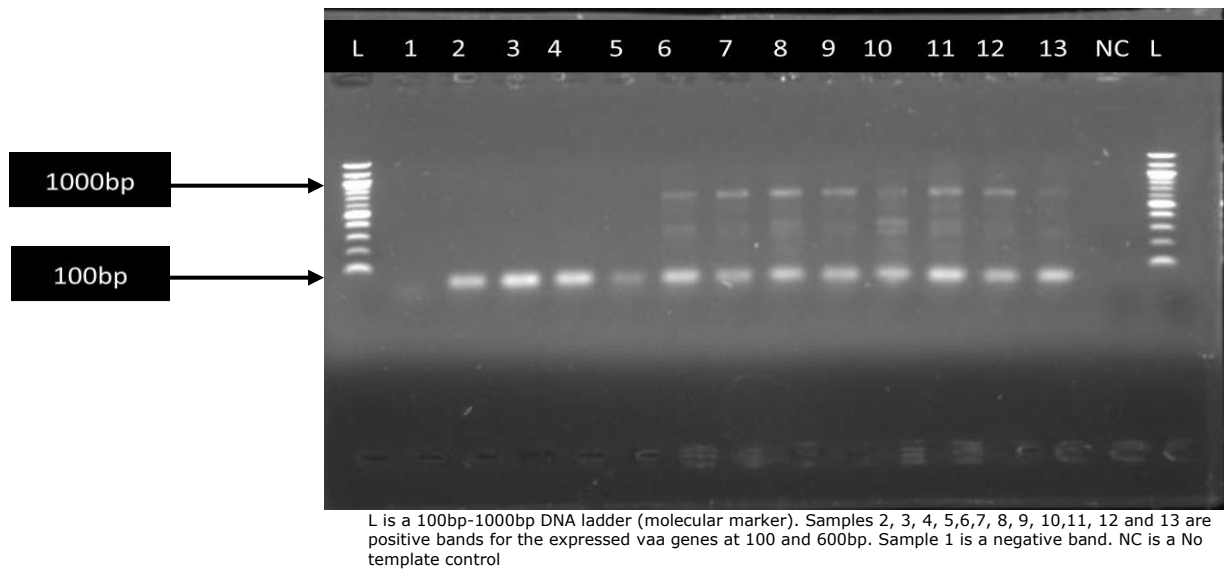
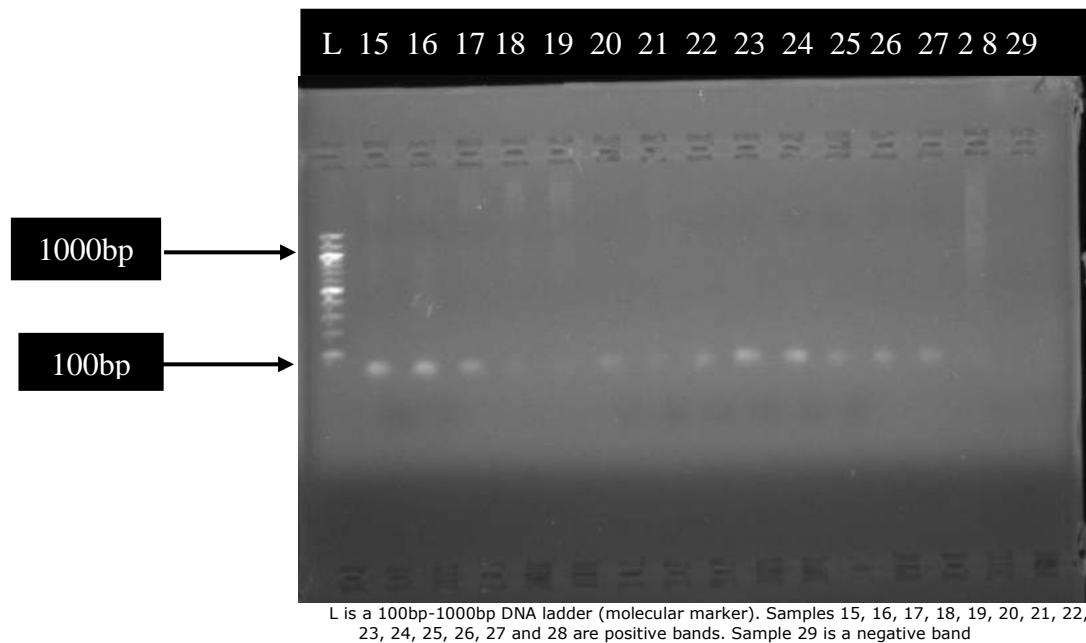
Table 3: Distribution of virulent genes among the four variants of *Mycoplasma hominis* isolates with respect to source

Clonal type	Vaa genes					P120 genes				
	Infertile women		Pregnant women		p value	Infertile women		Pregnant women		p value
	Freq	Pos	Freq	Pos		Freq	Pos	Freq	Pos	
MHS43 (n=10)	2	2	8	4	0.47	2	1	8	5	1.00
MHSWP2 (n=4)	3	1	1	1	1.00	3	2	1	1	1.00
MHBS (n=3)	3	3	0	0	1.00	3	3	0	0	1.00
MHKC87 (n=3)	1	1	2	2	1.00	1	0	2	2	0.33
<b>Total (n=20)</b>	<b>9</b>	<b>7</b>	<b>11</b>	<b>7</b>		<b>9</b>	<b>6</b>	<b>11</b>	<b>8</b>	

Freq =Frequency of occurrence; Pos=Number positive

Table 4: Virulent genes carriage among the four *Mycoplasma hominis* phylogenetic groups

Strains/Virulent genes	Total vaa gene	Total p120 gene	vaa gene only	p120-gene only	vaa + p120
MHS43 (10)	6	6	3	3	3
MHBS (3)	3	3	0	0	3
MHSWP2 (4)	2	3	1	2	1
MHKC87 (3)	3	2	1	0	2
<b>Total</b>	<b>14</b>	<b>14</b>	<b>5</b>	<b>5</b>	<b>9</b>

Plate 1: Gel electrophoresis picture of PCR amplicon of *vaa* genesPlate 2: Gel electrophoresis picture of PCR amplicon of *p120* gene

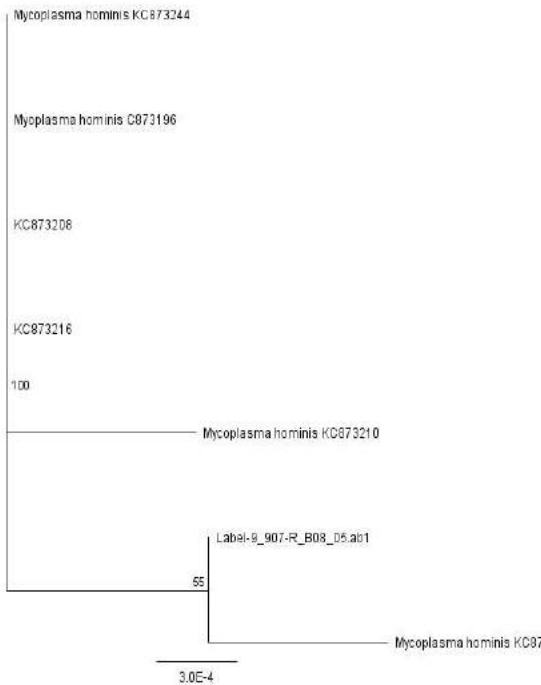


Fig. 3.1: Phylogenetic tree for *Mycoplasma hominis* strain KC87

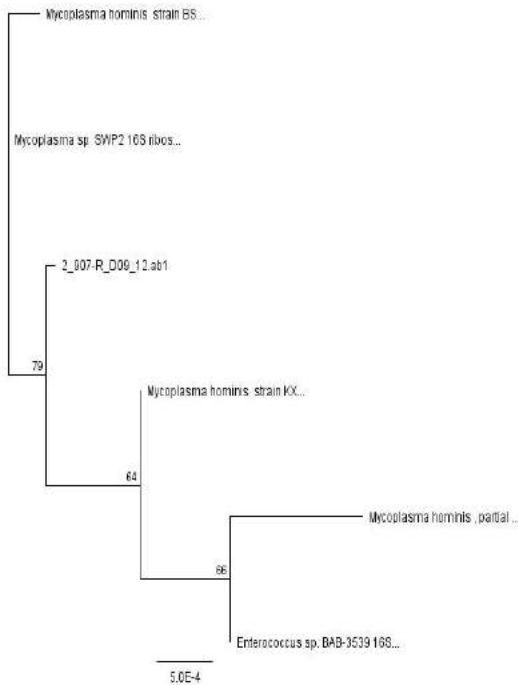


Fig. 3.2: Phylogenetic tree for *Mycoplasma hominis* strain BS

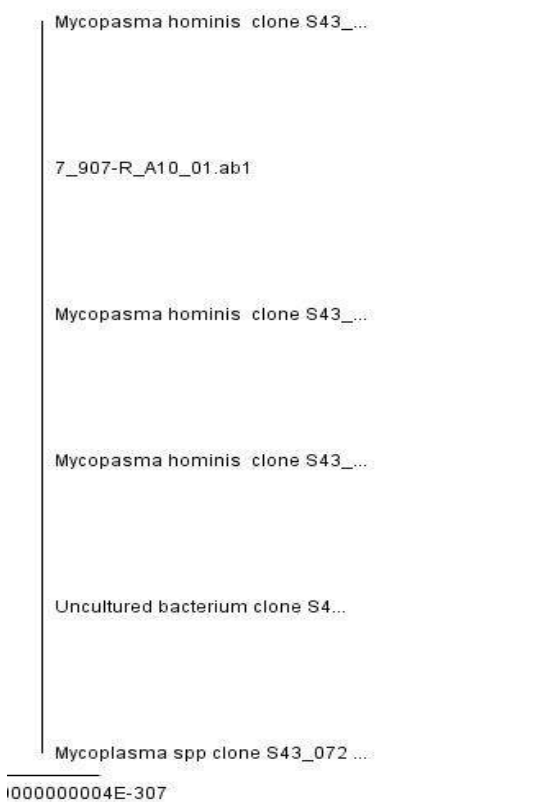


Fig. 3.3: Phylogenetic tree for *Mycoplasma hominis* strain S43

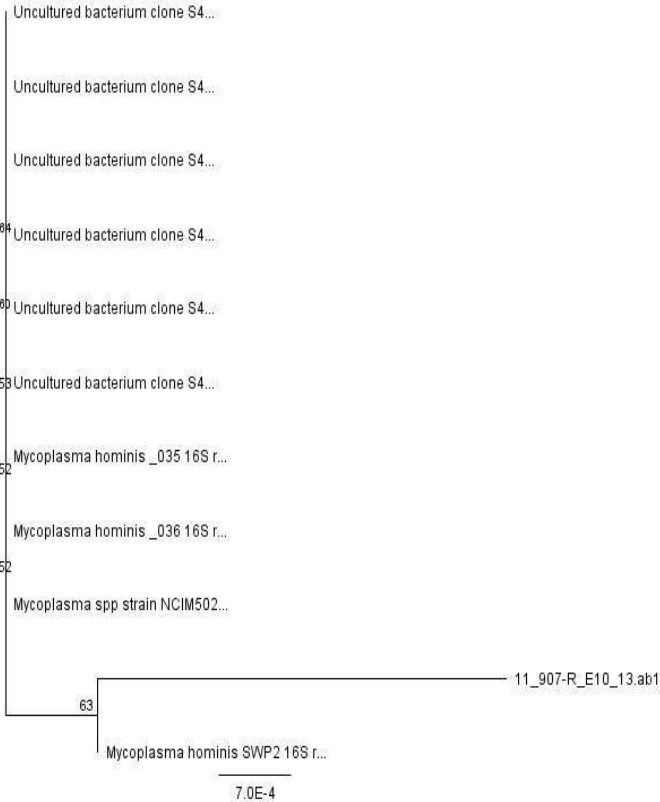


Fig. 3.4: Phylogenetic tree for *Mycoplasma hominis* strain SWP2



## Discussion:

The 20 *M. hominis* isolates yielded four phylogenetic variants. This finding is in agreement with other studies that made use of molecular typing methods. Using RFLP, Jironkin et al., (15) reported high levels of genetic heterogeneity among *M. hominis* and Sogaard et al., (16) examined six-house-keeping gene sequences to investigate evidence of genomic recombination in *M. hominis* to show a high degree of variability between these genes. Ferandon et al., (17) used multiple locus variable number tandem repeat (VNTR) analysis (MLVA) molecular typing system for the discrimination of 210 French *M. hominis* clinical isolates. In their study, they described 40 MLVA types, revealing a high genetic heterogeneity among this specie. Their result showed that a high level of intragenic and intergenic recombination occurred in *M. hominis* and these recombination levels are presumably important for the adaptation potential of this specie (15).

Although in our study, strains MHS43 and MHKC87 appeared to be more frequently associated with pregnant than infertile women, and strain MHBS and MHSWP2 appeared to be more frequently associated with infertile than pregnant women, the difference in frequency of occurrence was not significant ( $p>0.05$ ). This implies that none of the *M. hominis* variant is uniquely associated with infertility, which could be an indication that pathogenicity of each strain is host dependent. According to Christensen (18), comparisons of 12 genital isolates with other strains from different origins demonstrated that the genital isolates varied as much from each other as strains of non-genital origin. This agrees with the findings in this study although it was originally suggested that strains isolated from the same anatomical region should reveal a higher degree of similarity than strains isolated from another anatomical sites (19).

While 70% (14/20) of the *M. hominis* isolates was found to carry p120 virulent gene in our study, 30% (6/20) did not possess this gene. This shows that p120 gene, though more conserved and unique among *M. hominis* than *vaa* gene, was not present in all the isolates. Nyvold et al., (13) classified the p120 gene of *M. hominis* into 4 classes based on restriction endonuclease cleavage patterns of the hyper-variable domains. The different classes of p120 observed by Nyvold et al., (13) may well explain why 30% of *M. hominis* were found to be without p120 gene in our study. Mardassi et al., (20), in their study of patients with urogenital infections, discovered that the p120

protein undergoes substantial level of genetic variability at its surface exposed region. Part of the reason for the absence of p120 gene in a few isolates could be due to the use of primers constructed from the hyper-variable regions which are highly discriminatory unlike primers from the conserved regions which have the capacity to amplify p120 gene from all isolates. Mardassi et al., (20) reported that in a larger sample of *M. hominis* recovered from patients with urogenital infections, p120' protein, a variant of p120, also undergoes substantial level of genetic variability at its surface exposed region. Orville et al., (21) reported that the highly antigenic p120 gene displays a hyper-variable region due to accumulation of mutations while the *vaa* gene product displays both size variation and frameshift mutation to create variant products.

Thirty percent (30%) of the *M. hominis* isolates screened lacked *vaa* gene. For the sake of evolution and genome down-sizing, and possibly through gene truncation and activities of mobile genetic element (MGE), this is very possible and some of these isolates might have lost theirs to the environment. The detection of *vaa* gene in only 70% of *M. hominis* in our study disagrees with the assertion of Henrich et al., (22) that *vaa* genes are carried by all *M. hominis* species. The *vaa* locus has previously been described as a highly dynamic region of the *M. hominis* genome (3). It has been proposed that the mechanism underlying variation in the *vaa* gene is intra-species recombination whereby variable regions of *vaa* are exchanged resulting in a variable and dynamic 'hot-spot' in *M. hominis* genome (23). Based on Southern blot analysis and sequencing of the *vaa* gene, Henrich et al., (22) also proved the mechanism of variability to be based on specific truncations of the *vaa* gene. In addition to truncation, a coincidental duplication of some gene segments was also detected. Their work provided evidence for the genetic basis of a further variation in the *M. hominis vaa* adhesin

Vladislav et al., (24) identified 15 *M. hominis* isolates containing various versions of the *vaa* genes characterized by different amounts of homologous replaceable cassettes. They also determined a hyper-variable region of the *vaa* gene connected with an area encoding the immuno-significant part of the *Mycoplasma vaa* protein. This region is essential for immune recognition, persistence of *Mycoplasma* in humans, and colonizing host cells. It is noteworthy that presence of *vaa* gene, whether truncated or not, does not necessarily mean it is expressed. Hence, presence of *vaa* gene does not correlate with cytoadherence any-

more. Evidence abound that *M. hominis* can revert to other surface proteins such as p120, in instances where *vaa* gene is no longer functional (22).

Our study provides evidence that primers from *vaa* gene may no longer be suitable in screening for *M. hominis*. The shortfall in using *vaa* gene as a target for screening for *M. hominis* include omission of *vaa*-negative *M. hominis* giving rise to false negative, and lastly, the possibility of evading detection even in isolates that possess *vaa* gene, since other researches have proven that more genetic variability occur in *vaa* gene. The presence of *vaa* gene have been previously reported to be a prerequisite for virulence of *M. hominis* but the finding of our study not only indicated that it is no longer common to all *M. hominis* as speculated, but also not exclusive to perceived virulent strains. This was confirmed by the presence of *vaa* gene among strains (strain MHS43) that showed no apparent pathogenicity due to source of isolation, although this association was found to be non-significant, which implies that pathogenicity of *M. hominis* has to do with more than just possessing *vaa* gene, which means that issue of gene expression is of utmost importance. However, it is also important to note that variation or absence of this surface-exposed antigen may prevent the eradication of *M. hominis* by the host immune response (25), which is an added pathogenicity potential.

There is no significant variability among the virulent genes with regards to source, which confirms the findings of Rakhmatulina et al., (26) who demonstrated a potential effect of the *vaa* surface protein on the virulence of opportunistic *M. hominis* pathogen. They opined that certain variants of *vaa* are more frequently associated with clinical manifestations of inflammatory diseases while others are associated with clinically healthy people. Hence, pathogenicity may not just be about presence of *vaa* gene but of the *vaa* variant and gene expression. This implies that the strains from different sources do not vary significantly with respect to virulent genes of interest. This by implication means that any difference in the pathogenicity of these variants does not lie in the presence or absence of virulent genes of study. On the other hand, 100% (2/2) of strain MHKC87 from pregnant women had *vaa* and p120 indicating pathogenicity to be host-dependent. However, there is no conclusive evidence to this assertion yet because no further screening was carried out on the pregnant women because pregnancy was generally taken as evidence of fertility, but cases abound where pregnant

women may have one inflamed or blocked fallopian tubes and may have conceived through one tube (27). However, there was no such record and pregnancy does not annul the possibility of sequelae of reproductive tract infections. Thus, pregnancy may mask any underlying impact of the organism in the individual.

Other strains displayed inconsistent pattern both within and among strains, apart from strain MHBS which showed strain homology in pattern both in the source of isolation and how the virulent genes were carried. MHBS appeared not to have lost any of its adherence capability and this could have a profound influence on its pathogenicity potential. It could mean that strain MHBS is the most virulent among the four identified strain and that this strain is less subject to mutations and variabilities. Just like it is easy to shed of genes not serving it, it is just as well easy and possible for any *Mycoplasma* strain to pick up a gene from its environment to fortify itself in a new environment due to lack of cell wall, and this singular factor appeared to be the greatest weapon exploited by the species in promoting its pathogenicity potentials. Allen-Daniels et al., (27) identified a gene in *M. hominis* that was significantly associated with bacterial burden in the amniotic fluids suggesting that it could play a role in survival or fitness in this niche. The limitation of our study includes the small sample (isolate) size and the absence of adequate clinical information of the study population where the participants were selected from.

## Conclusion:

The differences in pathogenicity of *M. hominis* may be linked to heterogeneity within the specie and lack of homogeneity in the virulence factors as witnessed both in the intra specie and intra strain levels. This finding has become a foundational ground for re-examining the scientific fact that *vaa* gene is exclusive to *M. hominis* and has by extension, call for review of the use of primer construct from *vaa* gene as biomarkers for direct molecular screening of samples for *M. hominis*.

For more effective and meaningful progress and assertions in the determination of *M. hominis* strains and its pathogenicity, there should be a globally accepted standardized method of strain characterization. This will enable a uniformity of approach and outcome across the globe. Hitherto, there are just a conglomeration of different methodologies which hinders streamlined outcome and comparison.

## Contribution of authors:

CCP was involved in material preparation, data collection, analysis, and writing of the first draft of the manuscript. Other authors participated in review of the manuscript draft. All authors read and approved the final manuscript.

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## Original Article

## Open Access

## Prevalence and risk factors associated with canine dermatophytoses among dogs in Kwara and Osun States, Nigeria

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### Abstract:

**Background:** Dermatophytosis (ringworm) is a zoonotic fungal skin infection caused predominantly by *Microsporum canis*, *Microsporum gypseum* and *Trichophyton* spp. It is highly transmissible and, while normally self-limiting, could be problematic due to its potential to cause disease in certain human populations. The occurrence and associated risk factors of dermatophytoses in dogs presented at three veterinary clinics in Osogbo, and Ilorin, Nigeria between July and November 2019 were investigated in this study.

**Methodology:** This was a descriptive cross-sectional study of 325 dogs with lesions suggestive of dermatophytosis, selected by simple random sampling from three veterinary clinics in Osogbo and Ilorin, purposively selected for the study due to high patronage of the veterinary hospitals by dog owners. Using conventional mycological sampling techniques, plucked hairs and skin scrapings were obtained from the dogs. The samples were emulsified in 10% potassium hydroxide, examined microscopically for fungal elements and cultured using standard mycological procedures. Information on dog demographic characteristics and risk factors for dermatophytosis were collected using structured questionnaire. The association between risk factors and demographic variables with the occurrence of dermatophytoses was determined using Chi-square test (with Odds ratio and 95% confidence interval) and  $p$  value  $< 0.05$  was considered statistically significant.

**Results:** Positive cultures for dermatophytes were obtained from samples of 48 (14.8%) dogs with *M. canis* 37.5% (18/48), *M. gypseum* 27.0% (13/48) and *T. mentagrophytes* 8.3% (4/48). Other fungi identified were *Aspergillus flavus* 12.5% (6/48) and *Malassezia canis* 12.5% (6/48). The age distribution of positive dogs were  $< 1$  year (50.0%,  $n=24$ ), 1-3 years (29.2%,  $n=14$ ) and  $> 3$  years (20.8%,  $n=10$ ), while the risk factors associated with dermatophytosis included sex of dogs ( $p=0.0428$ ), history of dermatophytosis ( $p<0.0001$ ), clinical presentation ( $p<0.0001$ ) and lesion type, especially kerion and pustular lesions ( $p=0.0297$ ).

**Conclusion:** These findings established the occurrence of dermatophytosis in dogs kept for companionship (i. e., pets), security and breeding purposes in the two States in Nigeria. Our findings underscore the need for routine mycological investigations in dogs to facilitate early detection of cases and prompt institution of treatment interventions, thereby preventing zoonotic transmission of dermatophytes to their owners, handlers and veterinarians.

**Keywords:** Dermatophytosis; ringworm; dogs; risk factors

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## Prévalence et facteurs de risque associés aux dermatophytoses canines chez les chiens dans les États de Kwara et d'Osun, au Nigeria

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## Résumé:

**Contexte:** La dermatophytose (teigne) est une infection cutanée fongique zoonotique causée principalement par *Microsporum canis*, *Microsporum gypseum* et *Trichophyton* spp. Il est hautement transmissible et, bien qu'il soit normalement autolimitatif, il pourrait être problématique en raison de son potentiel à provoquer des maladies dans certaines populations humaines. La survenue et les facteurs de risque associés des dermatophytoses chez les chiens présentés dans trois cliniques vétérinaires à Osogbo et Ilorin, au Nigeria entre juillet et novembre 2019 ont été étudiés dans cette étude.

**Méthodologie:** Il s'agissait d'une étude transversale descriptive de 325 chiens présentant des lésions évocatrices de dermatophytose, sélectionnés par simple échantillonnage aléatoire dans les cliniques vétérinaires de deux hôpitaux, sélectionnés à dessein pour l'étude en raison de la forte fréquentation des hôpitaux vétérinaires par les propriétaires de chiens. En utilisant des techniques d'échantillonnage mycologique conventionnelles, des poils épilés et des raclures de peau ont été obtenus chez les chiens. Les échantillons ont été émulsifiés dans de l'hydroxyde de potassium à 10%, examinés au microscope pour les éléments fongiques et cultivés en utilisant des procédures mycologiques standard. Des informations sur les caractéristiques démographiques des chiens et les facteurs de risque de dermatophytose ont été recueillies à l'aide d'un questionnaire structuré. L'association entre les facteurs de risque et les variables démographiques avec la survenue de dermatophytoses a été déterminée à l'aide du test du chi carré (avec rapport de cotes et intervalle de confiance à 95 %) et la valeur de  $p < 0,05$  a été considérée comme statistiquement significative.

**Résultats:** Des cultures positives pour les dermatophytes ont été obtenues à partir d'échantillons de 48 (14,8%) chiens avec *M. canis* 37,5% (18/48), *M. gypseum* 27,0% (13/48) et *T. mentagrophytes* 8,3% (4/48). Les autres champignons identifiés étaient *Aspergillus flavus* 12,5% (6/48) et *Malassezia canis* 12,5% (6/48). La répartition par âge des chiens positifs était  $< 1$  an (50,0%,  $n=24$ ), 1-3 ans (29,2%,  $n=14$ ) et  $> 3$  ans (20,8%,  $n=10$ ), tandis que les facteurs de risque associés à la dermatophytose incluait le sexe des chiens ( $p=0,0428$ ), les antécédents de dermatophytose ( $p<0,0001$ ), la présentation clinique ( $p<0,0001$ ) et le type de lésion, en particulier les lésions kérien et pustuleuses ( $p=0,0297$ ).

**Conclusion:** Ces résultats ont établi la présence de dermatophytose chez les chiens élevés pour la compagnie (c'est-à-dire les animaux de compagnie), la sécurité et la reproduction dans l'un des États du nord et du sud du Nigéria. Nos résultats soulignent la nécessité d'enquêtes mycologiques de routine chez les chiens pour faciliter la détection précoce des cas et la mise en place rapide d'interventions thérapeutiques, empêchant ainsi la transmission zoonotique des dermatophytes à leurs propriétaires, maîtres-chiens et vétérinaires.

**Mots clés:** Dermatophytose; teigne; chiens; facteurs de risque

## Introduction:

Dermatophytosis is a superficial fungal infection of the stratum corneum of the epidermis and keratinized tissues including the skin, hair and nails of humans and animals. It is considered as the most commonly encountered fungal infection in veterinary practice affecting a wide range of domestic and wild animals (1,2,3), and is recognized as a major skin disease of zoonotic importance (4,5). Dermatophytosis is caused by a unique group of fungi known as the dermatophytes which can be categorized into one of three genera; *Trichophyton*, *Microsporum* and *Epidermophyton*. While over 20 species of dermatophytes have been found in domestic animals, the most common in dogs are *Microsporum canis*, *M. gypseum* and *Trichophyton* spp (6)

Transmission of dermatophytes occurs rapidly via direct contact with infected animals, fomite-associated contact with infectious spores and sometimes air-borne (7). The close interaction between humans and animals have allowed for easy transmission of dermatophytes to humans through direct contact with cutaneous lesions of infected animals, with *M. canis*, *Tinea capitis* and *T. corporis*

being the most commonly isolated from humans (8).

Generally, dermatophytes are classified into three ecological groups based on their major natural reservoirs. Those found in the soil, animals and humans are categorized as geophilic, zoophilic and anthropophilic, respectively (9). The zoophilic dermatophytes may precipitate zoonoses in humans with these human infections resulting most often from direct contact with an infected animal or through indirect contact with contaminated environments (10), such as fungus-bearing hair and scales from infected animals. According to Akpolat et al., (11), the prevalence of superficial mycoses caused by zoophilic dermatophytes is significant worldwide, especially in the tropical countries with warm and humid climate, crowded living and poor sanitary conditions (12).

Dermatophytosis often occurs when the host immune system is unable to generate sufficient cell-mediated immune response capable of eliminating the fungi from the host system (13). While the infection is more prominent in younger animals, especially those below one year of age, older animals are less susceptible as incidences of acquired immune response have previously been reported in

them (14). Apart from age, other factors including breed of animal, population density, management system, season of the year, climatic and socio-economic conditions, and natural reservoirs have been reported to play significant roles in determining the prevalence and causative agents of dermatophytosis in different animal species worldwide (15,16).

In recent years, there has been an upsurge in keeping of dogs as pets or for security purposes in Nigeria, with majority of these animals cohabiting and feeding with their owners and household members (17,18,19). In this way, these animals could serve as potential sources of zoonotic diseases including dermatophytoses to their owners and handlers. Despite the existence of several published works on human dermatophytoses in Nigeria, reports of the disease in animals, especially dogs, are limited. Nweze (20) and Chah et al., (21) have previously reported 49.5% and 12.5% prevalence rates of dermatophytosis, respectively in dogs in Nigeria. Moreover, Adebisi and Oluwayelu (21) reported the fact that dermatophytes of dogs probably play a significant role in the epidemiology of human dermatophytosis. Our study was therefore conducted to identify the causative fungi and risk factors associated with occurrence of dermatophytosis in dogs presenting to the veterinary clinics in Osogbo and Ilorin, the capital cities of Osun and Kwara States of Nigeria, respectively.

## Materials and method:

### Study setting and design:

This was a descriptive cross-sectional study carried out in three veterinary clinics; two veterinary clinics located in Osogbo, Osun State, Nigeria and the Small Animal Clinic of the Veterinary Teaching Hospital, University of Ilorin, Kwara State, Nigeria.

### Sample size and dog participants selection:

A purposive sampling technique was used to select a Veterinary Teaching Hospital clinic from Kwara State, and two veterinary clinics in Osun State, due to high patronage of these clinics by dog owners. A total of 325 dogs (125 from Osun State and 200 from Kwara State) with lesion suggestive of dermatophytosis were selected for the study by simple random sampling technique from each of the selected veterinary clinics between July and November 2019.

### Ethical approval:

Ethical approval for the study was obtained from the Ministry of Agriculture and Natural Resources, Osun State and the Animal Care and Use Research Ethics Committee,

Faculty of Veterinary Medicine, University of Ibadan (UI-ACUREC/19094).

### Data and sample collection:

A structured questionnaire was administered to obtain information on the dogs' demographic characteristics and possible risk factors for dermatophytosis from the animal owners and veterinary clinicians. Following detailed examination of the lesions under good illumination and cleaning of the affected area with 70% ethanol, skin scrapings of dogs were collected with sterile scalpel blade from the active edge of the lesions. Hair samples were collected by removing dull broken hairs from the margin of the lesion using sterile tweezers.

The samples were placed in small paper envelopes in separate polythene bags, and transported as dry packages (6) to the Teaching and Research Laboratory of the Department of Medical Microbiology, Ladoke Akintola University of Technology, Osogbo, where they were processed for direct microscopy and culture.

### Direct microscopy, fungal isolation and identification:

Each sample was divided into two portions; one portion was used for direct microscopic examination and the other was used for culture. Direct microscopic examination was performed by placing plucked hair strands or skin scrapings on a clean glass slide and 30 µL of 15% potassium hydroxide (KOH) added. The slide was covered with a coverslip, heated gently for a few seconds and allowed to cool at room temperature for 15 minutes. Thereafter, the wet preparation was carefully examined under both low (10×) and high (40×) power light microscope objectives to observe the fungal elements and diagnostic morphology.

Irrespective of the results of direct microscopy, the samples were cultured on Sabouraud's Dextrose Agar (SDA) containing 0.5 mg/mL chloramphenicol and 500 mg/L cycloheximide. The plates incubated at room temperature (25-30°C), and examined daily for fungal growth for up to four weeks (11). Suspected fungal colonies were sub-cultured on SDA slants to obtain pure cultures, which were then sub-cultured on Potato Dextrose Agar (PDA) (Oxoid, UK) to facilitate distinctive spore formation. The PDA plates were incubated at room temperature for up to 4 weeks. Identification of the isolated fungi was based on colonial morphology as well as microscopic features observed after staining with lactophenol cotton blue using standard mycological identification procedures (22).

### Statistical analysis:

Statistical analysis of data was performed



med using the Statistical Package for Social Sciences (SPSS) version 25.0 with comparison of proportions and Chi-square test (with Odds ratio and 95% confidence interval) to determine association of the risk factors with the prevalence of dermatophytosis in the dogs.

## Results:

A total of 325 dogs were examined; 125 from Osogbo and 200 from Ilorin. Of these, 35 (10.8%) were positive for fungal elements by direct microscopy while 48 (14.8%) yielded cultures positive for dermatophytes. Out of the 48 positive cultures, 37.5% (n=18) were identified as *M. canis*, 27.1% (n=13) as *M. gypseum* and 8.3% (n=4) as *T. mentagrophytes*. Other fungi identified include *Aspergillus flavus* 12.5% (n=6), and *Malassezia canis* 12.5% (n=6) (Table 1). It is noteworthy that 18 (37.5%) of the 48 culture-positive

samples were negative by direct microscopic examination.

Furthermore, out of the dog samples from Osogbo (n=125) and Ilorin (n=200) screened for dermatophytes, isolation rates of 16.0% (20/125) and 14.0% (28/200) respectively were obtained (OR=0.8547, 95% CI =0.4583-1.594,  $p=0.7386$ ). Of the 48 dermatophyte-positive samples, 8 (16.7%) were from dogs kept for companionship (i. e. as pets), while 29 (60.4%) and 11 (22.9%) were kept for security and breeding purposes, respectively ( $\chi^2=0.0159$ ,  $p=0.992$ ) (Table 2).

The association of risk factors with the prevalence of dermatophytosis in the dogs is represented in Table 3. It shows that sex ( $p=0.0483$ ), clinical presentation of dermatophytosis ( $p<0.0001$ ), dermatophytic lesion type, especially kerion and pustular lesions ( $p=0.0297$ ), and previous history of dermatophytosis in the dogs ( $p<0.0001$ ) were significantly associated prevalence of dermatophytoses.

Table 1: Dermatophytes and other fungi isolated from skin lesions of dogs in Kwara and Osun States, Nigeria

Organism	Ilorin	Osogbo	Total	Percentage (%)
<i>M. canis</i>	11	8	19	37.5
<i>M. gypseum</i>	7	6	13	27.1
<i>T. mentagrophytes</i>	3	1	4	8.3
<i>Aspergillus flavus</i>	4	2	6	12.5
<i>Malassezia canis</i>	3	3	6	12.5
<b>Total</b>	<b>28</b>	<b>20</b>	<b>48</b>	<b>100</b>

Table 2: Distribution of dermatophytes based on purpose for keeping dogs

Purpose	Number of dogs	No of positive dogs (%)	$\chi^2$	$p$ value
Companionship	56	8 (14.3)	0.01598	0.9920
Security	194	29 (15.0)		
Breeding	75	11 (14.7)		
<b>Total</b>	<b>325</b>	<b>48 (14.8)</b>		

Table 3: Risk factors associated with dermatophytosis in dogs from Kwara and Osun States, Nigeria

Variables	Parameters	Frequency	Positive (%)	Negative (%)	OR (95% CI)	$\chi^2$	p value
Location	Ilorin	200	28 (14.0)	172 (86.0)	0.8547 (0.4583-1.594)	0.113	0.7401
	Osogbo	125	20 (16.0)	105 (84.0)			
Period	July	65	9 (13.8)	56 (86.2)		4.058	0.3893
	August	65	10 (15.4)	55 (84.6)			
	September	65	12 (18.5)	53 (81.5)			
	October	65	5 (7.7)	60 (92.3)			
	November	65	12 (18.5)	53 (81.5)			
Breed	Caucasian	48	5 (10.4)	43 (89.6)		10.144	0.3389
	Rottweiler	64	7 (10.9)	57 (89.1)			
	German Shepherd	71	8 (12.7)	55 (87.3)			
	Lhasa Apso	18	1 (5.6)	17 (94.4)			
	Cane Corso	16	4 (25.0)	12 (74.0)			
	Boerboel	2	0	2 (100.0)			
	Samoyed	12	4 (33.3)	8 (66.7)			
	Mongrel	43	9 (20.9)	34 (79.1)			
	Labrador	2	0	2 (100.0)			
	Mastiff	2	0	2 (100.0)			
Sex	Female	168	18 (10.7)	150 (89.3)	0.508 (0.2704-0.9543)	1.616	0.0428*
	Male	157	30 (19.1)	127 (80.9)			
Age	1-9 months	141	24 (17.0)	117 (82.9)		3.881	0.1436
	1-3 years	135	14 (10.4)	121 (89.6)			
	≥ 3 years	49	10 (20.4)	39 (79.6)			
Purpose	Companionship	56	8 (14.3)	48 (85.7)		0.02014	0.9900
	Security	194	29 (14.9)	165 (85.1)			
	Breeding	75	11 (14.7)	65 (85.3)			
Location of lesion	Face	116	20 (17.2)	96 (82.8)		3.063	0.3819
	Back	89	12 (13.5)	77 (86.5)			
	Side	93	10 (10.8)	83 (89.3)			
	Leg	27	6 (22.2)	21 (77.8)			
Description of lesion	Alopecia	160	22 (13.8)	138 (86.3)		12.399	0.0297*
	Kerion	1	1 (100.0)	0			
	Pustular	1	1 (100.0)	0			
	Itchy	60	7 (11.7)	53 (88.3)			
	Scaly	53	9 (16.9)	44 (83.0)			
	Others	50	8 (16.0)	42 (84.0)			
Presented for dermatophytosis	Yes	48	48 (100.0)	0		325.0	<0.0001*
	No	249	0	249 (100.0)			
	Others	28	0	28 (100.0)			
Previous history	Yes	89	2 (2.3)	87 (97.7)		19.419	<0.0001*
	No	223	46 (20.6)	177 (79.4)			
	Others	13	0	13 (100.0)			
Previous medication	Acaricide	84	9 (10.7)	75 (89.3)		6.541	0.2570
	Antibiotics	74	10 (13.5)	64 (86.5)			
	Ointment	68	9 (13.3)	59 (86.8)			
	Honey	2	1 (50.0)	1 (50.0)			
	Anti-inflammatory	38	9 (23.7)	29 (76.3)			
	Ivermectin	59	10 (16.9)	49 (83.1)			
Housing type	Inside house	115	10 (8.7)	105 (91.3)		1.830	0.4006

	Outside House	52	8 (15.4)	44 (84.6)		
	Kennel	158	20 (12.7)	138 (87.3)		
Frequency of sanitation	Weekly	41	2 (4.9)	39 (95.1)	4.607	0.099
	Monthly	118	22 (18.6)	96 (81.4)		
	Scarcely	166	24 (14.5)	142 (85.5)		
Frequency of grooming	Weekly	42	5 (11.9)	37 (88.1)	3.194	0.2025
	Monthly	200	35 (17.5)	165 (82.5)		
	Scarcely	83	8 (9.6)	75 (90.4)		
<b>Total</b>		<b>325</b>	<b>48 (14.8)</b>	<b>277 (85.2)</b>		

\*: statistically significant:  $\chi^2$ : Chi square; OR: Odds ratio; CI: Confidence interval

Fig 1 represents the typical dermatophyte lesion on the skin of the dog infected with *M. canis* showing scales and skin crusts (A), with typical yellowish tinge on Sabouraud Dextrose Agar (B and C), and spindle shaped macroconidia on lactophenol cotton blue stain.

Fig 2 shows inflammation of the skin and alopecia in dogs infected with *T. mentagrophytes* (A), with growth and reverse growth on SDA (B and C) and typical macroconidia on lactophenol cotton blue stain (D).

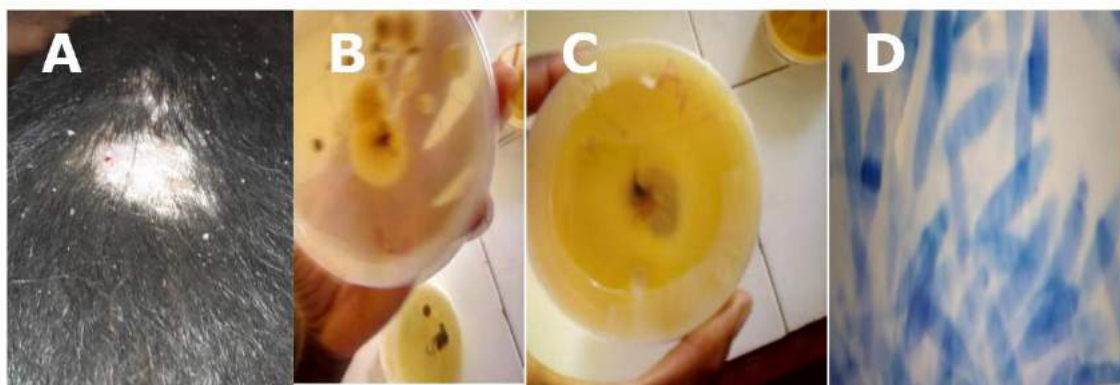


Fig 1: A: Dog skin showing alopecia and scaly lesion, B: *Microsporum canis* growth on Sabouraud Dextrose Agar, C: Reverse growth of *Microsporum canis* on Sabouraud Dextrose Agar, D: Macroconidia of *Microsporum canis* stained with lactophenol cotton blue

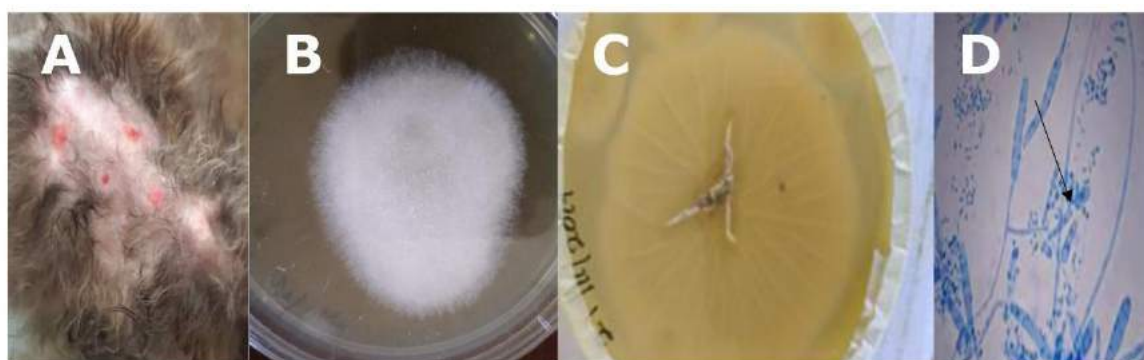


Fig 2: A: Dog skin showing alopecia and inflammation, B: *Trichophyton mentagrophytes* whitish cottony growth on Sabouraud Dextrose Agar, C: Reverse growth of *Trichophyton mentagrophytes* on Sabouraud Dextrose Agar, D: Macroconidia of *Trichophyton mentagrophytes* stained with lactophenol cotton blue

## Discussion:

Dermatophytosis has been recognized as a major public health hazard worldwide and has become endemic in some countries, especially in Africa. In Nigeria, it is a major etiology of skin infection in domestic animals and humans, especially children (19,23,24). This study was designed to investigate the occurrence and distribution of dermatophytes in dogs with suspected dermatophytosis and the prevalence of infection in relation to some epidemiological variables in Osogbo, Osun State and Ilorin, Kwara State, Nigeria.

Although the distribution of zoophilic dermatophytosis is worldwide, its incidence varies with the type of animal and geographical location. Murmu et al., (25) reported that the incidence of dermatophytosis in cats was higher (55.5%) than in dogs (37.8%) in Kolkata, India. However, in the present study, we obtained 14.8% prevalence of dermatophytosis in the dogs. This relatively low prevalence rate is comparable to the 8.2%, 18.7%, 12.5% and 8.2% reported by Khosravi and Mahmoudi (26), Seker and Dogan (27), Chah et al., (20) and Sigirci et al., (13) in symptomatic dogs in Iran, Western Turkey, South-eastern Nigeria and Istanbul, Turkey, respectively. According to previous workers (14,15, 24), differences in the prevalence of dermatophytoses can be attributed to age and breed of the animal, clinical and living conditions of the animal, geographical location, season of sampling, socio-economic conditions, and whether the animal is confined or free-roaming, among others.

The isolation of dermatophytes in dogs used essentially for companionship (i. e. as pets), security and breeding purposes in our study is of public health significance as they can serve as source of infection to their human owners, handlers, or veterinarians (28). Previous studies have established a direct correlation between dermatophytoses in dogs and humans. For instance, in a study in Italy, Cafarchia et al., (29) conducted a dermatophytic search in dogs and cats whose owners were either diagnosed with tinea corporis caused by *M. canis* or those without dermatophytosis. They found *M. canis* in 53.6% of cats and 36.4% of dogs whose owners had tinea corporis, and in only 14.6% of cats and in none of the dogs whose owners were not infected. They concluded that these pets should be recognized as a major source of dermatophytosis for humans even when they are asymptomatic.

Moreover, Adesiji et al., (30) recently reported a significant association between the playing habits of children and acquisition of dermatophytoses. Their study revealed that children who played with animals were at a

higher risk of dermatophytoses compared with children who did not play with animals. As the contact between humans and pets is becoming more intimate, dermatophytoses have become significant public and animal health concern globally. It is believed that the incidence of mycotic zoonoses such as dermatophytoses is increasing because of such direct inter-species interaction as is the possibility of re-infection due to indirect spread of spores in the environment (29,31,32).

Three major species of dermatophytes were identified in our study with *M. canis* being the most prevalent, followed by *M. gypseum* while *T. mentagrophytes* was the least prevalent. Other fungi detected include *Aspergillus flavus* and *Malassezia canis*. It has been reported that *M. canis*, *M. gypseum* and *T. mentagrophytes* are the fungi responsible for more than 95% of all dermatophytoses cases in companion animals (33). Further, analysis of the results of the present study revealed a statistically significant association between the breed of dogs and occurrence of dermatophytosis as German Shepherd breed had the highest prevalence of dermatophytic infections while Lhasa Apso breed had the least prevalence (34). It has been reported that dog breed plays a significant role in the acquisition of dermatophytosis. For example, there have been reports of superficial dermatophytosis and subcutaneous dermatophytic infections in Yorkshire terrier dogs caused mainly by *M. canis* in Brazil and Italy (29,35,36). Also, Cafarchia et al., (29) reported a higher prevalence of geophilic dermatophytes in pure breed dogs compared to cross-breed dogs.

A few studies also showed that dermatophytosis occurs more often in female animals than in males (37,38). However, our findings revealed that the prevalence of 19.1% for dermatophytosis in the male dogs was significantly higher than 10.7% in the female dogs (OR=0.5080, 95% CI=0.2704-0.9543,  $p=0.0483$ ), although other workers did not detect any association or correlation between sex and occurrence of dermatophytoses (27, 33). Some studies have indicated that age could be a factor in the incidence of dermatophytoses in domestic animals. Mattei et al., (33) determined that animals younger than one-year-old appeared to be susceptible to dermatophytoses. This is clearly at variance with the results from our study which showed that there was no statistically significant relationship ( $p=0.144$ ) between the age of the animals and the prevalence of dermatophytoses. Our result corroborates the findings of Seker and Doğan (27) who detected no statistically significant difference between the animal age groups and prevalence rate in a similar study. It has been reported that the higher susceptibility of young animals to dermato-

phytoses may be related to immunological immaturity, the deficiency of fungistatic sebum or linoleic acid, biochemical exchange on the skin, being of the anagen phase of hairs and physiological situation (7).

Other factors significantly associated with dermatophytosis in our study include previous history of dermatophytosis in the dogs ( $p < 0.0001$ ), clinical presentation of dermatophytosis ( $p < 0.0001$ ) and dermatophytic lesion type, especially kerion and pustular lesions ( $p = 0.0297$ ). However, the housing types ( $p = 0.4006$ ) and frequency of sanitation ( $p = 0.099$ ) were not significantly associated with the prevalence of dermatophytosis. This contrasts with previous studies that report housing type and sanitation as two factors critical to the incidence of dermatophytosis. For instance, in identifying the predispositions for the development of dermatophytoses in cats and dogs, Moriello et al., (24) underlined being puppies and kittens, lifestyle, free-roaming animals and warm locations as risk factors. Seker and Dogan (27) also opined that in addition to age, animal management and living conditions were risk factors for the acquisition of dermatophytoses by dogs. Moreover, our findings clearly showed that anatomical sites of lesions from where the dermatophytes were obtained had no significant association with the disease prevalence ( $p = 0.3819$ ), agreeing with previous studies which showed that pets harbor dermatophytic spores in both their hair coats and skin (27,39).

In conclusion, the results of this study revealed that dermatophytoses occur commonly in dogs in the study area, and that *M. canis* is the most prevalent fungus encountered. These findings, especially in dogs kept for companionship (i.e., as pets), security and breeding purposes, have significant implications for animal-to-human transmission of the disease and underscore the fact that canine dermatophytoses is a potential public health hazard in Nigeria. Routine screening of dogs followed by prompt treatment with appropriate anti-fungal agent in cases where infection is established are advocated to reduce incidence of dermatophytosis in dogs and prevent subsequent spread from these animals to humans.

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## Contributions of authors:

AYO conceived, designed, collected the data, and wrote the manuscript; ODO participated in design, coordination and critical review of the manuscript; and AJO collected data, coordinated sample collection from animals and reviewed the manuscript. All authors read and approved the final manuscript.

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Authors declare no conflict of interest

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## Original Article

## Open Access

## Bacteriological assessment of crab (*Pachycheles pubescens*) and dog whelk (*Nucella lapillus*) shellfishes from mesotidal estuarine ecosystem

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### Abstract:

**Background:** Shellfishes are susceptible to a wide variety of bacterial pathogens, most of which are capable of causing disease in humans but are considered to be saprophytic in nature. Bacteriological diversity of shellfishes depends on the fishing grounds, habitats and environmental factors around them. This study assessed the bacteria associated with shellfishes, *Pachycheles pubescens* (crab) and *Nucella lapillus* (dog whelk) harvested from mesotidal estuarine ecosystem.

**Methodology:** The bacteriological assessment of crab (*Pachycheles pubescens*) and dog whelk (*Nucella lapillus*) harvested from Okwano Obolo estuary in Eastern Obolo local government area (LGA), Akwa Ibom was evaluated. The density of heterotrophic and potential pathogens was determined using standard analytical procedures. The pure bacterial isolates were grouped into recognizable taxonomic units and characterized to their generic level.

**Results:** The mean (and range) total heterotrophic bacterial count (THBC), total coliform count (TCC), faecal coliform count (FCC), *Salmonella-Shigella* count (SSC) and total *Vibrio* count (TVC) of the crab samples (log<sub>10</sub> cfu/g) for the crab samples are; 4.281±0.085 (4.18-4.39); 4.187±0.078 (4.11-4.30); 4.115±0.081 (4.00-4.20); 4.076±0.058 (4.00-4.14); and 4.114±0.085 (4.00-4.23) respectively ( $p=0.003915$ ). For the dog whelk samples, the mean (and range) THBC, TCC, FCC, SSC and TVC are 4.232±0.095 (4.11-4.36); 4.185±0.095 (4.04-4.28); 4.082±0.068 (4.00-4.18); 4.062±0.055 (4.00-4.15) and 5.155±0.062 (4.08-4.23) respectively ( $p=0.028856$ ). Bacterial species isolated from the crab and dog whelk samples included *Salmonella*, *Bacillus*, *Shigella*, *Corynebacterium*, *Pseudomonas aeruginosa* and *Vibrio* (which was the most frequently isolated bacteria pathogen from both samples in 80%).

**Conclusion:** Some of the bacteria species especially *Vibrio*, *Salmonella* and *Shigella* isolated from the crab and dog whelk samples are known human pathogens, that can pose serious health risk if these seafoods are not properly cooked before consumption.

**Keywords:** Bacteria; *Pachycheles pubescens*; *Nucella lapillus*; estuary

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## Évaluation bactériologique des mollusques et crustacés du crabe (*Pachycheles pubescens*) et du buccin (*Nucella lapillus*) de l'écosystème estuarien mésotidal

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## Résumé:

**Contexte:** Les mollusques et crustacés sont sensibles à une grande variété de pathogènes bactériens, dont la plupart sont capables de causer des maladies chez les humains, mais sont considérés comme étant de nature saprophyte. La diversité bactériologique des coquillages dépend des zones de pêche, des habitats et des facteurs environnementaux qui les entourent. Cette étude a évalué les bactéries associées aux coquillages, *Pachycheles pubescens* (crabe) et *Nucella lapillus* (buccin) récoltées dans l'écosystème estuarien mésotidal.

**Méthodologie:** L'évaluation bactériologique du crabe (*Pachycheles pubescens*) et du buccin (*Nucella lapillus*) récoltés dans l'estuaire d'Okwano Obolo dans la zone de gouvernement local d'Obolo oriental (LGA), Akwa Ibom a été évaluée. La densité des pathogènes hétérotrophes et potentiels a été déterminée à l'aide de procédures analytiques standard. Les isolats bactériens purs ont été regroupés en unités taxonomiques reconnaissables et caractérisés à leur niveau générique.

**Résultats:** La moyenne (et la plage) du nombre total de bactéries hétérotrophes (THBC), du nombre de coliformes totaux (TCC), du nombre de coliformes fécaux (FCC), du nombre de *Salmonella-Shigella* (SSC) et du nombre total de *Vibrio* (TVC) des échantillons de crabe ( $\log_{10}$  ufc/g) pour les échantillons de crabe sont;  $4,281 \pm 0,085$  (4,18-4,39);  $4,187 \pm 0,078$  (4,11-4,30);  $4,115 \pm 0,081$  (4,00-4,20);  $4,076 \pm 0,058$  (4,00-4,14); et  $4,114 \pm 0,085$  (4,00-4,23) respectivement ( $p=0,003915$ ). Pour les échantillons de buccins, la moyenne (et la plage) THBC, TCC, FCC, SSC et TVC sont de  $4,232 \pm 0,095$  (4,11-4,36);  $4,185 \pm 0,095$  (4,04-4,28);  $4,082 \pm 0,068$  (4,00-4,18);  $4,062 \pm 0,055$  (4,00-4,15) et  $5,155 \pm 0,062$  (4,08-4,23) respectivement ( $p=0,028856$ ). Les espèces bactériennes isolées des échantillons de crabe et de bulot comprenaient *Salmonella*, *Bacillus*, *Shigella*, *Corynebacterium*, *Pseudomonas aeruginosa* et *Vibrio* (qui était la bactérie pathogène les plus fréquemment isolées des deux échantillons dans 80%).

**Conclusion:** Certaines des espèces de bactéries, en particulier *Vibrio*, *Salmonella* et *Shigella* isolées des échantillons de crabe et de buccin, sont des agents pathogènes humains connus, qui peuvent poser de graves risques pour la santé si ces fruits de mer ne sont pas correctement cuits avant consommation.

**Mots clés:** Bactéries; *Pachycheles pubescens*; *Nucella lapillus*; estuaire

## Introduction:

Shellfish comprises a variety of exoskeleton-bearing aquatic invertebrates including crustaceans such as lobsters, prawn crabs, and shrimps. Shellfish, in general, contains appreciable quantities of digestible proteins, essential amino acids, bioactive peptides, long chain polyunsaturated fatty acids, vitamin B and minerals, including copper, zinc, inorganic phosphate, sodium, potassium, selenium, iodine, and also other nutrients, which offer health benefits to the consumers. Bacteria, ubiquitous in the marine environment, constitute an important component of the coastal microbial communities. They play unique role in nutrient cycling, trophic dynamics of aquatic food webs and acts as a disease agent in human (1).

Estuaries provide good breeding sites for shellfishes and other fishes. Environmental parameters such as temperature, salinity, dissolved oxygen, and turbidity play a role in the filtration activity, thereby affects the retention of bacteria by bivalves (2). These factors determine the distribution and composition of bacterial communities. During the past decades, outbreaks of seafood associated infections have caused illness and death (3). The most prevalent microorganisms that contaminate the shell fishes are bacteria introduced into water bodies through human activities. Janina et al., (1) reported that shellfishes are able to harbor microorganisms in their environment.

Shellfishes are filter feeder organisms

that selectively strain small particles of phytoplankton, zooplankton, and inorganic matters and accumulate a diversity of other contaminants from surrounding waters (4). The number and type of pathogenic bacteria present in marine or estuarine water depends on seasonal, climatic and anthropogenic factors (5). Bivalve shellfish have been known as bio-indicators of aquatic contamination with heavy metal and pesticides for decades (6).

Bacterial pollution in shellfishes is a common challenge in almost all the coastal areas of developing countries. Shellfish-borne infectious diseases are generally transmitted through faeco-oral route, as such, the shellfish must be examined to ensure that pathogenic bacteria are not present (7). The main hazard associated with consumption of shellfishes arises from contamination of their habitat water, especially if eaten raw or lightly cooked. These circumstances make the shellfish an important vector of food borne diseases, thereby presenting a significant human health risk (8). A study (9) showed that consumption of raw or undercooked seafood is recognized as health risk to consumers. Faecal pollution enhances the occurrence of shellfish diseases and the population at risk include individuals with immunocompromised disorders such as patients with cancers, chronic liver and kidney diseases (10,11). Shellfishes have high nutritive base and serve as a good medium for the growth of microorganisms that can lead to food poisoning such as salmonellosis and vibriosis, when con-

sumed (12).

There has been a significant concern about the safety of shellfishes for human consumption. Despite extensive efforts to assure the safety supply of shellfishes, the incidence of infection and mortality have been increasing (13). Therefore, bacteria associated with shellfish are of paramount importance due to the widespread consumption of shellfishes by the populace at large. This study assesses the bacteria pathogens associated with shellfishes.

## Materials and method:

### Study setting

The study location is Okwano-Obolo in Eastern Obolo, a coastal settlement in Akwa Ibom State of the Niger Delta region of Nigeria. The region lies within latitude 4° 44' N, longitude 8° 41' E and latitude 4° 42' N, longitude 8° 42' E bordering the Atlantic Ocean. It is a fishing settlement characterized by different shellfishes including soft, dark, mudflats, crabs, dog whelks, and periwinkles. The ecosystem is equally used for petroleum and exploration activities (14).

### Sample collection

Edible shellfishes; crabs (*Pachycheles pubescens*) and dog whelks (*Nucella Lapillus*) were randomly obtained from the local fishermen in Etekwun, a fishing settlement in Okwano-Obolo of Eastern Obolo, Akwa Ibom State, Nigeria. Five samples (30g each) of each shellfish were collected and placed in ice-cooled chest and transported to the microbiology laboratory of the University of Uyo for analysis.

### Isolation and enumeration of bacterial isolates

Isolation and enumeration of heterotrophic bacteria and potential pathogens were determined using standard analytical techniques as described by Harrigan and McCance (15). The samples were first macerated to paste using sterile ceramic mortar and pestle. Serial dilution of the samples was done to achieve a reduction in microbial population in the sample by weighing 1g of each sample and transferring into test tubes containing 9ml sterile water. A ten-fold serial dilution was carried out aseptically on each of the sample as previously described (16).

Heterotrophic bacterial counts in the samples were determined by the pour plate technique (15) on nutrient agar plates. The isolation and enumeration of potential pathogens was carried out using three different media; thiosulfate-citrate-bile salt-sucrose agar (TCBS) for *Vibrio* species, mannitol salt agar (MSA) for

*Staphylococcus aureus* and eosin methylene blue agar (EMBA) for faecal coliforms. Heterotrophic bacteria plates were incubated at 28±2°C for 24 hours, TCBS and MSA plates at 37°C for 48 hours, and EMBA plates at 35°C for 4 hours, followed by 14 hours at 45°C. Discrete colonies on the culture plates were enumerated and recorded as colony forming units per gram (CFU/g) of the sample.

### Identification of bacterial isolates

Discrete colonies were sub-cultured in nutrient agar plates to obtain pure cultures. Bacteria isolates were identified based on cultural, morphological and biochemical characteristics. Relevant conventional biochemical tests included catalase, coagulase, oxidase, citrate utilization, sugar fermentation, motility, methyl red and Voges Proskauer tests as described in Bergey's Manual of Determinative Bacteriology (17).

### Statistical analysis

Data obtained from the microbiological assessment of the samples were analysed on SPSS package version 2.0 by one-way analysis of variance (ANOVA) with Duncan multiple range test for post-hoc determinations of significant differences ( $\alpha < 0.05$ ).

## Results:

Table 1 shows the mean total heterotrophic bacterial count (THBC), total coliform count (TCC), faecal coliform count (FCC), *Salmonella-Shigella* count (SSC) and total *Vibrio* count (TVC) of the crab samples (log10) of 4.281±0.085 (range 4.18-4.39); 4.187±0.078 (range 4.11-4.30); 4.115±0.081 (range 4.00-4.20); 4.076±0.058 (range 4.00-4.14); and 4.114±0.085 (range 4.00-4.23) respectively ( $p = 0.003915$  ANOVA). For the dog whelk samples, the mean THBC, TCC, FCC, SSC and TVC are 4.232±0.095 (range 4.11-4.36); 4.185±0.095 (range 4.04-4.28); 4.082±0.068 (range 4.00-4.18); 4.062±0.055 (range 4.00-4.15) and 5.155±0.062 (range 4.08-4.23) respectively ( $p = 0.028856$  ANOVA) (Table 2).

The isolated bacteria pathogens from the shellfish samples are *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella* Typhi, *Shigella* sp, *Corynebacterium* sp, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Proteus vulgaris* (Table 3). The most frequently isolated bacteria pathogen from the crabs is *Vibrio cholerae* in 80% of the samples while the least frequently isolated bacteria were *Bacillus* sp, *S. aureus* and *E. coli* in 20% of the samples. *Pseudomonas aeruginosa*, *Shigella* sp and

*Salmonella* sp were isolated in 60% while *Corynebacterium* sp and *Proteus vulgaris* in 40% of the crab samples. The most frequent bacterial isolate from the dog whelk samples is also *V. cholerae* in 80%, and the least frequent are *Pr-*

*oteus vulgaris*, *Corynebacterium* sp, *S. aureus* and *E. coli* in 20%. *Shigella* sp and *Salmonella* sp were isolated in 60% while *P. aeruginosa* and *Bacillus* sp in 40% of the samples (Table 3).

Table 1: Bacterial loads of crab shellfish (*Pachycheles pubescens*)

Sample	THBC (Log <sub>10</sub> CFU/g)	TCC (Log <sub>10</sub> CFU/g)	FCC (Log <sub>10</sub> CFU/g)	SSC (Log <sub>10</sub> CFU/g)	TVC (Log <sub>10</sub> CFU/g)
Crab 1	4.398	4.301	4.079	4.146	4.230
Crab 2	4.322	4.204	4.176	4.079	4.114
Crab 3	4.279	4.114	4.000	4.000	4.079
Crab 4	4.230	4.114	4.114	4.114	4.146
Crab 5	4.176	4.204	4.204	4.041	4.000
Mean	4.281	4.187	4.115	4.076	4.114
SD	0.085	0.078	0.081	0.058	0.085

THBC = Total Heterotrophic Bacterial Count; TCC = Total Coliform Count; FCC = Faecal Coliform Count; SSC = *Salmonella/Shigella* Count; TVC = Total *Vibrio* Count. CFU/g = Colony forming unit/gram, SD = Standard Deviation

Table 2: Bacterial loads of dog whelk shellfish (*Nucella lapillus*)

Sample	THBC (Log <sub>10</sub> CFU/g)	TCC (Log <sub>10</sub> CFU/g)	FCC (Log <sub>10</sub> CFU/g)	SSC (Log <sub>10</sub> CFU/g)	TVC (Log <sub>10</sub> CFU/g)
Dog whelk 1	4.362	4.279	4.114	4.146	4.230
Dog whelk 2	4.279	4.255	4.176	4.041	4.146
Dog whelk 3	4.230	4.204	4.000	4.079	4.114
Dog whelk 4	4.176	4.146	4.079	4.041	4.204
Dog whelk 5	4.114	4.041	4.041	4.000	4.079
Mean	4.232	4.185	4.082	4.062	4.155
SD	0.095	0.095	0.068	0.055	0.062

THBC = Total Heterotrophic Bacterial Count; TCC = Total Coliform Count; FCC = Faecal Coliform Count; SSC = *Salmonella/Shigella* Count; TVC = Total *Vibrio* Count; CFU/g = Colony forming unit/gram; SD = Standard Deviation

Table 3: Frequency of bacteria isolates from shellfish (crab and dog whelk) samples

Bacterial isolates	Crab samples (n=5)	Dog whelk samples (n=5)
<i>Pseudomonas aeruginosa</i>	3 (60.0)	2 (40.0)
<i>Shigella</i> sp	3 (60.0)	3 (60.0)
<i>Vibrio cholerae</i>	4 (80.0)	4 (80.0)
<i>Salmonella</i> sp	3 (60.0)	3 (60.0)
<i>Bacillus</i> sp	1 (20.0)	2 (40.0)
<i>Corynebacterium</i> sp	2 (40.0)	1 (20.0)
<i>Escherichia coli</i>	1 (20.0)	1 (20.0)
<i>Staphylococcus aureus</i>	1 (20.0)	1 (20.0)
<i>Proteus vulgaris</i>	2 (40.0)	1 (20.0)

## Discussion:

Crab and dog whelk are highly nutritious, but susceptible to contamination and spoilage. Bacteriological assessment of samples of crab and dog whelk shellfishes from Okwan-Obolo estuary of Anambra State, Nigeria, shows that the shellfishes harbor various bacterial pathogens, which may have arisen from the contamination and faecal pollution in the ecosystem. Our findings align with those of Leight et al., (18) who reported that pathogenic and indicator bacteria such as the family *Enterobacteriaceae* in aquatic resources originates from the contamination of the water due to human waste products.

Food-borne diseases constitute a serious public health problem at a global level, and seafoods such as crabs are mainly related to outbreaks and cases of diseases including food poisoning. In our study, a high frequency of isolation of *Vibrio* sp was recorded in crab and dog whelk shellfishes. *Vibrio cholerae* is an important microorganism in human, clinical and food safety. The bacterium is capable of developing resistance to antimicrobials and producing toxins thereby generating different disease types including food poisoning from consumption of contaminated seafood (19). *Vibrio cholerae* and *S. aureus* are considered pathogens of cosmopolitan distribution and their main reservoir is human beings (19).

Different measures have been developed and established for the control and prevention of food contamination such as implementation of good hygienic practices, quality assurance system and microbiological criteria for

the acceptance of products for consumption with emphasis on the hygiene of food handlers and storage conditions. *Escherichia coli* in seafood is considered a sanitary case and is a risk to consumers, if related to pathogenic strains especially diarrheagenic *E. coli* (20). However, the presence of non-pathogenic *E. coli* in shellfish is of public health interest since this bacterium is recognized as an indicator organism of faecal contamination suggesting the presence of other enteric pathogens. To ensure that consumed seafood does not harbor *E. coli*, some key measures have to be put in place such as maintaining the microbiological water quality, adequate hygiene conditions in handling process and bacteriological safety during all processes. The isolation of *E. coli* from the crab samples in this study points to the unsanitary status of the seashore where the samples were collected. The isolation of *Vibrio* sp from the samples is in agreement with the study of Vogan et al., (21) who reiterated that crabs such as Dungeness crab (*Cancer magister*), rock crab (*Cancer irroratus*) and tanner crab (*Chionoecetes opilio*) have been found to harbor a variety of Gram-negative and Gram-positive bacteria, and that gills of the fishes were the most common reservoirs of these organisms, including food-borne pathogens such as *Vibrio*, *Aeromonas*, *Acinetobacter*, *Klebsiella* and *Pseudomonas* species.

Another bacterium of public health importance is *Salmonella* specie, the causative agent of salmonellosis, a disease that is characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhoea (22). The predominance of *Salmonella*

in crab in this study is associated with human and animal actions in the beach being an enteric bacterium from flora of both humans and animals (23,24). *Staphylococcus aureus*, *Shigella* and *Salmonella* are pathogens carried by human, and their occurrence in the crab and dog whelk shellfishes can be attributed to improper handling and cross contamination by humans. Considering the large consumption of shellfishes, it is important to create awareness to the public on the health risk of improperly cooked shellfishes. The pathogenic organisms isolated are known to be responsible for many illnesses including infantile diarrhoea, blood stream infections and meningitis by *E. coli* and *S. aureus*, salmonella food poisoning and acute gastroenteritis caused by *Salmonella* species (25). Some of these bacteria are also indigenous bacteria flora in water body, hence are common and widely distributed in the aquatic environment. Maintenance of bacteriological standard for the aquatic ecosystem, good sanitary practices and further processing (preservation) before consumption is necessary to improve the quality of edible shellfishes.

## Conclusion:

Crabs and dog whelk from Okwano-Obolo estuary of Akwa Ibom State, Nigeria, harbor pathogenic bacteria including *Vibrio*, *Salmonella* and *Shigella* implicated in many disease conditions in humans, especially in persons with underlying medical or immunocompromising conditions. It is therefore important that shellfishes harvested from this estuary be properly processed before consumption. A better estuarine management should be framed to prevent transmission of pathogenic bacteria in water and the distributed shellfishes. Proper hygiene should be encouraged and toilet facilities provided in order to reduce contamination of the crab and dog whelk with bacteria associated with human faecal deposits at the sea-shore.

## Contributions of authors:

AN and NA conceptualized the study; AN, NA, CU, DU and EU designed the laboratory methods; SA, AN, and EU collected the data; SA, AN, NA, CU and DU performed laboratory analysis of samples; AN, NA, CU, DU, EU and SA analysed the data; AN, NA, CU, DU, EU performed data validation; AN and SA curated the data and wrote the initial manuscript draft; AN, NA and CU revised the manuscript, and AN supervised the project. All authors agreed to the final manuscript.

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## Conflict of interest:

Authors declared no conflict of interest

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## Short Communication

## Open Access

## Antibiotic susceptibility of uropathogenic *Escherichia coli* isolates in a hospital setting in Ouagadougou, Burkina Faso: A twelve-year retrospective analysis

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### Abstract:

**Background:** *Escherichia coli* is the main bacterium responsible for uncomplicated urinary tract infections (UTI). The increasing frequency of antibiotic resistance in *E. coli* isolates from UTI poses concern in their therapeutic management. The aim of this study is to describe the current antibiotic resistance profile of *E. coli* clinical isolates at the Yalgado Ouedraogo University Hospital of Ouagadougou (CHUYO), Burkina Faso, with a view to revising the treatment protocols for bacterial UTI.

**Methodology:** This was a retrospective review and descriptive study of all *E. coli* isolates from febrile UTI at CHUYO from January 2010 to December 2021. During this period, two techniques were used to perform antibiotic susceptibility test; agar diffusion from 2010 to 2018 and commercial liquid susceptibility testing from 2019 to 2021 using the BD Phoenix M50 automated system. The detection of ESBLs was performed using the Expert System of the automated system or a synergy test combining an amoxicillin-clavulanic acid and a 3<sup>rd</sup> generation cephalosporin (3GC) disc.

**Results:** A total of 2055 non-repetitive strains of *E. coli* were isolated from UTI over the period of study, with 62.0% (1274) of isolates resistant to 3GC. Resistance to 3GC by ESBL production was the most dominant mechanism in 83.1% of cases (1059/1274). Among the 781 (38.0%) 3GC-susceptible isolates, there were high resistance rates to ampicillin (75.2%) and cotrimoxazole (60.2%), but these isolates retained full susceptibility to imipenem (carbapenem) and fosfomycin. As for the 3GC-resistant strains, there was high resistance to cotrimoxazole (93.0%) and ciprofloxacin (90.3%) but relatively low to medium resistance to gentamicin (56.0%) and amikacin (26.3%), and low resistance to nitrofurantoin (10.0%), fosfomycin (6.1%) and imipenem (4.2%). About one third (31.7%) of all the isolates tested were resistant to both ceftriaxone and gentamicin.

**Conclusion:** In view of the results, the implementation of rapid diagnostic tools such as the  $\beta$ -lactamase test to guide empirical antibiotic therapy is essential for an early and efficient management of febrile UTI at the local level in Burkina Faso.

**Keywords:** Urinary tract infection, susceptibility, resistance, *Escherichia coli*,  $\beta$ -lactamase, Burkina Faso

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## Sensibilité aux antibiotiques des isolats d'*Escherichia coli* uropathogènes en milieu hospitalier à Ouagadougou, Burkina Faso: une analyse rétrospective sur douze ans

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## Résumé:

**Contexte:** *Escherichia coli* est la principale bactérie responsable des infections des voies urinaires (IU) non compliquées. La fréquence croissante de la résistance aux antibiotiques dans les isolats d'*E. coli* issus d'infections urinaires pose problème dans leur prise en charge thérapeutique. Le but de cette étude est de décrire le profil actuel de résistance aux antibiotiques des isolats cliniques d'*E. coli* au Centre Hospitalier Universitaire Yalgado Ouedraogo de Ouagadougou (CHUYO), Burkina Faso, en vue de réviser les protocoles de traitement des IU bactériennes.

**Méthodologie:** Il s'agissait d'une revue rétrospective et d'une étude descriptive de tous les isolats d'*E. coli* provenant d'infections urinaires fébriles au CHUYO de janvier 2010 à décembre 2021. Au cours de cette période, deux techniques ont été utilisées pour effectuer un test de sensibilité aux antibiotiques; diffusion sur gélose de 2010 à 2018 et tests commerciaux de sensibilité liquide de 2019 à 2021 à l'aide du système automatisé BD Phoenix M50. La détection des BLSE a été réalisée à l'aide du Système Expert de l'automate ou d'un test de synergie associant un amoxicilline-acide clavulanique et un disque de céphalosporine de 3ème génération (C3G).

**Résultats:** Un total de 2055 souches non répétitives d'*E. coli* ont été isolées d'UTI au cours de la période d'étude, avec 62,0% (1274) d'isolats résistants au C3G. La résistance aux C3G par production de BLSE était le mécanisme le plus dominant dans 83,1% des cas (1059/1274). Parmi les 781 (38,0%) isolats sensibles au C3G, il y avait des taux élevés de résistance à l'ampicilline (75,2%) et au cotrimoxazole (60,2%), mais ces isolats conservaient une sensibilité totale à l'imipénème (carbapénème) et à la fosfomycine. Quant aux souches résistantes au C3G, il y avait une résistance élevée au cotrimoxazole (93,0%) et à la ciprofloxacine (90,3%) mais une résistance relativement faible à moyenne à la gentamicine (56,0%) et à l'amikacine (26,3%), et une faible résistance à la nitrofurantoïne (10,0%), la fosfomycine (6,1%) et l'imipénem (4,2%). Environ un tiers (31,7%) de tous les isolats testés étaient résistants à la fois à la ceftriaxone et à la gentamicine.

**Conclusion:** Au vu des résultats, la mise en place d'outils de diagnostic rapide comme le test de la  $\beta$ -lactamase pour guider l'antibiothérapie empirique est indispensable pour une prise en charge précoce et efficace des IU fébriles au niveau local au Burkina Faso.

**Mots clés:** Infection urinaire, sensibilité, résistance, *Escherichia coli*,  $\beta$ -lactamase, Burkina Faso

## Introduction:

Urinary tract infection (UTI) is one of the most common infections in the world, and one of the first reasons for consultation, making the urine cytobacteriological examination by far the most prescribed bacteriological examination(1). The Gram-negative bacilli (GNB), especially *Enterobacteriaceae*, occupy important place among the bacteria responsible for UTI (2). *Escherichia coli* is both a commensal of the human gastrointestinal tract and the main pathogen responsible for UTI, representing 60-80% of uropathogenic enterobacteria in both the developed and developing countries (3,4). Its high frequency in infections makes it a priority for surveillance and assessment of its level of antibiotic resistance.

The last decades have been marked by a steady increase in antibiotic resistance in clinical *E. coli* strains due to the emergence and dissemination of CTX-M ESBLs worldwide (5,6). The frequent use of carbapenems which are reserved antibiotics for the treatment of severe *E. coli* ESBL infections, is associated with the emergence of carbapenem-resistant strains through production of carbapenemases, membrane impermeability or efflux mechanisms (7).

The third generation cephalosporins (3GC), either as monotherapy or in combination with an aminoglycoside (such as gentamicin), are used in the presumptive treatment

of febrile UTI at Yalgado Ouedraogo University Hospital of Ouagadougou (CHUYO), Burkina Faso. These therapeutic protocols developed on the basis of recommendations from other countries are not always in line with the microbial ecology of our countries. The aim of this study therefore was to describe the antibiotic resistance profiles of *E. coli* strains isolated from UTIs over the last decade to verify the validity of these first-line treatment protocols and to propose adaptive solutions.

## Materials and method:

### Study setting design and period of study:

This was a retrospective study over the period of 12 years (1 January 2010 to 31 December 2021) on all patients who presented with UTI and confirmed by a urine culture in the Bacteriology-Virology Department of the CHUYO.

### Inclusion and exclusion criteria:

Patients whose urine samples met the criteria for UTI were included in this study; leukocyturia greater than  $10^4$  leukocytes /ml and bacteriuria greater than or equal to  $10^3$ ,  $10^4$  or  $10^5$  CFU/ml, depending on the uropathogenic nature of the bacterial species identified (8,9,10,11). All patients with a negative or positive urine on cytobacteriological examination but associated with candidiasis were excluded. Clearly identified duplicates were also excluded (isolation of the same species

with the same susceptibility profile from the same patient).

#### Culture and identification of bacteria:

Bacteria were isolated on CLED and EMB or URSELECT® agar media, depending on their availability. From 2010 to August 2019, bacterial identification was mainly performed by conventional methods including the use of API20E or minimal galleries depending on the availability of inputs. From September 2019 to December 2021, identification and susceptibility testing was performed on the BD Phoenix M50 (Becton Dickinson; New Jersey).

#### Antibiotic susceptibility testing and resistance detection:

With the exception of antibiotic susceptibility tests performed after the acquisition of the BD Phoenix automated system from 2019, all other tests were performed by the agar diffusion method. The following antibiotic discs (ThermoFisher Diagnostics, Oxoid, France) were used; ampicillin (AM) 10µg, amoxicillin-clavulanic acid (AMC) 20-10µg, ceftriaxone (CRO) 30µg, ceftazidime (CAZ) 10µg, cefepime (FEP) 30µg, ceftazidime (FOX) 30µg, imipenem (IMP) 10µg, gentamicin (GN) 10µg, amikacin (AK) 30µg, ciprofloxacin (CIP) 5µg, trimethoprim-sulfamethoxazole (SXT) 1.25 - 23.75µg, nitrofurantoin (NR) 100µg, and fosfomycin (FOS) 200µg.

ESBL screening was performed by a synergy test involving a central disc of amoxicillin/clavulanic acid 30 mm from a disc of 3GC (ceftazidime, ceftriaxone) or 4GC (cefepime). For liquid antibiotic susceptibility testing, ESBL detection was performed using the BD Phoenix M50 Expert System (Becton Dickinson; New Jersey) but the cartridges used for antibiotic susceptibility testing did not allow the detection of carbapenemases or cephalosporinases (ampC). The interpretation of the susceptibility test was performed according to the 2019 guideline of the European Committee for Antimicrobial Susceptibility Testing

#### Statistical analysis:

The data for this study were entered

into Excel 2013 and analysed with EPI-INFO 7.2.4.0. Tables and graphs were produced. The Chi-square test used to measure association between the different proportions, with value of  $p < 0.05$  considered as statistical significance.

#### Ethical considerations:

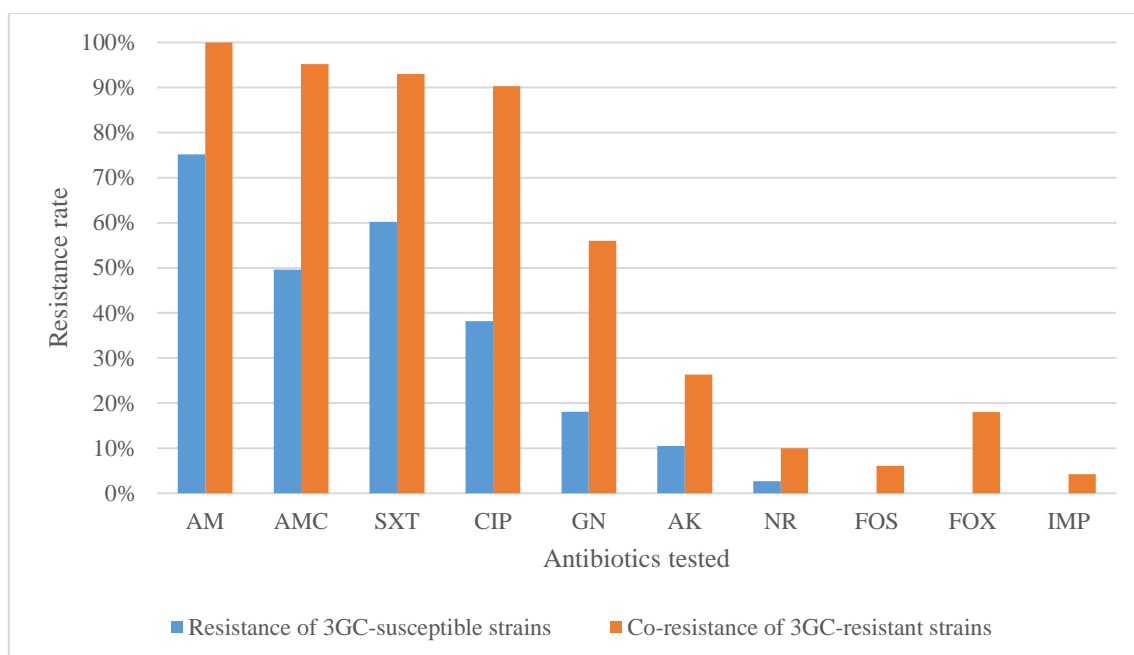
Personal data collected from patients were not disclosed.

#### Results:

A total of 2947 family *Enterobacteriaceae* were isolated out of which 2055 (69.7%) were non-repetitive *E. coli* isolates. A total of 1274 (62%) *E. coli* isolates were resistant to 3GCs while 781 (38%) were susceptible. Resistance by beta-lactamase production (78.4%, 1611/2055) was the main mechanism of resistance, of which 51.5% (1059/2055) were by ESBLs and 26.9% (552/2055) penicillinases. Resistance to 3GC by ESBL production was the dominant mechanism (1059/1274; 83.1%).

Among the 781 3GC-susceptible isolates, the rate of resistance was high to ampicillin (75.2%) and cotrimoxazole (60.2%), moderate resistance to amoxicillin/clavulanic acid (49.6%) and ciprofloxacin (38.2%), and low resistance to gentamicin (18.1%), amikacin (10.5%) and nitrofurantoin (2.7%). These isolates showed no resistance to carbapenems, cephamycins and fosfomycin (Fig 1).

The 3GC-resistant isolates showed a high resistance rate to amoxicillin-clavulanic acid (95.2%), cotrimoxazole (93.0%) and ciprofloxacin (90.3%), moderate resistance rates to aminoglycosides, with a large disparity between gentamicin (56%) and amikacin (26.3%). However, the resistance rate was low to ceftazidime (18%), nitrofurantoin (10%), fosfomycin (6.1%) and imipenem (4.2%) (Fig 1). Of the 1800 isolates tested for susceptibility to both gentamicin and ceftriaxone, one third of were resistant to both antibiotics and less than 30.0% of the isolates were susceptible to both (Table 1).



AM: ampicillin, AMC: amoxicillin-clavulanic acid, SXT: cotrimoxazole, CIP: ciprofloxacin, GN: gentamicin, AK: amikacin, NR: nitrofurantoin, FOS: fosfomycin, FOX: ceftiofur, IMP: imipenem

Fig 1: Comparison of the resistance rates of 3GC-resistant versus 3GC-sensitive *Escherichia coli* isolates to different antimicrobial agents

Table 1: *In vitro* efficacy of combined ceftriaxone and gentamicin against *Escherichia coli* isolates

Phenotypes	Number of isolates	Percentage
CRO <sup>S</sup> and GN <sup>R</sup>	243	13.5
CRO <sup>R</sup> and GN <sup>S</sup>	448	24.9
CRO <sup>S</sup> and GN <sup>S</sup>	538	29.9
CRO <sup>R</sup> and GN <sup>R</sup>	571	31.7
<b>Total</b>	<b>1800</b>	<b>100.0</b>

GN: Gentamicin; CRO: Ceftriaxone; S: Susceptible; R: Resistant

## Discussion:

The resistance profiles of uropathogenic *E. coli* isolates vary considerably in time, and space. The knowledge of the local epidemiology and its evolution is necessary for the choice of an effective and adapted empirical antibiotic therapy. The result of our study showed a predominance of *E. coli* (69.7%) in UTIs caused by enterobacteria. The predominance of *E. coli* in the bacteriological profile of UTIs has been reported in several studies in proportions ranging from 60% to more than 80% of enterobacteria (4,12). This predominance can be explained by the presence of virulence factors such as adhesins (fimbriae or pili) and toxins in the strains responsible for extra-intestinal pathologies (13). The role of type 1 pili of *E. coli* in lower UTI such as cystitis has been widely documented. Indeed, its adhesin, FimH, attached to the top of the pili, specifically binds to D-mannose residues attached to

the surface of membrane glycoproteins lining perineal and bladder cells resulting in bacterial persistence and enhanced inflammatory response to infection (14).

The main mechanism of antibiotic resistance in *E. coli* observed in this study was enzymatic resistance through the production of beta-lactamases. Indeed, analysis of the data shows that resistance by ESBL production (51.5%) and penicillinases (26.9%) were the most frequent mechanisms. Similar results were reported in Antananarivo, Madagascar on uropathogenic *E. coli* strains with respective frequencies of 22.5% of ESBL-producing strains against 55.9% of penicillinase-producing strains, 50% of which was high-level resistance penicillinases (15).

Aminopenicillin (ampicillin and amoxicillin/clavulanic acid) resistance was the most common, with a rate of 75.2% among 3GC-susceptible isolates for ampicillin and 49.6% for amoxicillin/clavulanic acid, with 100% and

95.0% among 3GC-resistant isolates respectively. Penicillinase resistance in *E. coli* has long been described and is most commonly associated with TEM penicillinases (15,16). However, the emergence and spread of this acquired resistance is thought to be a consequence of the overuse of aminopenicillins through selection pressure.

About 62% of the *E. coli* isolates were resistant to 3GCs and in 83.1% of cases, this resistance was associated with ESBL production. Sbiti et al., (17) reported high rates of 3GC resistance of up to 80% among ESBL producing strains. The emergence and spread of ESBLs in *E. coli* is thought to be due to the advent of CTX-M ESBLs in the 1990s, which are genetically supported by a plasmid, but also due to the selection pressure caused by the overuse of antibiotics (6). The high incidence of resistance to 3GCs considerably reduces the therapeutic options and maintains a continuous increase in the prescription of carbapenems which retain good activity in most cases (18). Indeed, over the study period 1.9% of *E. coli* isolates were resistant to imipenem. These data show the emergence of pan-resistance phenotype to beta lactam antibiotics, often combining several mechanisms, including production of ESBLs or the co-production of ESBLs and carbapenemases, as well as impermeability of bacteria to antibiotics (19). The use of rapid diagnostic tools such as the  $\beta$ -lacta test (Bio-Rad, France) for the early detection of bacteria resistant to 3GCs through production of beta-lactamases will allow an appropriate choice of first-line antibiotics and the preservation of last-resort drugs (20).

The rate of resistance to fluoroquinolones (ciprofloxacin) was very high in 3GC resistant isolates (90.3%) in contrast to the 3GC-sensitive strains (38.2%). Similar results were reported by Sbiti et al., (17) and El Bouamri et al., (21) who reported in their studies, rates of ciprofloxacin co-resistance ranging from 82% to 92.5% in ESBL producing strains. The co-resistance of ESBL-producing strains is thought to be linked to the presence of mobile *qnr* genetic elements (A, B, S alleles) and especially the *aac(6')-Ib-cr* gene which most often co-exists with CTXM-type ESBL genes and confers dual aminoglycoside-piperazinylamine-quinolone resistance (22). In addition, the high prescription of fluoroquinolones in the treatment of UTIs caused by enterobacteria, in this case *E. coli*, is not to be outdone (12). High-level resistance in all fluoroquinolones is most often related to cumulative mutations in DNA gyrase A or B (*gyrA/gyrB*) and *parC* (23).

Almost all family *Enterobacteriaceae* are naturally susceptible to aminoglycosides. Most of the observed resistance is attributable to the acquisition of modifier enzymes. Aminoglycoside co-resistance in our study varied significantly depending on the molecule and

the bacterial phenotype towards beta-lactams. Indeed, 3GC-resistant isolates had relatively high rates of associated resistance, 56.0% for gentamicin versus 18.1% for amikacin. The 3GC-sensitive isolates had resistance rates to aminoglycosides of 26.3% and 10.5% for gentamicin and amikacin respectively. Belmonte et al., (24) and Sbiti et al., (17) reported that 67.2%-75% of 3GC-resistant strains of family *Enterobacteriaceae* through ESBL production were resistant to gentamicin compared to 6%-18% for amikacin. However, a low rate of resistance to amikacin (14.3%) was observed overall in our study. This pattern was reported by Sbiti et al., (17) and Nana et al., (1) with rates of 6.1% and 10.4% respectively. The high variability of resistance to gentamicin and amikacin is thought to be associated with aminoglycoside acetylases of the AAC(3) type that spare amikacin and/or AAC(6') with amikacin AAC(6')-I and gentamicin AAC(6')-II as substrates. The high resistance to gentamicin in our study is thought to be due to the presence of AAC(3) and/or AAC(6')-II acetylases.

Analysis of combined *in vitro* efficacy of ceftriaxone and gentamicin commonly used as empirical treatment for febrile UTIs showed that less than 30% of *E. coli* isolates were sensitive to both drugs, compared to 31.7% of cases of associated resistance. This combined antibiotics, long considered as the empirical antibiotic therapy of choice during invasive infections in many departments of the CHUYO, is now experiencing enormous limitations due to the multi-resistance of bacteria, in this case *E. coli*.

## Conclusion:

*Escherichia coli* is the main uropathogenic bacterium observed at the CHUYO in Ouagadougou, Burkina Faso. The production of beta-lactamases is its main mechanism of resistance to beta-lactams. Antibiotics for the treatment of uncomplicated UTIs, notably fosfomycin and nitrofurantoin remained active on *E. coli* isolates in the present study but about one third of the isolates were resistant to both ceftriaxone and gentamicin, which are generally used in synergy for therapy of febrile UTIs at UHYO.

The implementation of rapid diagnostic tools such as the  $\beta$ -lacta test (Bio-Rad, France) for rapid identification of resistance, will guide empirical antibiotic therapy, which is essential for early and effective management of febrile UTIs at local level in Burkina Faso.

## Contributions of authors:

HK was involved in data compilation, analysis, writing of the manuscript; MO was involved in data compilation; MK, YDC, KO,

RO, MS and CG were involved in sample analysis; LS was involved in supervision of the project and correction of the manuscript; ASO was involved in coordination of the surveillance of antibiotic resistance throughout the national territory and correction of the manuscript; and IS was involved in correction of the manuscript. All authors approved the final manuscript submitted.

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## Conflicts of interest:

Authors declare no conflict of interest

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Copyright AJCEM 2023: <https://dx.doi.org/10.4314/ajcem.v24i2.12>**Case Series****Open Access****Malaria treatment failure after Artemisinin-based combination therapy: A case series of children managed at a private tertiary hospital in southwest Nigeria**<sup>1,2\*</sup>Fatunla, O. A. T., <sup>3,4†</sup>Irek, E. O., <sup>1,2</sup>Oyebanji, A. H., <sup>2</sup>Adisa, S. O., <sup>5</sup>Jesulana, T. E., and <sup>2</sup>Ajibola, T. O.<sup>1</sup>Department of Paediatrics, Faculty of Clinical Sciences, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria<sup>2</sup>Department of Paediatrics, Afe Babalola Multi-System Hospital, Ado-Ekiti, Nigeria<sup>3</sup>Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria<sup>4</sup>Department of Medical Microbiology and Parasitology, Afe Babalola Multi-System Hospital, Ado-Ekiti, Nigeria<sup>5</sup>Department of Internal Medicine, Afe Babalola Multi-System Hospital, Ado-Ekiti, Nigeria\*Correspondence to: [odunayofatunla@abuad.edu.ng](mailto:odunayofatunla@abuad.edu.ng); +2348034397539; ORCID: <https://orcid.org/0000-0002-2839-1491>

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**Abstract:**

Malaria treatment failure is the inability to clear parasitaemia after antimalarial drug administration. There are reports of treatment failure with artemisinin-based combination therapy (ACT) in Nigeria but few reported among children. We report three paediatric cases of treatment failure with ACT admitted at a private tertiary hospital in Nigeria in early 2022. All three were 'under-fives' admitted for open-heart surgery, major flame burns, and cerebral malaria respectively. They had symptomatic *Plasmodium falciparum* infection but one had mixed *P. falciparum* and *Plasmodium vivax* infections. Cases 1 and 2 were initially given oral artemether-lumefantrine while case 3 received intravenous artesunate. Despite appropriate antimalarial drug compliance, all the 3 still had fever with heavy parasitaemia. They subsequently received intravenous quinine, with improvement within the first 24 hours of therapy, and no longer had fever at the fourth week of follow-up. Although ACT resistance was not established, poor drug quality may have contributed to treatment failure. There is a need for pharmacovigilance of anti-malarial in Nigeria.

**Keywords:** Artemisinin; Quinine; Malaria; *Plasmodium vivax*; Treatment failure; Nigeria

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## Résumé:

L'échec du traitement du paludisme est l'incapacité à éliminer la parasitémie après l'administration d'un médicament antipaludique. Il y a des rapports d'échec de traitement avec la thérapie combinée à base d'artémisinine (ACT) au Nigeria, mais peu ont été signalés chez les enfants. Nous rapportons trois cas pédiatriques d'échec de traitement avec ACT admis dans un hôpital tertiaire privé au Nigeria au début de 2022. Tous trois étaient des « moins de cinq ans » admis pour une chirurgie à cœur ouvert, des brûlures graves par la flamme et un paludisme cérébral, respectivement. Ils avaient une infection symptomatique à *Plasmodium falciparum* mais un avait des infections mixtes à *P. falciparum* et *Plasmodium vivax*. Les cas 1 et 2 ont initialement reçu de l'artéméter-luméfantrine par voie orale tandis que le cas 3 a reçu de l'artésunate par voie intraveineuse. Malgré une bonne observance des médicaments antipaludiques, tous les 3 avaient encore de la fièvre avec une forte parasitémie. Ils ont ensuite reçu de la quinine par voie intraveineuse, avec une amélioration dans les 24 premières heures de traitement, et n'avaient plus de fièvre à la quatrième semaine de suivi. Bien que la résistance à l'ACT n'ait pas été établie, la mauvaise qualité des médicaments peut avoir contribué à l'échec du traitement. Il y a un besoin de pharmacovigilance des antipaludéens au Nigeria.

**Mots clés:** Artémisinine; Quinine; Paludisme; *Plasmodium vivax*; Échec du traitement; Nigeria

## Introduction:

Malaria treatment failure poses a threat to malaria control and global eradication. Treatment failure in malaria is the inability to clear malaria parasitaemia or prevent recrudescence in an individual after administration of an anti-malarial (1). Early treatment failure is parasitaemia with symptoms of malaria occurring within three days of commencement of treatment while late clinical failure is presence of parasitaemia with symptoms of malaria occurring on any day between day 4 and day 28 in patients who did not meet criteria for early treatment failure (2). The symptoms include fever (axillary temperature of  $\geq 37.5^{\circ}\text{C}$ ), danger signs or features of severe malaria (2,3). If an individual still has parasitaemia on any day between day 7 and day 28 but without fever, it is termed late parasitological failure parasitaemia (2).

There are many factors that can cause malaria treatment failure, these include incorrect dosing, poor drug compliance, poor drug quality, drug interactions and drug resistance (1,4). There are reports of resistance to all the groups of anti-malarial drugs including artemisinins recommended by the World Health Organization (WHO) for the treatment of uncomplicated malaria (1,5). In Nigeria, reports of malaria treatment failure with artemisinin-based combination therapy (ACT) have been documented (1,6–8), but there are very few reported incidences among children, who have the highest malaria burden.

Between January and March 2022, three paediatric patients were managed for malaria treatment failure associated with artemisinin-based combination therapy (ACT) at the Afe Babalola University Multi-System Hospital, Ado-Ekiti (AMSH), a privately owned tertiary facility in Southwest Nigeria. We report these cases and the management challenges, and suggest ways

of circumventing treatment failure of malaria in children.

## Case presentations:

### Case 1:

Case 1 is KO, a 7-month-old boy who had open heart surgery for tetralogy of Fallot in January 2022 in AMSH. He had blood transfused intra-operatively and was noticed to have developed intermittent fever a week after the surgery while on admission. He was conscious and otherwise clinically stable post-operatively except for fever that ranged from  $37.5^{\circ}\text{C}$  to  $38.9^{\circ}\text{C}$ . Blood film for malaria parasite revealed presence of trophozoites of *Plasmodium falciparum*. He weighed 5kg and was therefore administered oral combination of artemether 20mg and lumefantrine 120mg at 0 hour, then 8 hours after first dose then twice daily for a total duration of 3 days. Fever still persisted despite completion of antimalarial. Blood culture sample was obtained but yielded no growth.

Four days after the completion of the oral ACT, a quick bedside rapid diagnostic test (Onsite® Rapid Test, CTK Biotech, USA) done was positive for *P. falciparum* and *P. vivax*. This was corroborated by microscopic blood film that revealed trophozoites of mixed species of *P. falciparum* and *P. vivax*, with a parasite density of 5,820 parasites/ $\mu\text{L}$  [white blood cell (WBC) count of 9,700 cells/ $\mu\text{L}$ ]. He was then commenced on intravenous (IV) quinine 50mg 8-hourly delivered in intravenous 10% dextrose in water. Fever subsided within 12 hours of commencement of quinine and no parasite was seen in the repeat blood film performed afterwards. He received six IV doses of quinine and was discharged on oral quinine 50mg three times a day to complete 21 doses (including the IV formulations). He was followed up for four weeks and remained fever-free throughout the period.



**Case 2:**

Case 2 is TA, a 33-month-old girl who was on admission for major flame burns with inhalational injury in February 2022, developed intermittent fever on the 7<sup>th</sup> day of admission with highest temperature reading of 37.8°C at that time. She was conscious with an estimated weight of 15kg and was given oral combination of artemether 40mg and lumefantrine 240mg at 0 hour, then 8 hours after first dose then twice daily for a total duration of 3 days. The fever subsided from the second day of commencing the ACT until the last dose of ACT was taken.

Three days afterwards, on the 12<sup>th</sup> day of admission, she developed fever again with a temperature range of 37.8°C to 40.0°C. Repeat blood film revealed trophozoites of *P. falciparum* and was given three doses of IV artesunate 40 mg at 0, 12<sup>th</sup> and 24<sup>th</sup> hour. Blood culture sample taken at this time yielded growth of *Escherichia coli* sensitive to piperacillin-tazobactam, imipenem and levofloxacin, and was consequently given IV imipenem 250mg 6hrly. Nevertheless, fever still persisted after the third dose of IV artesunate, and despite IV antibiotics temperature was as high as 40.0°C. Another blood sample was then obtained for blood film microscopy which still revealed >10 parasites/high power field trophozoites of *P. falciparum*, with a parasite density of 20,849 parasites/μL (WBC count of 16,100 cells/μL). Subsequently, IV quinine 150mg 8hourly was administered in IV 10% dextrose in water. Fever subsided after the second dose of IV quinine. She received 6 doses intravenously and 15 oral doses of syrup quinine 150mg three times a day. She spent another four weeks on admission for burns but remained fever-free throughout.

**Case 3:**

Case is IJ, a 19-month-old boy referred to AMSH in March 2022. He had been on admission at the referring health facility for a week, where he had presented with a week history of fever, and had one episode of generalized tonic-clonic seizure that lasted for 15 minutes which was aborted at the referring facility. He subsequently lapsed into unconsciousness and remained so at presentation in AMSH. The referral note stated that he had microscopy done that revealed hyper parasitemia, for which he was given IV artesunate 30mg stat, at 12<sup>th</sup> and 24<sup>th</sup> hour and then daily for six days. His cerebrospinal fluid (CSF) was also analyzed but was said to be normal, nonetheless, he was given IV antibiotics at the referring facility.

At admission in AMSH, he was still unconscious, with a Glasgow Coma Score (GCS) of 8, febrile with a temperature of 39.1°C and

weighed 8kg. There was no evidence of raised intracranial pressure and no sign of meningeal irritation. Thick blood film showed trophozoites of *P. falciparum*, with parasite density of 30,562 parasites/μL (WBC count of 23,600 cells/μL) but the CSF findings were essentially normal. His cranial magnetic resonance imaging (MRI) revealed only cerebral oedema.

He was nursed as an unconscious patient and managed for cerebral malaria with IV quinine 160mg loading dose then 80mg 8hourly, administered in 10% dextrose water. Fever began to subside after six hours of commencement of quinine and GCS improved to 10 within 24 hours of therapy. He remained fever-free and regained full consciousness on the fourth day of admission after receiving 9 doses of intravenous quinine, and was subsequently commenced on oral quinine 80mg three times a day to complete a total of 21 doses of quinine. However, he had some neurologic deficits including cortical blindness and cortical deafness with regression of previously attained milestone that resulted from cerebral malaria. On the eighth day of admission, there were improvements with his vision and hearing and he was subsequently discharged home. He maintained normal temperature even at the fourth week of follow-up.

**Clinical and laboratory characteristics of the patients:**

The blood films for malaria parasites of all the three patients were made and read by one of the authors (a consultant medical microbiologist at AMSH). Cases 1 and 3 had early treatment failure while case 2 had late clinical failure. The blood glucose measurements of all the patients were normal during parenteral quinine administration and thereafter. One of the cases who could communicate verbally (this excludes the infant and the patient managed for cerebral malaria), did not complain of tinnitus or hearing impairment. All the three cases had auditory assessments that were grossly normal, besides the case with cerebral malaria who had cortical deafness that improved over time. None of the cases had history to suggest glucose-6-phosphate dehydrogenase (G6PD) deficiency, neither did any of them exhibit clinical features to suggest hemolysis induced by quinine administration.

**Discussion:**

This case series is a report of three cases of malaria treatment failure managed at the department of paediatrics of a private tertiary hospital in southwest Nigeria. These cases

were managed within the first three months of the year 2022, during the dry season, when malaria transmission is supposedly low (9). All three patients were younger than five years but only one of them had severe malaria. This buttresses the observation that the burden of malaria is high among the 'under-fives' (10).

All the patients were initially given artemisinin-based medications as first line treatment according to the WHO recommendation, even in the management of *P. vivax* infection (5). The poor responses to this first line treatment in this case series may be due to various factors including the use of poor-quality medicine. Poor-quality drugs (counterfeit or sub-standard) have been documented as causes of treatment failure and development of resistance (5). In addition to this, some practices such as the indiscriminate use of ACT for malaria prophylaxis as observed in a rural community in southwest Nigeria (11) or the over-diagnosis and presumptive treatment of clinical malaria (5) may contribute to development of resistance to artemisinins. There are several studies that have inferred a possibility of artemisinin resistance in Nigeria (6–8), nonetheless, artemisinin-resistance is yet to be established in this part of the world (5). There is a need for effective pharmacovigilance in the community to curb the development of artemisinin resistance, based on possible adulteration of antimalarials.

There are alternatives to the use of ACTs, which include primaquine, combination of full doses of parenteral artesunate and parenteral quinine for severe malaria, and new combinations of artesunate and pyronaridine (5). Primaquine is known to achieve radical cure in cases of *P. vivax* and to clear gametocyte stages of *P. falciparum* very rapidly (2,5,12). Unfortunately, this drug is not routinely prescribed in Nigeria (13) and was not available for use at the time. Primaquine unavailability may be due to lack of diagnostic tools for *P. vivax* and reduced expertise, hence under-diagnosis of non-falciparum plasmodia species.

The new combination of artesunate and pyronaridine was not available either, hence quinine (initially parenteral then oral) was administered to all the three patients that demonstrated malaria treatment failure to artemisinin-based treatment and good responses were achieved. Besides, this new combination is also an ACT and there is a possibility of cross-resistance if this drug was used assuming genetic mutations in ACTs were responsible for the treatment failure. There is a need for molecular analyses for artemisinin-resistance in Nigeria so that policies regarding malaria treatment failure can be reviewed as necessary.

Although, chloroquine has good coverage of *P. vivax*, its use for malaria treatment is prohibited in Nigeria (14,15) because of drug resistance. It may become imperative to cautiously review the role of chloroquine in the treatment of *P. vivax* infection given the recent increasing reports of *P. vivax* infection in Nigeria (16–19). Quinine, on the other hand, is an anti-malarial that has been in use for over a century, yet there is dearth of reports of quinine-resistance in recent times since the early observations during the first world war (20). Perhaps side effects of quinine, such as bitter taste of the oral formulation, hypoglycaemia, tinnitus, hearing impairment, among others and the cumbersome of IV administration deter the abuse of this medication. In addition, the WHO recommendation of the artemisinins as first line anti-malarial may have preserved the effectiveness of quinine in the treatment of malaria. All the patients in this case series tolerated quinine therapy with good response and none had any untoward side effect. This case series supports the use of quinine in the treatment of children with malaria treatment failure as recommended by the WHO (4,5).

## Conclusion:

This case series reports malaria treatment failure among three children managed at a private tertiary hospital in Nigeria, one of whom had mixed *P. falciparum* and *P. vivax* infection. There were remarkable clinical and parasitological improvements with the use of quinine in managing malaria treatment failure in the three children. Although, inherent drug resistance to ACTs administered could not be ruled out completely, there is a possibility that poor drug quality via adulteration contributed to the treatment failure seen in these patients.

There is a need for pharmacovigilance in the country, to promptly mitigate factors that contribute to malaria treatment failure. There should be more studies to review the effectiveness of ACTs in the treatment of malaria especially in the wake of reports of treatment failures. There is also a need to ease the availability of primaquine for the increasing prevalence of *P. vivax* infections in Nigeria.

## Consent for publication:

Written informed consent for publication was obtained from the parents of the three children.

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### Authors' contributions:

All authors have made substantial contributions to this manuscript. OATF, EOI and AHO designed the study. OATF drafted the manuscript and all authors were involved revising it. EOI prepared and interpreted all the peripheral blood films. All authors were involved with data retrieval. All authors read and approved the final version of the manuscript, and agree to be personally accountable for their respective contributions.

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### Conflicts of interests:

Authors declare no conflict of interests.

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## **AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY**

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