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All correspondence to Editor-in-Chief
Department of Medical Microbiology,
LAUTECH Teaching Hospital,
PMB 4007, Ogbomoso, Nigeria
Email: afrcjem@gmail.com and ajcem2019@gmail.com
Mobile: +234 (0) 8033436344
OR
Department of Medical Microbiology,
College of Health Sciences,
LAUTECH, PMB 4400, Osogbo, Nigeria
E-mail: sstaiwo@lautech.edu.ng

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**Review Article****Open Access****Antimicrobial resistance in Madagascar: a review of the current situation and challenges**

Rasamiravaka, T.

Laboratory of Biotechnology and Microbiology, Department of Applied and Fundamental Biochemistry,
Faculty of Sciences, University of Antananarivo (UA), BP 906, Antananarivo 101, MadagascarCorrespondence to: travaka@yahoo.fr**Abstract:**

Antimicrobial resistance (AMR) is a growing public health threat worldwide occurring in a wide range of pathogenic bacteria. It is encouraging that governments of countries around the world are beginning to pay attention to the issue of AMR that serves to undermine the future of modern medicine. However, each country solution approaches to this issue will differ in terms of magnitude and response capacity. Madagascar is a low-income country and one of the poorest countries in the world with poor environmental hygiene practices and easy availability of antimicrobial drugs without medical prescription. These particular contexts certainly influence the spread of multi-drug resistant bacteria. This review presents reported data on AMR from 2001 to 2018 in Madagascar among the World Health Organization (WHO) priority human pathogens, and determined the scope and magnitude of the AMR problems in the particular context of this low-income country, which could help in formulating effective response strategies for control of AMRs in Madagascar.

Key words: Antimicrobials, Madagascar, Multi-drug resistance, Prevalence

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Résistance aux antimicrobiens à Madagascar: bilan de la situation actuelle et des défis

Rasamiravaka, T.

Laboratoire de biotechnologie et de microbiologie, Département de biochimie appliquée et fondamentale,
Faculté des sciences, Université d'Antananarivo (UA), BP 906, Antananarivo 101, MadagascarCorrespondance à: travaka@yahoo.fr**Abstrait:**

La résistance aux antimicrobiens (RAM) est une menace croissante pour la santé publique dans le monde, qui se produit dans un large éventail de bactéries pathogènes. Il est encourageant de constater que les gouvernements des pays du monde commencent à prêter attention à la question de la RAM qui sert à saper l'avenir de la médecine moderne. Cependant, chaque approche de solution de pays à ce problème sera différente en termes d'ampleur et de capacité de réponse. Madagascar est un pays à faible revenu et l'un des pays les plus pauvres du monde avec de mauvaises pratiques d'hygiène environnementale et des médicaments antimicrobiens facilement disponibles sans prescription médicale. Ces contextes particuliers influencent certainement la propagation de bactéries multirésistantes. Cette revue présente les données rapportées sur la RAM de 2001 à 2018 à Madagascar parmi les agents pathogènes humains prioritaires de l'Organisation mondiale de la santé (OMS), et a déterminé la portée et l'ampleur des problèmes de RAM dans le contexte particulier de ce pays à faible revenu, ce qui pourrait aider à formuler des stratégies de réponse efficaces pour le contrôle des RAM à Madagascar.

Mots-clés: Antimicrobiens, Madagascar, Multi-pharmacorésistance, Prévalence**Introduction:**

Antimicrobial resistance (AMR) is increasing worldwide and represents a major

threat to public health (1). Indeed, in the European Union and the United States, antibiotic resistance is reported to cause over 25,000 and 23,000 deaths per year respect-

ively (2). In India, over 58,000 babies were reported to have died in one year as a result of infection caused by resistant bacteria passed from the mothers to babies (3). Thus, AMR is a worldwide concern and governments around the world are beginning to pay attention to this colossal issue.

Madagascar is the fourth largest island in the world located in the Indian Ocean (IO) off the eastern coast of southern Africa and east of Mozambique. Malagasy population is estimated around 25 million with various and mixed culture demise by Malayo-Indonesian, African and Arab ancestry. Living on an isolated island, Malagasy people are not sheltered from this AMR threat. Although no study has shown evidence that linked infectious disease death and antibiotic resistance, several studies report the presence of multi-drug resistant (MDR) bacteria in Malagasy community (4-7).

Recently, Gay et al., (4) reviewed the antimicrobial resistance in IO including Madagascar and pointed out the threatening presence of AMR in IO, although the report was to be interpreted with caution. However, particular context of each country that may constitute main factors contributing to the development of antibiotic resistance have not been addressed. For instance, Madagascar is a low-income country and one of the poorest countries in the world with poor environmental hygiene practices and high availability of antimicrobial drugs without medical prescription.

Tackling AMR progression surely represents a big challenge that needs deep context analysis and concerted efforts of local authorities and large panel of researchers in

different areas of expertise. This review summarizes epidemiological knowledge and trends of AMR in Madagascar from 2001 to 2018 focusing on WHO antibiotic-resistant "priority pathogens" for research and development (R & D) for new antibiotics (8). The particular socio-economic background of the country and the management of bacterial infections are also discussed.

Methodology:

We conducted a bibliographic search for relevant available articles obtained through match searches using Google Scholar and PubMed. Relevant information was obtained for phenotypic and genotypic profiles (if available) of resistance in bacteria defined as 'critical', 'high' and 'medium priority' by the WHO (Table 1). Related terms such as 'prevalence', 'resistance', 'antimicrobial', 'antibiotic', 'epidemiology', 'Madagascar', 'African', and 'Indian Ocean' were also used to search for additional data.

We considered only strains isolated from humans and excluded strains from animals, food and food products. Considering the low rate of publication by Malagasy researchers, unpublished results presented in international congresses and seminars were included. However, data reported through national doctorate and/or master defenses (n=10) which were also referenced in Google results and/or Malagasy University websites were excluded. In total, 24 published articles and international congresses and seminars reports were included in the review.

Table 1: WHO priority pathogens list for Research and Development (R & D) of new antibiotics (5)

Priority	Pathogen
1: Critical	<i>Enterobacteriaceae</i> , carbapenem-resistant, ESBL-producing <i>Acinetobacter baumannii</i> , carbapenem-resistant <i>Pseudomonas aeruginosa</i> , carbapenem-resistant
2: High	<i>Staphylococcus aureus</i> , methicillin-resistant, vancomycin-intermediate and resistant <i>Enterococcus faecium</i> , vancomycin-resistant <i>Helicobacter pylori</i> , clarithromycin-resistant <i>Campylobacter</i> spp., fluoroquinolone-resistant <i>Salmonellae</i> , fluoroquinolone-resistant <i>Neisseria gonorrhoeae</i> , cephalosporin-resistant, fluoroquinolone-resistant
3: Medium	<i>Shigella</i> spp., fluoroquinolone-resistant <i>Streptococcus pneumoniae</i> , penicillin-non-susceptible <i>Haemophilus influenzae</i> , ampicillin-resistant

WHO 'critical priority' pathogens in Madagascar:

Enterobacteriaceae: carbapenem - resistant; ESBL-producing

The primary cause of resistance among members of the family Enterobacteriaceae is β -lactamase production. In recent years, β -lactamases have extensively diversified due to the extensive use of β -lactams in hospitals (9). One of the most threatening β -lactamase is the extended-spectrum β -lactamase (ESBL) which confers resistance in Enterobacteriaceae to β -lactam antibiotics and carbapenems, except to cephamycin, but inhibited by clavulanic acid (10).

In Madagascar, ESBL producing Enterobacteriaceae (ESBL-PE) was first isolated in urinary tract infections between 2004 and 2006 (11). Following that period, high fecal carriage of ESBL-PE was identified in both community (Table 2) and hospitals (Table 3). For instance, a prevalence of 21.3% was reported in two hospitals of Antananarivo from 2006 to 2008 (12). The same trend (21.2%) was reported in a pediatric hospital in 2008 (5) whereas 10.1% was reported by Herindrainy et al., (6) in community setting investigation in 2009. Between 2013 and 2014, 18.5% of rectal colonization by ESBL-PE was estimated

among pregnant women at delivery (7). Another study conducted among patients, healthcare workers and students reported 7.1% of Enterobacteriaceae nasal carriage resistance to third generation cephalosporin (3GC) in patients at admission (13). A study conducted in neonatal units of two different hospitals in Antananarivo from 2012 to 2013 reported the presence of ESBL-PE in early neonatal infection (12.9%). Due to the lack of carbapenems, these infections were treated with extended spectrum cephalosporins resulting in high mortality rate of 45% (14).

ESBL-PE in Madagascar mostly belong to the CTX-M-15 type (12,15) which is widely distributed worldwide (16), followed by SHV-12 type, whereas one New Delhi metallo- β -lactamase-1 (NDM-1)-producing Enterobacteriaceae (*Klebsiella pneumoniae*) was isolated among pregnant women in the community (12). Additionally, the first carbapenem resistant Enterobacteriaceae (CRE) was reported in a community survey of uropathogens implemented in 2011–2013 (17). Imipenem resistance rate was 40% for *K. pneumoniae*, 15% for *Enterobacter cloacae* and 2.3% for *Escherichia coli*. However, the small sample size for this study could not reflect global resistance patterns. Overall, 5.7% (871/15,100) of the population studied had ESBL-PE isolated.

Table 2: Evolution of antibiotic resistance of Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from the community between 2001 and 2018 in Madagascar

Town, location	Population (sex, age or mean age)	Date	Population number	Study design	Sample type	ESBL strains	CRE strains	CRP	CRA	Resistant to CIPRO	References
Antananarivo	M/F (Mean age 33 years old)	2004–2006	6884	Laboratory surveillance	urine	29	NI	NI	NI	120	23
Multi-location ^a	M/F (under 5 years old)	2008–2009	2692	Cross-sectional study	stool	7	NI	NI	NI	7 ^b	24
Antananarivo	M/F (Mean age 28 years old)	2009	484	Cross-sectional study	stool	53	0	NI	NI	31	6
Antananarivo	M/F (1 to 89 years old)	2011–2013	335	Laboratory surveillance	urine	79	15	NI	0	154	17
Antananarivo	M/F (Age not communicated)	2006–2007	79	Laboratory surveillance	Various bacteriological specimens	NI ^d	0	0	0	NI	15
Antananarivo (Moramanga)	Pregnant women (mean age 26 years old)	2013–2014	356	Cohort study	Stool	66	1 ^c	NI	NI	24 ^b	7
Antananarivo	M/F students, health care workers and patients (15 to 84 years old)	2015	1710	Cohort study	nasal	50	0	0	0	12 ^b	13
Antananarivo	Pregnant women 17 to 38 years old	2015	146	Cross-sectional study	Urine	11	1	0	NI	NI	46

^a14 locations: Antananarivo, Mahajanga, Tulear, Moramanga, Ihosy, Maeva Ambatondra, sambava, fianara, Antsiranana, Tsiroanomandry, Morondava, Tolangaro, Toamasina, ^bamong ESBL strains only, ^cNDM-1 producing Enterobacteriaceae (*Klebsiella pneumoniae*), ^dRakotonirina et al. (2013) conducted a study in both community and hospital population, however the number of ESBL patient in community have not been communicated see Table 3 for global number of ESBL carriage; M/F: male and female; NI: not identified; ESBL: Extended spectrum β -lactamase; CRE: carbapenem resistant Enterobacteriaceae; CRP: Carbapenem Resistant *Pseudomonas aeruginosa*; CRA: Carbapenem Resistant *Acinetobacter baumannii*; CIPRO: Ciprofloxacin

Acinetobacter baumannii; carbapenem-resistant

In Madagascar, the epidemiological situation of resistance of *A. baumannii* is difficult to estimate due to limited samples. In the first decade of this century (2006–2008), a prevalence of *A. baumannii* of 8.8% was reported in infections diagnosed at hospital; the resistance to ceftazidime (62.0%) and imipenem was particularly high (45.7%) (9).

A study conducted by Andriamanantena et al., (18) among patients from four hospitals (three public and one private) in Antananarivo reported that 44% of collected strains between 2006 and 2009 were resistant to imipenem and 94.3% to ceftazidime, with multi-drug resistant OXA-23-producing *A. baumannii* phenotype, while no resistance to carbapenem was reported by Rasamiravaka et al., (17) among ten uropathogenic isolates in the community between 2011 and 2013.

Pseudomonas aeruginosa: carbapenem-resistant

Data in regard of *Pseudomonas* is very rare as this strain is not commonly isolated from infected patients. In 2006–2008, *P. aeruginosa* isolates showed moderate resistance to penicillins (piperacillin 12.8% and ticarcillin 31.9%) but still susceptible to ceftazidime and imipenem (9).

WHO 'high and medium priority' pathogens in Madagascar:**Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate and resistant**

In Madagascar, the prevalence of

MRSA increased from 2001 to 2014 as shown in Table 4 and 5. Beginning from rates of 0 to 6% in year 2000 and through the first decade of the twenty first century, nasal carriage rate of MRSA in the community increased to 14.8% in 2011 (19, 20). Data collected by Andrianarivelo et al., (21) seems to corroborate the trend in community acquired as well as hospital acquired strains (13.8% and 15.7% respectively). In most of the studies, risk factors analysis revealed that history of hospitalization, recent antibiotic intake and frequent contact with animals and livestock workers/veterinarians increase the risk of MRSA nasal carriage.

Indeed, populations constantly in contact with animals are the most MRSA carriers and this is particularly relevant among Malagasy pig and poultry farmers (25% of MRSA carrier, 45/180) (22). Overall, resistance rates were higher for widely available drugs (23). Increasing rate of resistance to gentamicin (42.9%) and vancomycin (7.1%) was observed in MRSA isolates (20). In the same line, an increase resistance rates to fluoroquinolones have been noticed (19–22). Considering all collected data, 3.1% (317/10,191) of studied populations have been in contact with MRSA strains.

Enterococcus faecium, vancomycin-resistant

In Madagascar, reported data of Enterococcus-vancomycin resistant (EVR) are very poor. In 2006–2008, rate of resistance to vancomycin in *Enterococcus* spp. was 3.3% (9) whereas in 2011–2013, one *E. faecalis* resistant to vancomycin (5.6%) was isolated during an uropathogenic survey (17).

Table 3: Evolution of antibiotic resistance of Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from hospitals between 2001 and 2018 in Madagascar

Town, location	Population (sex, age or mean age)	Date	Population number	Study design	Sample type	ESBL strains	CRE strains	CRP	CRA	Resistant strain to CIPRO	References
Antananarivo	M/F (mean age 37 years old)	2006–2008	651	Laboratory surveillance	Various bacteriological samples	53	4	2	22	139	12
Antananarivo	M/F children (mean age 53 months) and medical staff	2008	474	Cohort study	stool	215	0	NI	NI	NI	5
Antananarivo	M/F (Age not communicated)	2006–2007	830	Laboratory surveillance	Various bacteriological samples	239 ^a	0	0	0	NI	15
Antananarivo	Newborn (0 to 2 days)	2012–2013	303	Cross-sectional study	Blood and gastric samples	51	0	0	0	8 ^b	14
Antananarivo	M/F Children (age not communicated)	2015	55	Cross-sectional study	Rectal swab	4	NI	NI	NI	4 ^b	47
Antananarivo	M/F Newborns and Children (1 months to 15 years)	2016	101	Cross-sectional study	Rectal swab	14	NI	NI	NI	NI	48

^aglobal number of ESBL carriage among hospital and community population, ^b among ESBL strains only; M/F: male and female; NI: not identified; ESBL: Extended Spectrum -lactamase; CRE: Carbapenem Resistant Enterobacteriaceae; CRP: Carbapenem Resistant *Pseudomonas aeruginosa*; CRA: Carbapenem Resistant *Acinetobacter baumannii*; CIPRO: ciprofloxacin

Table 4: Evolution of Methicillin Resistant *Staphylococcus aureus* isolated from community between 2001 and 2018 in Madagascar

Town, locality	Population (sex, age or mean age)	Dates	Population number	Study design	Sample type	Number of MRSA carrier (%)	Resistance rates to other antibiotics (%)					References
							ERY	LIN	GEN	VAN	FLUO	
Antananarivo	M/F (mean age 34 years old)	2001–2005	506	Laboratory surveillance	Various bacteriological samples	33 (6.5)	33.3	19.4	11.1	0	13.9	23
Antananarivo	M/F (Mean age 33 years old)	2004–2006	6884	Laboratory surveillance	Urine	2 (0.02)	NI	NI	NI	0	100	11
Antananarivo	M/F (Mean age 33 years old)	2011	304	Cross-sectional study	Nasal swabs	45 (14.80)	66.67	31.11	4.4	NI	53.3	19
Antananarivo	Pregnant women (mean age 26 years old)	2015	146	Cross-sectional study	Urine	3 (6)	NI	NI	NI	0	NI	46
Antananarivo	M/F (1 to 89 years)	2011–2013	335	Laboratory surveillance	Urine	4 (1.2)	NI	NI	NI	0	NI	17
Antananarivo	M/F Veterinarian student (mean age 23 years old)	2013–2014	155	Cross-sectional study	Nasal swabs	14 (9.08)	64.28	NI	42.85	7.14	57.14	20
Antananarivo	M/F Farmer (mean age 23 years old)	2013–2014	180	Cross-sectional study	Nasal swabs	45 (25)	63.5	NI	30.5	0	47.5	22
Antananarivo	M/F (1 to 81 years)	2005–2014	282	Cross-sectional study	Various bacteriological samples	39 (13.83)	14.89	7.50	5.67	0	24.11	21

ERY: erythromycin; VAN: Vancomycin; FLUO: Fluoroquinolone; GEN: Gentamycin; LIN: Lincomycin; NI: not identified

Table 5: Evolution of Methicillin Resistant *Staphylococcus aureus* isolated from hospital between 2001 and 2018 in Madagascar

Town, locality	Population (sex, age or mean age)	Dates	Population number	Study design	Sample type	Number of MRSA carrier (%)	Resistance rates to other antibiotics (%)					References
							ERY	LIN	GEN	VAN	FLUO	
Antananarivo	M/F mean age 34 years old	2001–2005	68	Laboratory surveillance	Various bacteriological samples	3 (4.4)	33.3	19.4	11.1	0	13.9	23
Antananarivo	M/F mean age 27 years old	2006–2008	651	Laboratory surveillance	Various bacteriological samples	14 (2.1)	NI	NI	3.9	0	NI	9
Antananarivo	M/F 5 to 15 years	2016	156	Cross-sectional study	Nasal swabs	17 (13.49)	NI	NI	NI	0	59.02	22
Antananarivo	M/F 1 to 81 years	2005–2014	624	Cross-sectional study	Various bacteriological samples	98 (15.70)	14.13	9.15	6.57	0	22.44	21

ERY: erythromycin; VAN: Vancomycin; FLUO: Fluoroquinolone; GEN: Gentamycin; LIN: Lincomycin; NI: not identified

***Salmonella* spp., fluoroquinolone resistant**

Salmonella spp. are major fecal–oral food-borne pathogens found worldwide. Most human salmonellosis is associated with eating contaminated raw or undercooked chicken, eggs, pork, and contaminated water. In Madagascar, resistance rates of community associated *Salmonella* spp. among children in 2008–2009 was 0% for quinolones, low for 3GC (1.2% for ceftazidime and cefotaxime) and moderate for ampicillin (35.7%) and ticarcillin (35.7%) (24).

***Campylobacter* spp., fluoroquinolone resistant**

Campylobacter spp. are responsible for both gastro-enteritis and extra-intestinal diseases in which *C. jejuni* and *C. coli* are the most isolated species from patients with diarrhea, as reported by Randremanana et al., (25) in their studies conducted between

2010–2012 (70.1% and 23.6% respectively). The main source of infection in humans is cross-contamination from the environment including livestock animal contact and contamination from undercooked chicken, raw or unpasteurized milk (26). The rate of resistance in *Campylobacter* spp. was low in community children (from study conducted between 2008 and 2009) with overall resistance rate of 2.2% to ciprofloxacin (24) while *Campylobacter* spp. collected in 2005–2006 from chicken neck skin presented 5.5% resistance rate to ciprofloxacin (27).

***Shigella* spp., fluoroquinolone resistant**

Shigella spp. are commonly isolated from stool of dysenteric patients, particularly in developing countries (28). In Madagascar, resistance in *S. dysenteriae* started being reported by the end of the 1980s (29).

Indeed, 5 resistant strains to ampicillin, carbenicillin, streptomycin, chloramphenicol, tetracycline, sulphonamides and trimethoprim were isolated from Malagasy children in Tananarive and on the east coast of Madagascar between 1988 and 1989. In the first decade of the 21st century, high rates of resistance were reported for widely used drugs (for example 79.9% for trimethoprim-sulfamethoxazole, 62.8% for amoxicillin and 62.2% for ticarcillin) among strains isolated from community children but no resistance to ciprofloxacin was reported (24).

Resistance in *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Helicobacter pylori* and *Streptococcus pneumoniae*

A multicenter study conducted between 2004 and 2006 reported 125 cases of *Neisseria gonorrhoeae* infection in Antananarivo (30). All tested strains were susceptible to cephalosporins and fluoroquinolones. In the same way, a study conducted in three pediatric hospitals of Antananarivo from 1998 to 2000 assessing the role of *Haemophilus influenzae* meningitis in Antananarivo reported that among 160 cases of meningitis, *H. influenzae* type b were isolated from 27 cases. Antimicrobial susceptibility testing revealed that 42%, 29% and 22% of *H. influenzae* strains were resistant to chloramphenicol, amoxicillin and gentamicin respectively with no resistance to 3GC (31). Another study reported a prevalence of *H. influenzae* B to be 43% among 119 children with meningitis in the main hospitals of Antananarivo and no resistance to 3GC was observed (32).

Resistance to other high priority pathogens such as clarithromycin-resistance in *Helicobacter pylori* have not yet been reported probably due to the lack of isolated bacteria as they require special culture media and environmental conditions for growth. However, this is contrasting with the high seroprevalence of *H. pylori* infection of 36.2% and 82% among children and adult Malagasy population respectively (33,34). A case report communicated that patient infected by *H. pylori* present refractory profile to metronidazole and clarithromycin treatment, the first and second-line therapy against *H. pylori* infection. Authors underlined that susceptibility testing is overpriced for the concerned population (36). Likewise, only one study carried out in the main hospital in Antananarivo from 1998 to 2000 reported that all *Streptococcus pneumoniae* isolated from children with meningitis were sensitive to penicillin G (32) while *S. pneumoniae* was the first isolated bacteria pathogen (34-52%) responsible for meningitis in Madagascar (31, 36).

Discussion:

A glance at bacterial resistance in countries surrounding Madagascar revealed that MRSA rates are lower in Malagasy community compared to that of Mauritius and La reunion (38% and 13% respectively). Similarly, ESBL rate is estimated to be lower compared to Mauritius and La reunion (50% and 5.6% respectively) (4). However, the main AMR issues identified for Madagascar were ESBL and MRSA which is in agreement with their increase worldwide over the past decade (37). Nevertheless, the collected data should be interpreted with caution. Indeed, due to the diversity of study designs (diagnostic isolates versus systematic detection), antibiogram panels, different periods of time and sample collection, comparison of AMR patterns between territories may be difficult. This is one of the reasons we did not present meta-analysis and estimate the evolution of AMR in Malagasy community from 2001 to 2018.

Additionally, the studies were generally fractionated to limited population, for instance, all MRSA surveys were conducted in Antananarivo suggesting that the major population were natives of this town. However, healthcare facilities are very restricted in some regions such that people from rural areas go to urban city for care, suggesting that the studied populations may actually be from different areas of Madagascar. In any case, it is important to point out that data are concentrated in capital town of Madagascar. Indeed, if prevalence rate is lower in Antananarivo, it may be actually higher in the suburb and scrubland where access to water and healthcare facilities are lacking.

Only one study conducted in 14 districts of Madagascar by Institute Pasteur of Madagascar investigated the presence of ESBL-PE in stool samples from pediatric population (24). A longitudinal survey that take account of the native town of investigated population and cover at least the capital town of each Malagasy region will be necessary to really appreciate the magnitude of AMR among Malagasy population. Another critical limitation of studies carried out in Madagascar is the lack of molecular typing. Only few studies, principally carried out by Institute Pasteur of Madagascar reported molecular data in a multicenter study. In most studies, the presence of *mecA* gene in isolated MRSA strains was not verified. This is also valid for ESBL-PEs where very few studies investigated their molecular typing. This limitation is principally due to high cost of molecular analysis and the implementation

of PCR platform is difficult even in urban laboratory.

According to our academic experience, it is important to note that there are studies conducted by academic researchers which focused on AMR prevalence with phenotypic and genotypic profiles presented at doctorate and master defense. Regrettably, they have not yet been published in peer reviewed journals or communicated in international conferences due to the inability of local researchers to cover the publication fees (personal communication). Generally, they are able to publish their work only in association with international funders or academic institutes. This fact may explain the gaps in information on pathogens of major public health importance in Madagascar. However, it is encouraging to remark that some peer-review journals propose to waive the charge for publication in low-income countries for the promotion of research.

Socio-economic factors in relation to AMR in Madagascar

Madagascar is one of the poorest countries in the World. Indeed, more than three out of every four citizens of the country lived on less than \$1.90 a day in 2019 (38). There is poor access to water, and sanitation and hygiene facilities are totally absent in some areas and only about 35% of the population has access to clean water. Access to sufficient and safe sanitation facilities is vital for hygiene, disease prevention, and human health. Although Madagascar has rainfall and water resources, they are under-exploited and access to good drinking water is difficult even in the capital town of Madagascar (39,40).

The lack of access to water supply and sanitation has significant health impacts especially in propagation of infectious diseases and MDR organisms. In the same line, access to healthcare facility is difficult particularly in rural areas, where citizens have to walk over 20 miles on foot of rugged roads to reach the simplest healthcare facility where inadequate services are offered.

Antibiotic consumption and AMR infections in Madagascar

A direct relationship between antibiotic consumption, emergence, and dissemination of AMR has been demonstrated (41). A study conducted by Padget et al., (42) reported that children population estimates for antibiotic consumption were 29.3% in Antananarivo and 24.6% in Moramanga (a town 200 km from Antananarivo). In all investigated sites, the large majority of antibiotics were taken with prescription (92.2% and 87.0% for

Antananarivo and Moramanga respectively) and purchased in pharmacies (89.4% and 73.5% for Antananarivo and Moramanga respectively). Moreover, living in houses without modern toilets and age between 6 to 18 months were significantly associated with antibiotic consumption after adjusting for sites, and a higher density of public health structures was associated with lower antibiotic consumption levels, while a higher density of private pharmacies was associated with higher levels across sites. Importantly, most of the antibiotics can be purchased without medical prescription and regulations on the prohibition of dispensing non-prescription drugs exist only for particular cases (certain antibiotics) and particular period (e.g. plague epidemic of 2017) (43).

With regards to medical prescription, the physician role is based on the correct prescription of antibiotics although it is sometimes difficult to make an accurate diagnosis in low-income countries where diagnostic tools for bacterial infections are limited and only present in major cities. Furthermore, the risk of superinfection is high in localities where hygiene is very precarious and this context brings doctors to immediately establish antibiotic therapy even if they are aware of the viral origin of infection. Moreover, in rural localities, patients often travel more than 10 miles to reach a healthcare facility suggesting that patient who has received medication from the doctor will no longer return for follow up check as far as his condition does not really get worse. Aware of these different parameters and constraints, physicians generally opt for empirical antibiotic coverage.

The monitoring system for MDR bacteria is less well developed in Madagascar, mainly due to limited diagnostic infrastructures. Bacterial cultures are only taken after failure of empirical antibiotic therapy and the number of hospital infections is undoubtedly underestimated. This empiric antibiotic approach is dependent on local prevalence of bacteria species. Due to the lack of microbiological analysis, there is no national database for bacterial profiles of UTI or vaginal infections such that the European, and particularly French database, are used as guide for empirical therapy. Intriguingly, a study conducted by Rasamiravaka et al., (17) demonstrated that the bacterial profiles of UTI in Madagascar is not the same as presented by French recommendation. Indeed, there is an increase rate of Gram-positive cocci of up to 34% causing UTI while it is usually in the range of 10% in the French database. This point may lead to failure of empiric antibiotic therapy approach by using inappropriate antibiotics. Meanwhile, it can

also lead to increase antimicrobial resistance by selecting persistent or resistant bacteria. It is also important to note that although bacteriological analysis of UTI is important for diagnosis of bacterial infection and surveillance of bacterial susceptibility to common antibiotics, its systematic use is difficult to install because of high costs of reagents and consumables.

Conclusion:

This review highlights the prevalence of resistant strains, particularly the ESBL producers, which is far from negligible in Madagascar although the overall estimation is low. It seems necessary to set up strategy to monitor antibiotic resistance of greater magnitude. It is obvious that AMR spread is connected to the local socio-economic context indicating that tackling AMR spread does not fall only on physicians, pharmacists and microbiologists, but requires a high commitment of government with private partners in order to regulate antibiotic consumption, and improve healthcare facilities and hygiene access. Without radical improvement of accessibility and quality of healthcare as well as water, sanitation and hygiene facilities, the successful reduction of antibiotic resistance spread will remain utopian.

The main strategy should first focus on controlling the spread of resistant strains through; (i) proper use of antibiotics, (ii) training on the right prescription (national education in antibiotic use), and (iii) the fight against illicit sales. For antibiotics, they must be issued only under prescription. It is assumed that such measures will discourage self-medication which may decrease the number of customers of the pharmacists thus government supportive measures should be considered. Indeed, the government must reassure antibiotic distributors that prohibition of antibiotic delivery without prescription will have no negative impact in their business. Moreover, government should propose different measures to reduce distributors' fear such as subvention and/or drug tax reduction. These measures require the increase number of pharmacy and drug deposit to not damage the accessibility of the drug, which is virtually improved by their presence in grocery stores that are largely close to the population.

In parallel, accessibility to healthcare facilities should be improved and private pharmacies should be controlled in order to reduce antibiotic over consumption. Furthermore, control of antibiotic dispatching as well as circulation/distribution of antibiotics should be rigorous to avoid the circulation of fake

and poor-quality drugs. Indeed, some drugs placed on the market, despite having the active ingredients, may be under dosed or with poor bioavailability for the body resulting in decreased antibiotic concentration on the infectious sites. Such drugs must be tracked by the sanitary authorities. The free sale of authentic or non-genuine medication in general grocery stores must be prohibited, with only pharmacists and drug stores allowed to sell them. With regards to the difficulty of most Malagasy patients to pay for overpriced microbiological analysis due to high cost of reagents/consumables in medical laboratory, one alternative is the use of homemade reagents with the help of reference strains as quality control that may reduce charge of each test. For susceptibility testing, a proposed solution is to reduce the number of first line antibiotic disk testing to those really used by physicians and those detecting AMR. Thus, laboratory can propose cheaper price accessible to most households.

Other important keys to prevent AMR are national and international network in surveillance of MDR strains. It is encouraging that the "Mérieux Foundation (MF)" supports the setting up of a laboratory network for medical microbiology analysis and infectious disease surveillance. With the support of the Ministry of Public Health, MF generates national laboratories network called RESAMED in order to standardize practices, facilitate the flow of data and knowledge, and participate in the national health strategy, particularly on antimicrobial resistance surveillance. However, this type of support should be extended to applied research such as discovery of new antimicrobial compounds and in a fundamental aspect including accurate microbiological resistance mechanisms study. In this aspect, research and development (R & D) should be more innovative in the fight against bacterial infections by finding new original antibiotic with low risk of resistance from non-cultivable and telluric bacteria or original antimicrobial compounds targeting virulence ability of bacteria instead of their viability.

This latter approach does not intend to compete with the search of new antibiotics or to replace the use of available antibiotics but rather to reduce the use of the latter (for example decrease the dose of prescribed antibiotic while maintaining its full effectiveness) which hopefully could slow down the spread of MDR bacteria. Other interesting compounds are those with antibiofilm properties. Indeed, the ability of bacteria to form biofilm is one of the key mechanisms of microbial resistance to antibiotics as the biofilm matrix protects them against antimicrobials and immune

defense (44). It is promising that natural compounds have already been isolated from African and Malagasy plants with the ability to disrupt biofilm formation of *Pseudomonas aeruginosa* PAO1 and restore tobramycin effectiveness in *in vitro* experiments (45, 46).

To conclude, Madagascar is not adequately armed to actively tackle MDR progression. It is urgent that the government adopt the minimal requirement to contain MDR progression such as increasing accessibility to health care service and prohibiting delivery of antibiotic without medical prescription. With the re-emergence of Plague in 2017 in the country, a very strong counterattack by the antimicrobial prophylaxis of mass was instituted by the health authorities (more than one million doses of antibiotic to treat 100,000 people were distributed by the WHO) so we can expect an increase in the resistance rate in the coming years. As evoked by WHO "While more R and D is vital, alone, it cannot solve the problem. To address resistance, there must also be better prevention of infections and appropriate use of existing antibiotics in humans and animals, as well as rational use of any new antibiotics that are developed in future.....The lack of adequate surveillance in many parts of the world leaves large gaps in existing knowledge of the distribution and extent of this phenomenon" (8).

This review article provides an update on the baseline data and enlightenment of the magnitude of AMR in Madagascar and the particular context of the country which may explain the difficulty in developing standardized public health actions. Madagascar is under threat of AMR spread which needs urgent reaction of government and non-governmental entities concerned in preservation of public health.

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**Review Article****Open Access****Phytotherapy as an alternative for the treatment of human papilloma virus infections in Nigeria: a review**

^{*1}Yusuf, L., ^{1,2}Bala, J. A., ¹Aliyu, I. A., ¹Kabir, I. M., ¹Abdulkadir, S., ^{1,3}Doro, A. B., and ¹Kumurya A, S.

¹Microbiology Unit, Department of Medical Laboratory Science, Faculty of Allied Health Science, Bayero University, Kano, Nigeria, P.M.B 3011, Kano, Nigeria

²Virology Unit, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia

³Federal Medical Centre Katsina, PMB 2121, Katsina, Nigeria

*Correspondence to: iaaliyu.mls@buk.edu.ng; isahaa97@gmail.com

Abstract:

Human papillomavirus (HPV) has been incriminated as the causal agent of cervical cancer which has been rated as the second most common cancers among women in developing countries and seventh most common cancers in the developed world. In spite of the fact that HPV has been the major cause of cervical cancer, the dilemma lies in finding a cost-effective therapy. Approximately 291 million women are infected with HPV worldwide, 32% of whom are infected with HPV16 or HPV18. The estimated prevalence of HPV in sub-Saharan Africa is 24% and 11.7% globally. There have been studies reporting specific HPV prevalence rates in some part of Nigeria, with 37% in Abuja, 10% in Port Harcourt, and 26.3% in Ibadan. In the Nigeria population, awareness of HPV infections is low, HPV vaccines are inadequate, and the cost of HPV vaccination per person is beyond what an average citizen can afford. It has been suggested that herbal therapy such as *Echinacea* therapy reduces HPV replication and enhances the immune system. Although there is yet no scientific proof of the efficacy of *Echinacea* therapy against HPV infections, future emphasis should be placed on scientific research into this alternative therapy. There is need for more studies on development of antiviral agents against HPV, with a prospect of easy accessibility and affordability in Nigeria.

Keywords: Phytotherapy; HPV; Cervical cancer; Nigeria

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La phytothérapie comme alternative au traitement des infections à papilloma virus humain au Nigéria: une revue

^{*1}Yusuf, L., ^{1,2}Bala, J. A., ¹Aliyu, I. A., ¹Kabir, I. M., ¹Abdulkadir, S., ^{1,3}Doro, A. B., et ¹Kumurya A, S.

¹Unité de microbiologie, Département des sciences de laboratoire médical, Faculté des sciences connexes de la santé, Université Bayero, Kano, Nigéria, P.M.B 3011, Kano, Nigéria

²Unité de virologie, Département de pathologie et de microbiologie, Faculté de médecine vétérinaire, Universiti Putra Malaysia, Malaisie, 43400, Serdang, Selangor Darul Ehsan, Malaisie

³Centre médical fédéral Katsina, PMB 2121, Katsina, Nigéria

*Correspondance à: iaaliyu.mls@buk.edu.ng; isahaa97@gmail.com

Abstrait:

Le papillomavirus humain (HPV) a été incriminé en tant qu'agent causal du cancer du col de l'utérus, classé comme le deuxième cancer le plus fréquent chez les femmes dans les pays en développement et le septième cancer le plus fréquent dans le monde développé. Malgré le fait que le VPH ait été la principale cause de cancer du col de l'utérus, le dilemme réside dans la recherche d'une thérapie rentable. Environ 291 millions de femmes sont infectées par le VPH dans le monde, dont 32% sont infectées par le VPH16 ou le VPH18. La prévalence estimée du VPH en Afrique subsaharienne est de 24% et 11,7% dans le monde. Des études ont signalé des taux de prévalence spécifiques du VPH dans une partie du Nigéria, avec 37% à Abuja, 10% à Port Harcourt et 26,3% à Ibadan. Dans la population nigériane, la sensibilisation aux infections au VPH est faible, les vaccins contre le

VPH sont inadéquats et le coût de la vaccination contre le VPH par personne dépasse ce qu'un citoyen moyen peut se permettre. Il a été suggéré que la thérapie à base de plantes telle que la thérapie à l'échinacée réduit la réplication du VPH et renforce le système immunitaire. Bien qu'il n'y ait encore aucune preuve scientifique de l'efficacité de la thérapie à l'échinacée contre les infections au VPH, l'accent devrait être mis à l'avenir sur la recherche scientifique sur cette thérapie alternative. Il est nécessaire de poursuivre les études sur le développement d'agents antiviraux contre le VPH, avec une perspective d'accessibilité et de prix abordable au Nigéria.

Mots-clés: Phytothérapie; HPV; Cancer du col utérin; Nigeria

Introduction:

Human papillomavirus (HPV) is now recognised as the major aetiological factor of cervical cancer (CC), with more than 99.7% of the cases associated with prior oncogenic/high risk human papillomavirus (HrHPV). Infection with HrHPV is therefore, the primary risk factor for cervical cancer and the pre-cancerous, cervical intraepithelial neoplasia (1). As HPV infection is a sexually transmitted infection, cervical cancer is now known as sexually transmitted cancer by origin (2). While infection with HrHPV is prevalent among sexually active women, it is usually transient and neutralised within 2 years of infection (3). A prospective study of 3,282 women in the Netherlands found that about 34% of young women failed to clear HPV in 2 years (4). The reported persistent rate of infection among women in Mainland China (age group 16-69 years) was about 23% (5).

Human papilloma virus is one of the 6 human viruses identified as Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) (6,7). Worldwide, cervical cancer takes the lives of nearly 300,000 women a year, with more than 80% from developing countries (8). Global HPV data show that Africa has the highest prevalence of 22.1% (9), with the lowest prevalence of 2.2% in Sudan (10). Cervical cancer is the most common gynaecological cancers in Nigeria, and the leading cause of cancer deaths among Nigerian women (11), where it is responsible for the death of one woman every hour and more than 9,000 female patients annually (12).

Treatments for cervical HPV infections are restricted. The recommendations of the American Society for Colposcopy and Cervical Pathology (ASCCP) suggest that women with lesser cervical abnormalities such as HrHPV positive but cytologically negative, atypical Squamous Cell of Undetermined Significance (ASCUS), or Low Grade Squamous Intra-epithelial Lesion (LGSIL) should be diagnosed through genotyping and testing of HrHPV within twelve months (13). Nevertheless, according to recent studies, the risk of developing malignant lesions will be increased with chronic carcinogenic infection (14). If infection with HrHPV persists for more than a year, 21% of women progressed to CIN2 (15).

There has been debate over the treatment of HrHPV positive people. Several researchers in Taiwan have proposed cryotherapy as one of the LGSIL's treatment options, and a cohort study found that cryotherapy for women with LGSIL could minimize the incidence rate of CIN3+ by improving the clearance of HPV infections (16). In Nigeria some of the natural (herbal) remedies that have been used for the treatment of HPV infections include garlic, banana peel, apple cider vinegar, orange peel, potato, aloe vera, and pineapple. Although these have been used to treat warty HPV infections, they have not been used for HrHPV infections.

Methodology:

Online databases were searched for available relevant documents written in English language up till January 15, 2019. These included African Biomedical databases, Nigerian Scientific Journals database, HPV Information Centre Registry, and PubMed Central (NCBI). The keywords for search were 'HPV', 'traditional treatment of HPV in Africa', 'treatment of HPV in Nigeria', 'phytochemistry', 'HPV awareness and vaccine in Nigeria', and 'alternative medicine'.

We included original articles (cross sectional, prospective and randomized control trials), and review articles that provided information on current trends and meta-analysis of HPV infection and treatment in Nigeria and globally. Independent reviewers evaluated the eligibility of each document for consideration and eliminated the likelihood of bias. A total of 58 citations and 18 articles were found eligible for the review.

Prevalence of HrHPV infections in Nigeria

The prevalence of cervical HPV infection worldwide varies greatly, with some of the highest rates observed among African women. Approximately, two hundred and ninety-one million women are infected with HPV worldwide, 32% of whom are infected with HPV16 or HPV18 (9). The estimated prevalence of HPV infection in sub-Saharan Africa is 24%, and 11.7% globally (17). There have been studies reporting specific HPV prevalence rates in some parts of Nigeria, with 37% in Abuja, 10% in Port Harcourt, and

26.3% in Ibadan (18). In a population-based study conducted on women who have had sexual relations in their lifetime and their cervixes examined by PCR and liquid based cytology, a 14.7% rate of high-risk HPV was detected in two third of the women (19). In another study conducted in north-eastern Nigeria, 48.7% of the women participants were positive for HPV type 18, 13.2% for HPV16, and 18.5% for combined HPV 31, 33 and 35 (20).

In a similar prevalence study conducted in Ibadan, southwest Nigeria on 932 sexually active women aged 15 years and above, 19.7% had HrHPV types 16, 31, 35, and 58 as the main HPV types, while 3.2% each had HPV types 16 and 35 (11). In Kano, northwest Nigeria, a hospital-based study conducted on 50 women aged 18 years and above to determine the prevalence of HPV infection using PCR for HPV DNA detection, 76% of the study participants were positive for either HPV 16, 18 or both while 60.5% were co-infected with both HPV 16 and 18 (21). In Nigeria, HPV infection tends to be predominantly reported in patients with high grade intraepithelial lesion as shown in Fig 1, although about 8% of the patients who have normal cytology had HPV infection, with higher prevalence among younger age group. Some biological mechanisms, such as immaturity of the cervix, insufficient cervical mucus production and increased cervical ectopia in younger women and adolescents, have been postulated to make them more susceptible to HPV infections (22).

Preventive measures against cervical lesion in Nigeria

One of the most effective measures to prevent cervical cancer is to prevent HrHPV infection, and this can be achieved by administering vaccines such as Gardasil which target HPV 16, 18, 6 and 11 (23), Cervarix targeting HPV 16 and 18 (24), and Gardasil 9 targeting HPV 31, 33, 45, 52 and 58. These vaccines reduces the chances of being infected with HPV when given to women before exposure to sexual activities. Nevertheless, these vaccines still have limitations in their failure to protect other HPV types not present in the vaccines. However, it is the most favoured method of prevention.

The vaccinations were approved and launched in Nigeria in 2009, with only a few affluent people taking up between 0 to 49% (25). In the Nigeria population, awareness of HPV infections and uptake of HPV vaccines is low, and the cost of HPV vaccination per person is beyond what an average Nigerian can afford (26). In the parts of Nigeria where the level of education is high, the female children caregivers showed very low knowledge on the risk factors associated with HPV infections and cervical cancer (27). While HrHPV infections are being treated by these vaccines, warts caused by low risk HPVs often regress without treatment, although these viruses are difficult to manage, as infection can reappear in the same site or in other places. Some of the current therapy for HPV infections are shown in Table 1. Although these medications are effective, they require multiple applications on the lesions.

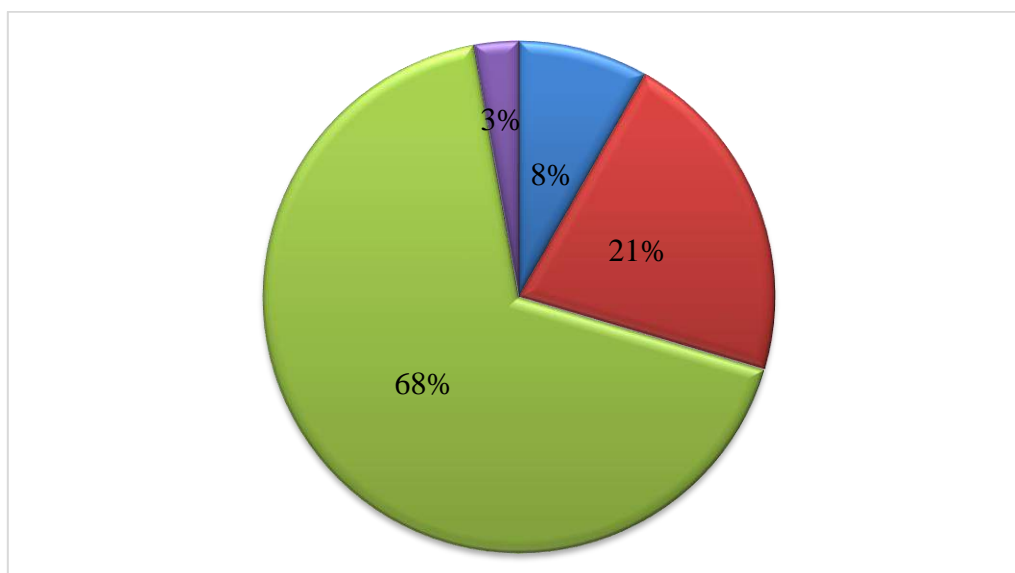


Fig 1.0: A pie chart showing Nigeria HPV prevalence in relation to Pap smear test

Blue colour represents the HPV prevalence in people with normal cytology, red colour shows prevalence of HPV in people with low grade intraepithelial lesion, and green colour depicts HPV prevalence in High grade intraepithelial lesion, while the purple colour is portraying the prevalence of HPV in cervical cancer patients.

Table 1: Current treatment for HPV infection and its associated problem

Current anti-HPV agents	Problem associated with the anti-HPV agents
Gardasil	Effective for only those infected with the virus
Cervarix	Inability to clear existing HPV infection
Imiquimod	Severe itching, hives, and long treatment time (4 months).
Condylox	Causes serious allergic reaction
Cidofovir	Expensive, side effects such as ocular disturbances
Interferon	Have limited efficacy for high grade HPV associated lesions
Podophyllotoxin	Not HPV-specific with up to 65% recurrence rate
Heparin	Has an anticoagulant effect
5-fluorouracil	Generates strong inflammatory response

HPV therapeutic vaccines

Human papillomavirus therapeutic vaccines are administered to treat pre-malignant and malignant lesions associated HPV, as opposed to Gardasil, Cervarix and Gardasil 9 vaccines, which are used to stimulate the development of L1 capsid protein neutralizing antibodies. HPV therapeutic vaccines are administered in order to target onco-proteins such as E6 and E7 proteins, which are expressed by HPV throughout their life cycle, although these therapeutic vaccines are still in phase II and III clinical trial stages. An example of HPV therapeutic vaccine that has passed the clinical phase III evaluation is the MVA E2 which targets HPV16, and contains the bovine papilloma virus E2 protein. Since the E2 protein regulates the expression of E6 and E7 onco-proteins, truncated E2 has negative regulatory gene to E6 and E7 promoter sequence. Hindering the E6 and E7 onco-proteins transcription in HPV infected cells thereby reduce progression to malignancy. Other HPV therapeutic vaccines are reported to have local mild to moderate side effects (28).

Development of new anti-HPV agents from natural products

The recent major developments in medical treatment and biomedical technology includes various antiviral agents developed and used to treat infectious diseases, yet such progress has also led to the development of viral resistance strain. Hence it is necessary to develop new antiviral agents with various kinds of antiviral actions. The quest for novel antiviral agents is based not only on synthetic substances, but also on natural compounds such as herbal pharmaceutical products (29).

Herbal products possess their own specific metabolites, which may identify variations between host and viral metabolism, contributing to antiviral activity. Most herbal pharmaceutical products can be easily obtained at a cheaper cost, and can be important for development of new antiviral agents with different antiviral activities than the current antivirals. The need to thoroughly investigate the causal agent associated with cases of cervical cancer is highly imperative, thus several diagnostic approaches are needed including the routine virological culture involving tissue culture screening (30-32). Viral isolation could be carried out on either embryonated chicken egg (33,34) as well as cultivation on a continuous cell culture systems (35-39). Often, these situations were synonymous with an approach to establish cultures that improved biological products such as insulin for diabetic remedy employing the goats' islets for xenotransplantation (40-43). Similar scenario stands in the selection of suitable cells for study of apoptosis (44-46).

The function of protein-receptor inhibitors used in *in silico* research is very important in drug design. Fig 2.0 summarises the steps to be taken to ensure herbal remedies are not only identified, but the mechanisms of actions can be properly understood. Recently countries like China have used detoxification therapy in the treatment of HPV, which tends to have positive effects on increasing HPV clearance rate, increasing the rate of CIN regression and influencing the proportion of certain immune cells, and also the level of cytokines in the genitalia following treatment (47).

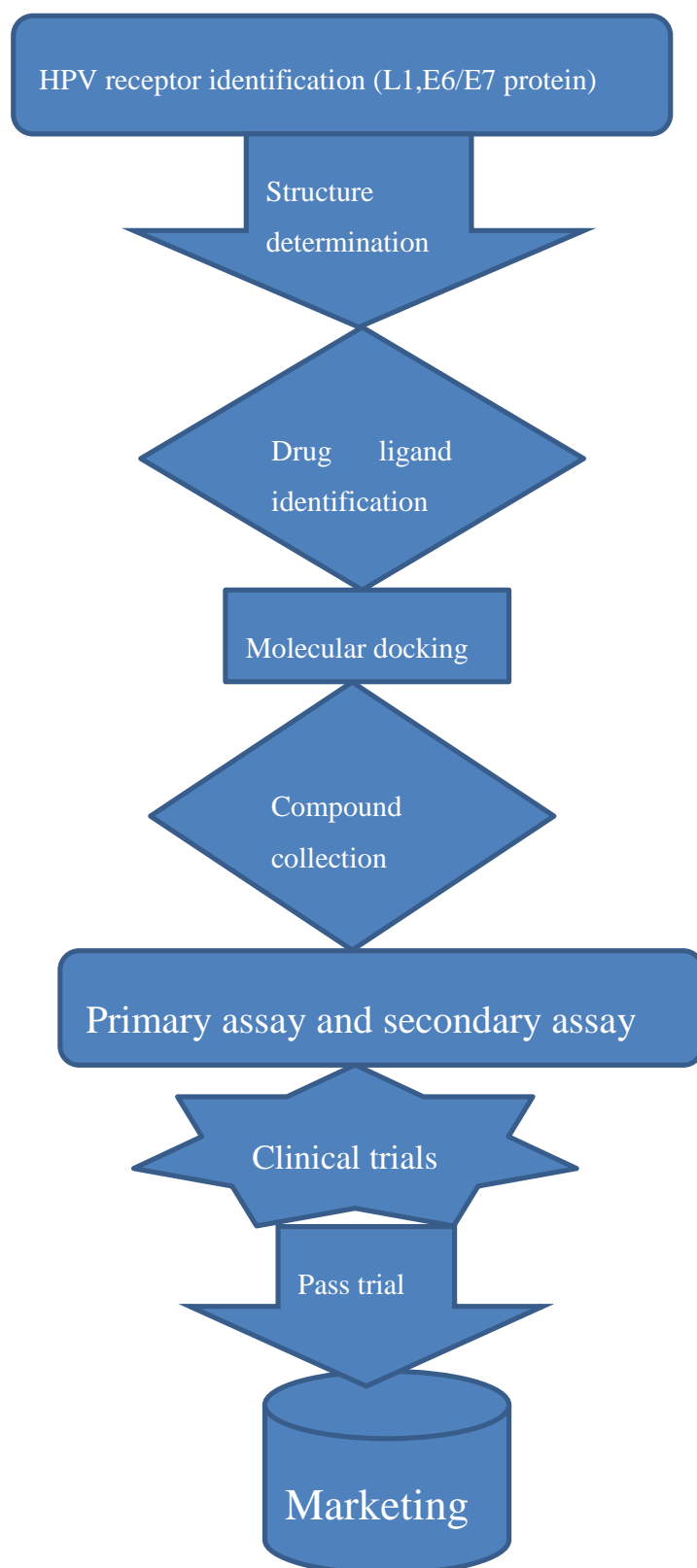


Fig 2.0: New anti-HPV agent drug testing algorithm

Potential herbal compounds as alternative for HPV infection therapy

Silymarin:

Silymarin is an herbal antioxidant that is believed to have anti-cancer and anti-HPV properties (48). It is contained in milk thistle as an active agent and, possesses antioxidant properties. It was reported to induce apoptosis by modulating Bcl-2 family proteins and also activating caspase 3 (48).

Curcumin (diferuloylmethane):

Curcumin is a polyphenol produced in the *Curcuma longa* plant, with a common name known as turmeric. For more than five decades, researches have shown that curcumin has onco-therapeutic effect with its antioxidative properties. Curcumin is a perennial herb which also possess anti-inflammatory properties and was reported to down-regulates AP-1 and nuclear factor kappa light-chain enhancer of activated B cells both of which plays major role in HPV transcription (49). One research showed the therapeutic potential of curcumin in HrHPV oral cancer cells, with reported ability to selectively inhibit E6 oncogene-mediated P53 degradation in HPV 16 positive cells in oral cancer throughout carcinogenesis (50). It also down-regulates the expression of uPA, COX2, NOS, LOX, MMP-9, and TNF chemokines (50).

Epigallocatechin gallate (EGCG):

Tea is one the world most consumed non-alcoholic beverages. The major tea catechins are epigallocatechingallate (EGCG), epicatechingallate (ECG), epicatechin (EC) and epigallocatechin (EGC) (51). EGCG represents over 40% of all green tea catechins and it plays a key role in the prevention of cancer, obesity, neurodegenerative disorders, and stroke (52). The cancer prevention capability of EGCG is largely supported by epidemiological, *invivo* and *invitro* studies (53). Despite adequate evidence of EGCG effects against liver, breast and prostatic cancers, its effects on cervical cancer prevention is however controversial and still inconclusive (54).

EGCG has been reported to induce mitosis (G1, G2, S and M) arrest in squamous Me180 cervical carcinoma cell at low concentration (0-25µg/mL) (55). Another study showed that EGCG also mediated G1 phase arrest in CaSki cells linked to HPV 16 and regulated gene expressions (56). The activation of epidermal growth factor receptor (EGFR) and its downstream target, extracellular signal regulated protein kinases (ERK1/2), is important during cancer cell proliferation (57). Through the level of P53 and CKI, EGCG inactivate EGFR and ERK1/2 protein kinases causing G1 arrest and

increased apoptosis in several cervical cell lines (57). Studies showed that EGCG inhibits the proliferation of cervical adenocarcinoma, due to its effects on PK-i67 and suppression of telomerase function, thereby inducing apoptosis (58). Current researches also reveal that EGCG through telomerase, a reverse transcriptase, telomerase which adds new DNA to the telomeres at the end of the chromosomes (58), and RNA polymerase III, could control the growth of cancer cells (58).

Carageenan

Carageenan prevents the binding of HPV virions to the basal cells of the cervix, thereby preventing viral pathogenesis.

Nordihydroguaiaretic acid

Nordihydroguaiaretic acid is an active compound from Lignan plant, which inhibits HPV SP1-mediated transcription.

Echinacea purpurea

Echinacea purpurea is one of the most favourite medicinal plants in the world, belonging to the family Astraceae. It is commonly used as a chemo-preventive and chemotherapeutic plant especially in upper respiratory tract. It is also used in treatment of cancer such as cervical cancer and serves as immunomodulator. Although investigators have noted the isolation and structural elucidation of its main compound, there is no conclusion about its mechanism of action (59).

The active ingredients are alkamide, caffeic acid and polysaccharides. The alkamide is reported to be responsible for plant immunomodulatory property which uses T-cell activation and enhances the production of TNF and IFN-γ. Its antiviral properties can also be linked to the fact that it inhibits hyaluronidases produced by viruses while the polysaccharide is responsible for its anti-inflammatory effects (28). Caffeic acid, however, is not present in all *Echinacea* species and could be used for plant extract authentication and quality control (59).

Immunomodulatory activity of Echinacea

The immunostimulant properties of the plant involve three mechanisms: phagocytosis activation, fibroblast stimulation, and increased respiratory activity resulting in increased leucocyte migration. There are different studies that have reported the *invivo* immunomodulatory and anti-inflammatory properties of *Echinacea purpurea* suggesting enhanced innate immunity when the plant is administered and increased immune response by stimulating macrophages, polymorphonuclear leucocytes (PMN), and natural killer (NK) cells (60). It is thus an effective

preventive measure for the treatment of various infectious diseases such as infections of the respiratory tract, wound and pelvis (61).

The complex chemical composition of *Echinacea* roots and herbs contain keto-alkenes, alkaloids, caffeic acid derivatives, polysaccharides and glycoproteins which are allegedly responsible for noted immunostimulatory and anti-inflammatory activities (60). The alkaloids have been tested and documented to be acting on type 2 cannabinoid receptors (CB2), and this is also hypothesized as possible mechanism for their immunomodulatory properties (62). One research has shown that N-alkaloids obtained from root and herbal tincture induced synergistic properties on CB2 and eventually contribute to the immunomodulatory effects along with interleukin 10 (63). They also inhibit both cyclo-oxygenase enzymes (COX-1 and COX-2) and 5-lipo-oxygenase (F-LO), enabling natural cell inhibition and anti-inflammatory activity (64). It is reported that the plant portion (polysaccharides) increases the production of interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF- α by macrophages, and also increase their phagocytic and microbicide activity, and cerebrospinal fluid secretion (65).

The expression of CD83, which is a marker of dendritic cell maturation, has been shown to be significantly enhanced by the plant's floral and root extracts, while the stem and leaf extracts can significantly decrease CD83 levels. In addition, studies have shown that the extracts from the plant's root and aerial section require up and down regulation of insulin-like growth factor 1 receptor (IGF-1R), respectively. In addition, the plant's root extract can control a variety of genes involved in immune cell activation or function including Chemokine (C-C motif) ligand 4 (CCL4), interleukin-7 receptor, nuclear factor of activated T-cells, cytoplasmic 2, T-box transcription factor, cytohesin-interacting protein (PSCDBP), integrin, alpha E (ITGAE), and intercellular adhesion molecule-1 (ICAM1), while CD34 and integrin beta-1 (ITG-B1) are down regulated by the aerial part of the plant extract in dendritic cells (DCs) (66).

Cytotoxic activity of Echinacea

It has been documented that the plant extract flower and cichoric acid inhibit both the human colon cancer cell lines, Caco-2 and HCT-16, in a dose dependent manner after 48 hours of exposure. It has also been reported that cichoric acid slows down the HCT-16 cell line telomerase activity, which could be presumed as the molecular mechanism of apoptosis induction (67). Nonetheless, an extract from the plant root known as n-hexane

that can be obtained from three species of *Echinacea* has been shown to have potent anticancer activity (68).

Lethal dosage of Echinacea in animal studies

Animal studies of various preparations of *Echinacea* species showed generally low toxicity (69). In acute toxicity test, the LD50 value was estimated at 2500 mg/kg in an intraperitoneal injection of the plant's polysaccharides fraction into female mice (69). In other studies, oral and intravenous LD50 values for plant juice were estimated to exceed 30g/kg and 10g/kg in mice, and 15g/kg and 5g/kg in rats (70).

Antiviral activity of Echinacea

In an *invitro* study, aqueous solution from the plant extract of *Echinacea purpurea* was confirmed to be active against both acyclovir resistant and susceptible strains of herpes simplex virus 1 (HSV 1) and herpes simplex virus 2 (HSV 2) (71), whereby, the plant root hexane extract and cichoric acid exhibited HSV 1 inhibition (72). Furthermore, cichoric acid has been reported to inhibit integrase activity of human immunodeficiency virus type 1 (HIV-1) (73). Mouse embryonic fibroblasts incubated with the plant juice and alcoholic root extract was immunized to influenza A2, herpes, and 24-hour infection of the vesicular stomatitis virus (74).

Normal preparation of the plant extract showed strong inhibition of the influenza viruses A/Victoria/75 (H3N2) and A/Puerto Rico/8/1934 (H1N1), avian strains A/Thailand/1(KAN-1)/2004 (H5N1) and A/FPV/Dutch/1927 (H7N7), and the pandemic novel influenza A (S-OIV) (H1N1) of swine origin in direct contact. Nonetheless, haem-agglutination (HA) assays showed that the preparation inhibited HA activity, and thus prevented the virus from entering the treated cells (75).

Conclusion:

This review highlighted the improvement in the design of anti-HPV, as well as potential therapy regimen for HPV infections in Nigeria and globally, using phytotherapeutic approaches. HPV being a major cause of cervical cancer, and cervical cancer being one of the leading causes of annual death in adult women, prevention of cervical cancer is possible by seeking a cost-effective treatment for the main causative agent, HrHPV. The main antiviral therapy against HPV is cidofovir (CDV), which in addition to being expensive, has side effects ranging from renal to ocular disturbances.

While alternative medicine approaches for therapy of cervical cancer ranges

from globally accepted homeopathic methods to the allopathic approach embraced by the Indians, Chinese, and Japanese to treat HPV-related diseases, their mechanisms of action are not established. Therefore, more studies on antiviral agents against HPV are required. It has been reported that herbal therapy such as *Echinacea* not only reduce viral replication but also enhance the immune system, with the prospect of easy accessibility and affordability in Nigeria. Although there is no scientific explanation for the efficacy of *Echinacea* therapy against HPV infections at present, there need for more researches in this area.

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Original Article

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Seroprevalence of and associated risk factors for *Leptospira interrogans* serovar Hardjo infection of cattle in Setif, Algeria

*¹Benseghir, H., ²Amara-Korba, A., ¹Azzag, N., ¹Hezil, D., and ¹Ghalmi, F.

¹Research Laboratory Management of Local Animal Resources, Higher National Veterinary School, El Alia, Oued Smar, 1615, Algiers, Algeria

²Leptospira unit, Pasteur Institute of Algeria, Rue 1 of Doctor LAVERAN, Hamma Anassers Algiers, Algeria

*Correspondence to: mazouni05@yahoo.fr

Abstract:

Background: Leptospirosis is a cosmopolitan zoonosis caused by *Leptospira interrogans* responsible for heavy loss both economically and in health, in humans and animals. This study was conducted to determine the seroprevalence and risk factors associated with *Leptospira interrogans* serovar Hardjo infection in cattle in the state of Setif, northeastern Algeria.

Methodology: Between the period 2015 and 2019, a total of 48 randomly selected herds of cattle were investigated, and 406 sera from apparently healthy cattle were analyzed using an indirect enzyme-linked immuno-sorbent assay (ELISA). In order to determine possible risk factors related to leptospirosis, a pre-validated questionnaire was administered to herd owners.

Results: The herd prevalence of *Leptospira interrogans* serovar Hardjo was 31.25% (15/48, 95% CI 19.95–45.33) while the cattle prevalence was 5.42% (22/406, 95% CI 3.61–8.07). Multivariable logistic regression analysis showed that the age of cattle between 3 and 6 years (OR = 9.25; $p < 0.03$), breeding herd size > 20 cows (OR = 13.65; $p < 0.01$), and semi-intensive management system (OR = 0.21; $p < 0.02$) were significantly associated with seropositivity to *Leptospira interrogans* serovar Hardjo.

Conclusion: We concluded from this study that *Leptospira interrogans* serovar Hardjo is circulating among cattle farms in the state of Setif, Algeria. Furthermore, we recommend more studies to be carried out to prove the infectivity and implementation good hygienic practices among cattle farms and people at risk.

Keywords: ELISA, herds, questionnaire, leptospirosis, prevalence, Algeria

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Séroprévalence et facteurs de risque associés de l'infection à *Leptospira interrogans* serovar Hardjo des bovins à Sétif, Algérie

*¹Benseghir, H., ²Amara-Korba, A., ¹Azzag, N., ¹Hezil, D., et ¹Ghalmi, F.

¹Laboratoire de recherche sur la gestion des ressources animales locales, École nationale supérieure de médecine vétérinaire, El Alia, Oued Smar, 1615, Alger, Algérie

²Unité Leptospira, Institut Pasteur d'Algérie, Rue 1 du Docteur LAVERAN, Hamma Anassers Alger, Algérie

*Correspondance à: mazouni05@yahoo.fr

Abstrait:

Contexte: La leptospirose est une zoonose cosmopolite causée par *Leptospira interrogans*, responsable de lourdes pertes économiques et sanitaires, chez l'homme et l'animal. Cette étude a été menée pour déterminer la séroprévalence et les facteurs de risque associés à l'infection à *Leptospira interrogans* serovar Hardjo chez les bovins de l'État de Sétif, dans le nord-est de l'Algérie.

Méthodologie: Entre 2015 et 2019, un total de 48 troupeaux de bovins sélectionnés au hasard ont été étudiés, et 406 sérums de bovins apparemment sains ont été analysés à l'aide d'un test d'immunosorbant indirect lié à une enzyme (ELISA). Afin de déterminer les facteurs de risque possibles liés à la leptospirose, un questionnaire pré-validé a été administré aux propriétaires de troupeaux.

Résultats: La prévalence du troupeau de *Leptospira interrogans* serovar Hardjo était de 31,25% (15/48, IC 95% 19,95–45,33) tandis que la prévalence bovine était de 5,42% (22/406, IC 95% 3,61–8,07). Une analyse de régression logistique multivariable a montré que l'âge des bovins entre 3 et 6 ans (OR=9,25; $p<0,03$), la taille du troupeau reproducteur >20 vaches (OR=13,65; $p<0,01$) et le système de gestion semi-intensif (OR=0,21; $p<0,02$) étaient significativement associés à la séropositivité pour le sérovar Hardjo de *Leptospira interrogans*.

Conclusion: Nous avons conclu de cette étude que *Leptospira interrogans* serovar Hardjo circule parmi les élevages bovins de l'Etat de Sétif, en Algérie. De plus, nous recommandons de mener plus d'études pour prouver l'infectivité et la mise en œuvre de bonnes pratiques d'hygiène dans les élevages bovins et les personnes à risque.

Mots-clés: ELISA, troupeaux, questionnaire, leptospirose, prévalence, Algérie

Introduction:

Leptospirosis is a worldwide zoonotic disease, representing a global public health problem, with high morbidity and mortality (1). It is an emerging infectious disease caused by a pathogenic bacterium, *Leptospira interrogans*, which infects humans as well as wild and domestic animals (2). The dissemination of this pathogen occurs mainly by exposition to urine of the major reservoirs (rodents) and other animals directly or indirectly via urine-contaminated water (1). *Leptospira* species have about 300 serovars clustered into 28 serogroups (3). Among all serovars, *Leptospira interrogans* serovar Hardjo is considered as the main serovar responsible for leptospirosis in cattle (4). Bovine leptospirosis is most often a chronic subclinical disease (5), mainly characterized by reproductive losses including abortions, stillbirth and miscarriages as well as reduced weight gain, mastitis, and decreased milk yield (6,7,8). However, laboratory tests, mainly serological methods, are used to confirm diagnosis (9).

The most widely reported risk factors for leptospirosis in bovine herds are age of cows, large herd size, presence of dogs in rural properties co-grazing with other infected animals, contaminated water sources, use of natural breeding, purchase of replacement heifers from infected herds, and dirty drenching equipment (10,11,12). Nevertheless, these risk factors are highly variable among different regions (13,14,15).

Several studies have been conducted in many countries of the world to determine the prevalence of *Leptospira* serovar Hardjo infection in cattle and have reported prevalence rates ranging from 3% to 50% at the animal level (16, 17). In Algeria, only one serological study on bovine leptospirosis has been conducted targeting a small number of cows in Algiers region (18). Therefore, the objectives of this study are to determine the seroprevalence of *Leptospira interrogans* serovar Hardjo infection in apparently healthy cows in the Setif region of Algeria, and to identify risk factors associated with its seropositivity.

Materials and method:

Study area

The study was carried out in the high plains of Setif in the Northeast of Algeria. This region covers approximately 6,550 km² and lies between the longitudes 4°73' - 6°02' and the northern latitudes of 35°61'-36°59'. The climate is semi-arid Mediterranean, characterized by cold rainy winters and hot dry summers. The average annual rainfall was 350 mm from 1984 to 2014. The study area contains approximately 161,952 cattle, including 79,354 dairy cows distributed in 4,465 dairy herds (Agricultural Services Direction of Setif 2015) (Fig. 1).

Study design and sampling

The study is descriptive cross sectional targeting small to medium sized cattle herds located in the Setif region of Algeria, conducted during the period 2015 to 2019. We calculated the sample size using the formula for simple random samples recommended by Thrusfield (19) which is $n = (1.96)^2 \frac{P_{exp}(1-P_{exp})}{d^2}$ where 'n' is the required sample size; 'Pexp' is expected prevalence; 'd' is desired absolute precision; and 1.96 is the Z value for the selected confidence level (95%). According to this formula, the minimum sample size for an infinite population was calculated to be 384 cows using an expected individual prevalence of 50% (as there were no previous studies in this region), a desired absolute precision of 5%, and a confidence level of 95%. The sample size was increased to 406 in order to increase the absolute precision and compensate for 5% attrition.

A total of 406 apparently healthy cows, from which blood samples were collected, were therefore selected by simple random sampling technique among the cattle population aged 3 and 13 years old from a total of 48 cattle herds (Fig. 1). The minimum number of cows to be tested from each herd was established as 10 (20), corresponding to the probability of detecting at least one seropositive animal per herd. For herds with less than 10 cows, all of them were selected for testing.

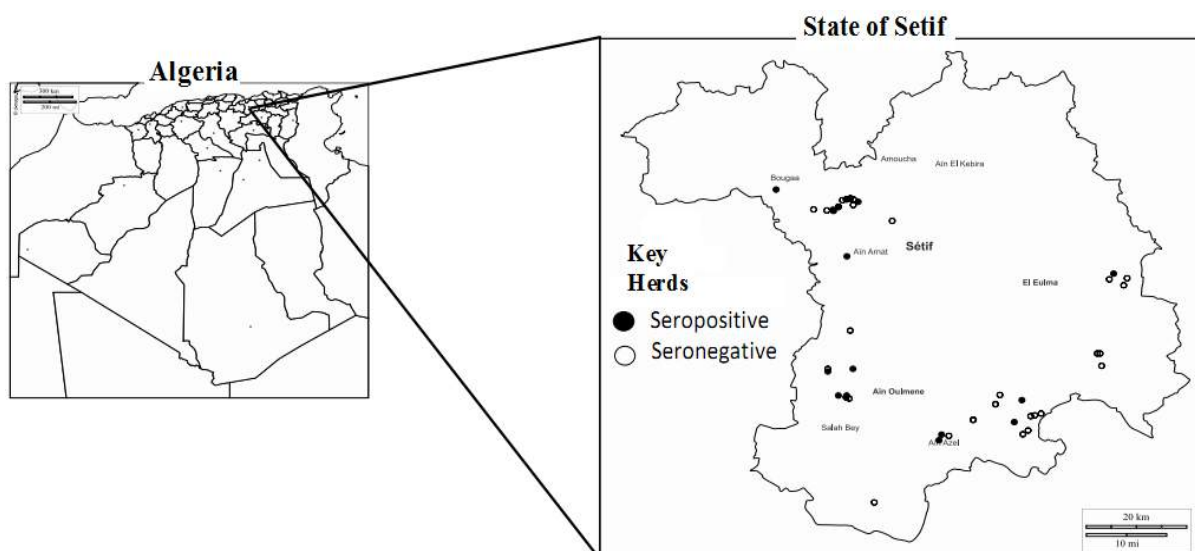


Fig.1: Map of Setif state in the northeastern Algeria, showing the coordinates of the sample locations and spatial distribution of cattle herds that were seropositive for *Leptospira interrogans* serovar Hardjo infection using Arc GIS software

Collection of samples and conservation

After complete physical examination of the enrolled apparently healthy cows, about 10 mL of whole blood was aseptically collected from each cow via the coccygeal vein into plain vacutainer sterile tubes using disposable needles. The tubes were then numbered and immediately transported, on ice, to the laboratory. Serum was obtained by centrifugation of clotted samples at 3000 g for 5 to 10 minutes. The sera were immediately transferred to Eppendorf® tubes and rapidly stored in a freezer at -20°C until laboratory analysis was performed.

Data collection

A pre-validated structured questionnaire was interview-administered to herd owners at the time of blood collection in order to obtain information on potential risk factors associated with *Leptospira interrogans* serovar Hardjo infection. The questionnaire included information related to the herds visited (management system, breeding type and herd size) and the cows enrolled (breed, age, pregnancy, body condition score and history of abortion).

Serological test

The sera were analyzed in the National Center for Biotechnology Research of Constantine (CRBt). For the detection of specific antibodies against *Leptospira interrogans* serovar Hardjo, we used an indirect ELISA test of the PrioCHECK™ *L. hardjo* Ab Strip Kit (Thermo Fischer Scientific, Netherlands) following the protocol prescribed by the manufacturer. The result of each sample was expressed as percent positivity (% PP), which was calculated according to the formula; % PP = (correct OD₄₅₀ test

sample/correct OD₄₅₀ reference serum1) x 100. Samples with a % PP greater than 45% were considered positive; % PP between 20 and 45% were considered as inconclusive, and those less than 20% were considered negative. Inconclusive results were considered negative in this study.

Statistical analysis

The relationship of the risk factors potentially associated with *Leptospira interrogans* serovar Hardjo seropositivity was evaluated in two steps; univariable and multivariable analyzes. In the univariable analysis, Chi-square or Fischer tests was used to examine the association of each variable to Hardjo sero-positivity (21). Variables with a $p \leq 0.20$ were subjected to multivariable logistic regression analysis (22), which was expressed by a significance level (p) of 5%, odds ratio (OR), standard error (SE) and 95% confidence interval (CI). The statistical analysis was performed using SPSS IDEM 23.0 software for Windows.

Results:

Overall seroprevalence of *Leptospira interrogans* serovar Hardjo

The results showed that of the 406 animals, 22 tested positive for *Leptospira interrogans* serovar Hardjo, with an individual prevalence rate of 5.42% (95% CI 3.61–8.07%), and 15 of the 48 herds tested positive, with herd seroprevalence rate of 31.25% (95% CI 19.95–45.33%) (Table 1). The seroprevalence rate of the cows per herd ranged from 7 to 33.3% (95% CI \pm 4.63, mean 17.90%, Q1 10%, median 20%, Q3 25%).

Table 1: Seroprevalence of *Leptospira interrogans* serovar Hardjo infection in individual cows and herds sampled in Setif, Algeria

Cow/herd	Number sampled	Number of positive samples	Seroprevalence rate (%)	95% confidence interval	
				Lower	Upper
Individual cow	406	22	5.42	3.61	8.07
Herd of cattle	48	15	31.25	19.95	45.33

Table 2: Seroprevalence and risk factors associated with *Leptospira interrogans* serovar Hardjo infection among cattle in Setif, Algeria during the period 2015 to 2019

Variables	Categories	Number of animal sampled	Number positive	Prevalence (%)	p value
Pregnancy	Yes	244	13	5.33	0.929
	No	162	9	5.65	
Age of cows (years)	3-6	286	21	7.34	0.008
	>6	120	1	0.83	
Body condition score	Good	156	11	7.05	0.244
	Medium	250	11	4.40	
Breeding type	Dairy	335	17	5.07	0.51
	Mixed	71	5	7.04	
Management system	Intensive	33	4	12.12	0.129
	Semi-intensive	353	18	5.10	
	Extensive	20	0	0.00	
Herd size	5-10	74	1	1.35	0.001
	10-20	196	4	2.04	
	>20	136	17	12.40	
Breed	Crossed	74	4	5.41	0.959
	Imported	318	17	5.35	
	Local	14	1	7.14	
History of abortion in previous years	Yes	31	3	9.98	0.28
	No	375	19	5.07	

Risk factors analysis

Table 2 shows the results observed for the risk factors. The univariable analysis showed significant association of Hardjo infection with both herd size ($p=0.008$) and age of cow ($p=0.001$). In the multivariable (Table 3) analysis, there was significant association between herd size and *Leptospira interrogans* serovar Hardjo sero-positivity. Herds with more than 20 cows were 13 times

more likely to contract leptospirosis than those with 10-20 and 5-10, therefore sero-positivity increases with the herd size. Also, cows under 6 years of age were more likely to be seropositive compared to cows over 6 years old (OR: 9, $p=0.03$). In addition, herds raised in semi-intensive system have lower risk of being seropositive to *Leptospira interrogans* serovar Hardjo compared with herds in the intensive system (OR 0.21, $p=0.02$).

Table 3: Multivariate analysis of risk factors for *Leptospira interrogans* serovar Hardjo infection of cattle in the State of Setif, Algeria

Risk factors	Standard Error (SE)	Odds ratio (OR)	P value	95% confidence interval	
				Lower	Upper
Semi-intensive system	0.68	0.21	0.02	0.06	0.82
Age of cows (3-6 years)	1.04	9.25	0.03	1.19	71.47
Herd size >20	1.02	13.65	0.02	1.58	117.7

Discussion:

The main objective of this study was to determine the prevalence and risk factors for *Leptospira interrogans* serovar Hardjo infection among herds of cattle in the state of Setif, northeast of Algeria. To our knowledge, this is the first major study to report the seroprevalence and risk factors of *Leptospira interrogans* serovar Hardjo infection in cattle in Algeria. Often, the diagnosis of bovine leptospirosis is complex and difficult. Apart from abortion, *Leptospira interrogans* infection in cows is often asymptomatic and unnoticed (23). Consequently, the use of serological tests remains the only alternative to screen animal for infection *in vivo*. ELISA is a commonly used method for initial serological screening for the detection of specific antibodies against *Leptospira interrogans* (9), because it is practical, rapid, inexpensive, and has higher throughput (24). Compared to the micro-agglutination test (MAT) (the reference serological method for leptospirosis detection), ELISA is characterized by a high sensitivity and specificity (9). For these reasons, we used indirect ELISA to determine the seroprevalence of specific antibodies against *Leptospira interrogans* serovar Hardjo natural infection in healthy unvaccinated cows, as vaccination against bovine leptospirosis is not a usual practice in Algeria.

In the present study, we report an individual seroprevalence rate of 5.42%, which is quite similar to 3.89% reported by the only study performed in Algiers region in Algeria (18). The rate in our study is also similar to what have been reported by studies in other countries such as Malaysia with 2.4% (25), Nigeria 8.44% (26), Laos 3% (27), Iran 5.61% (28), Trinidad 4.1% (29), Mexico 2.4% (30) and Turkey 2.8% (31). Contrariwise, higher prevalence rates have been reported in studies from Tanzania 14.76% (17), India 27.76% (32), Jordan 26.25% (13), Bangladesh 47.27% (14), New Zealand 45.6% (33), and extremely high rate in Brazil 83.3% (34). However, the rate in our study is still higher than 0.8% reported from Morocco (35)

and 1% from Spain (36). Similarly, in this study, the herd prevalence was 31.25%, which is much lower than rates reported in other countries such as Brazil with 100% (37), Jordan 92.3 % (13) and Ireland 82.29% (11). On the other hand, this rate is higher than 11% reported in Spain (36). These discrepancies in individual and herd prevalence rates between regions and countries can be attributed to several factors, such as local ecological factors, type of management and husbandry practices, levels of natural immunity and disease resistance among different breeds (13,14).

Another aspect of our work was to study the risk factors associated with *Leptospira interrogans* serovar Hardjo seropositivity. Indeed, univariable and multi-variable analyses showed that herd size, age of cows and management system are the major risk factors that have potential impact on the Hardjo seroprevalence in cattle. We observed that Hardjo sero-positivity increases with herd size (OR of 13 and seroprevalence rate of 12.40% for size > 20). In the literature, many studies have reported the same findings (11,14,38,39). This observation might be attributed to poor quality of sanitation facilities and the great difficulty in monitoring the hygienic measures on large-scale compared to small dairy farms, and the rapid spread of infection in overcrowded farms, which is a potential risk factor for higher prevalence of leptospirosis (14).

In this study, cows aged 3 to 6 years old were more likely to be infected by Hardjo (OR of 9 with seroprevalence 7.34%) compared to the other age category (more than 6 years old with seroprevalence of 0.83%). Indeed, Ijaz et al., (40) reported that age is a potential risk factor for *Leptospira interrogans* serovar Hardjo infection, where young animals were more likely infected than adults. This has been reported by others studies (11,41,42). Moreover, cows raised in semi-intensive system have lesser chance of becoming seropositive to the disease, with a seroprevalence of 5.10% (OR of 0.2), compared to those raised in an intensive

system, where the seroprevalence was 12.12%. This finding might be explained by the fact that farms in intensive system suffer from massive infestation by rodents (major reservoirs of leptospirosis), poor food storage (direct contact of rodents with cattle feed), and long-term direct contact between animals, which was observed by us during this study. Moreover, Dos-Santos et al., (38) attributed this problem to the poor husbandry practices and to the fact that infected animals in overcrowded and confined conditions increase the risk of contaminating the environment. In contrast to our findings, Ismail et al., (13) reported that cattle under semi-intensive system were more likely to be infected by leptospirosis. However, in their study, they investigated both Hardjo and Pomona serovars. For the last one, boars were the main reservoir, and were more in contact with semi-intensive farms and less likely to be in touch with animals in well-isolated herds under intensive system.

Conclusion

In conclusion, results of this study showed that *Leptospira interrogans* serovar Hardjo is present and circulating in apparently healthy cows in Setif state of Northeastern Algeria. Herd size, age of cows and as well as management system were key associated factors for leptospirosis in these herds. Therefore, we recommend very wide surveys in animals and humans all over the country to assess the real prevalence of leptospirosis in Algeria. We also recommend the implementation of hygiene practices and biosecurity measures in farms to reduce the spread of infection, as well as the use of vaccination in animals and people at risk.

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**Original Article****Open Access****Production and efficacy testing of live attenuated and inactivated vaccines against experimental *Salmonella* Kentucky infection in broiler chickens**

*¹Igomu, E. E., ²Fagbamila, I. O., ³Elayoni, E. E., ¹Pwajok, D., ¹Agu, G. C., ⁴Govwang, P. F., ⁵Msheliza, E. G., ⁵Oguche, M. O., and ⁶Mamman, P. H.

^{*1}Bacterial Vaccine Production Division, National Veterinary Research Institute, P.M.B. 01, Vom, Nigeria

²Bacterial Research Division, National Veterinary Research Institute, Vom, Nigeria

³Biological Science Department, Adamawa State University Mubi, Adamawa State, Nigeria

⁴Livestock Investigation Division, National Veterinary Research Institute, Vom, Nigeria

⁵Central Diagnostic Division, National Veterinary Research Institute, Vom, Nigeria

⁶Department of Veterinary Microbiology, Ahmadu Bello University, Zaria, Nigeria

*Correspondence to: elayonigomu@gmail.com; +2348032786224

Abstract:

Background: *Salmonella* Kentucky is of great veterinary and public health concern and exhibits the capacities to emerge as the most prominent *Salmonella* serotype in human disease. The objective of this study is to evaluate the efficacy of prepared live attenuated and inactivated vaccines against experimentally induced salmonellosis caused by *S. Kentucky* in broiler chickens.

Methods: Field isolate of *S. Kentucky* was obtained from the National Veterinary Research Institute Vom, Nigeria and used in the preparation of experimental vaccines. Broiler chickens purchased and ascertained to be free of *Salmonellae* were randomly distributed into three experimental groups of 20 chicks each. Five-bromouracil at a concentration of 1000µg/ml was used in attenuation of *S. Kentucky* in preparation of the live vaccine and 0.6% formaldehyde was used in inactivation of *S. Kentucky* for preparation of the inactivated vaccine. Group 1 chicks were immunized with the live vaccine subcutaneously at 0.5ml per bird, group 2 received 1ml of the inactivated vaccine subcutaneously, and group 3 served as unvaccinated/control.

Results: There was 100% faecal inhibition and 100% vaccinal efficacy in broiler chickens immunized with the live vaccine on day 56 of age, and 49.6% faecal inhibition and 82.8% vaccinal efficacy in broiler chickens immunized with the inactivated vaccine on day 56 of age when compared with the unvaccinated control. The vaccinated birds also showed higher antibody (IgY) titre levels from day 21 to day 56 of age ($p < 0.001$) on all sampling days when values were compared with the unvaccinated birds.

Conclusion: The interplay between vaccination protocol that includes administration of live attenuated or inactivated *S. Kentucky* vaccines and developed antibody (IgY) can reduce intestinal colonization by *S. Kentucky* and subsequent shedding of the serovar in faeces.

Keywords: *Salmonella* Kentucky, 5-bromouracil, IgY, Antibody, Vaccine

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Production et tests d'efficacité de vaccins vivants atténués et inactivés contre une infection expérimentale à *Salmonella* Kentucky chez des poulets de chair

*¹Igomu, E. E., ²Fagbamila, I. O., ³Elayoni, E. E., ¹Pwajok, D., ¹Agu, G.C., ⁴Govwang, P. F., ⁵Msheliza, E. G., ⁵Oguche, M. O., et ⁶Mamman, P. H.

*¹Division de la production de vaccins bactériens, Institut national de recherche vétérinaire, P.M.B. 01, Vom, Nigéria

²Division de recherche sur les bactérienne, Institut national de recherche vétérinaire, Vom, Nigéria

³Département des sciences biologiques, Adamawa State University Mubi, Adamawa State, Nigeria

⁴Division des enquêtes sur le bétail, Institut national de recherche vétérinaire, Vom, Nigéria

⁵Division centrale de diagnostic, Institut national de recherche vétérinaire, Vom, Nigéria

⁶Département de microbiologie vétérinaire, Université Ahmadu Bello, Zaria, Nigéria

*Correspondance à: elayonigomu@gmail.com; +2348032786224

Abstrait:

Contexte: *Salmonella* Kentucky est un grand problème vétérinaire et de santé publique et présente les capacités d'émerger comme le sérotype de *Salmonella* le plus important dans les maladies humaines. L'objectif de cette étude est d'évaluer l'efficacité des vaccins vivants atténués et inactivés préparés contre la salmonellose induite expérimentalement par *S. Kentucky* chez les poulets de chair.

Méthodes: L'isolat au champ de *S. Kentucky* a été obtenu auprès de l'Institut national de recherche vétérinaire de Vom (Nigéria) et utilisé dans la préparation de vaccins expérimentaux. Les poulets de chair achetés et confirmés exempts de salmonelles ont été répartis au hasard en trois groupes expérimentaux de 20 poussins chacun. Le cinq-bromouracile à une concentration de 1000µg/ml a été utilisé pour atténuer *S. Kentucky* dans la préparation du vaccin vivant et 0,6% de formaldéhyde a été utilisé dans l'inactivation de *S. Kentucky* pour la préparation du vaccin inactivé. Les poussins du groupe 1 ont été immunisés avec le vaccin vivant par voie sous-cutanée à 0,5ml par oiseau, le groupe 2 a reçu 1ml du vaccin inactivé par voie sous-cutanée et le groupe 3 a servi de vaccin/vaccin non vacciné.

Résultats: Il y avait 100% d'inhibition fécale et 100% d'efficacité vaccinale chez les poulets de chair immunisés avec le vaccin vivant au 56e jour, et 49,6% d'inhibition fécale et 82,8% d'efficacité vaccinale chez les poulets de chair immunisés avec le vaccin inactivé le 56e jour par rapport au témoin non vacciné. Les oiseaux vaccinés ont également montré des niveaux de titres d'anticorps (IgY) plus élevés du jour 21 au jour 56 ($p < 0,001$) tous les jours d'échantillonnage lorsque les valeurs ont été comparées avec les oiseaux non vaccinés.

Conclusion: L'interaction entre le protocole de vaccination qui comprend l'administration de vaccins *S. Kentucky* vivants atténués ou inactivés et les anticorps développés (IgY) peut réduire la colonisation intestinale par *S. Kentucky* et l'excrétion subséquente du sérovar dans les fèces.

Mots-clés: *Salmonella* Kentucky, 5-bromouracile, IgY, anticorps, vaccin

Introduction:

The global incidence of non-typhoidal *Salmonella* (NTS) gastroenteritis in 2010 was estimated to be 94 million cases, 80.3 million of which were via food-borne transmission, with 155,000 deaths (1,2). Non-typhoidal *Salmonella* can also cause severe extra-intestinal, invasive bacteremia, which is referred to as invasive non-typhoidal salmonellosis (INTS) (3). The most widely reported serovars associated with INTS across Africa were *Salmonella* serovar Typhimurium (serogroup B) and *Salmonella* serovar Enteritidis (serogroup D) (4, 5), but over the last decade, there have been a significant shift in the predominant *Salmonella* serovar associated with poultry and human infections (6,7).

Salmonella Kentucky is now widely spread across Africa, and a virulent strain of this serovar with multi-locus sequence type (MLST) ST198 which was reported to originate from Egypt, have been isolated in Canada, Europe and Asia, raising global public health concerns (7,8). This strain is known to be multi drug resistant (MDR) and has accumulated various chromosomal resistance determinants, encoding resistance to multiple antimicrobials

including β -lactam antibiotics, carbapenems, quinolones, aminoglycosides, co-trimoxazole (trimethoprim-sulfamethoxazole), and azithromycin (7, 9-12) causing infection in humans probably through consumption of contaminated foods especially poultry meat (12).

Molecular characterization of *S. Kentucky* (MLST sequence type ST198) shows a chromosomal genomic island carrying the resistance genes that confer resistance to these antimicrobials (7), and another matter of concern is the expanding livestock reservoir for this strain. Initially identified in African autochthonous poultry, it has subsequently been found in various animals and food items, and this has allowed for a widespread transmission and propagation of this virulent strain (12-17). In Africa, sources and modes of transmission of NTS are still poorly understood due to the lack of coordinated national epidemiological surveillance systems (18) but poultry flocks have been reported to contribute to its dissemination, as this strain has now been isolated from chickens in Ethiopia, Nigeria and Togo, and from turkeys in Morocco (7).

In Nigeria, there is a high rate of production of poultry and this signifies a high rate consumption of its products; however, this

also means a concomitant increase in the cases of *Salmonella*-associated gastroenteritis (19). This high rate consumption of poultry products can be attributed to its availability and acceptance across several ethnic, religion and diverse cultural backgrounds in the country (20-22). Several reports have documented the spread of *S. Kentucky* serovar across all the geopolitical regions in Nigeria (23-25), and Raufu et al., (26) identified the presence of the virulent *S. Kentucky* MLST type ST198 clone that encode genes resistant to multiple antimicrobials in Nigeria.

A reduction of the burden of *S. Kentucky* associated salmonellosis in humans will involve the elimination of the infection from poultry (27), and this may be achieved through the use of vaccination regimens that will prevent proliferation of the bacteria in poultry. In chickens, vaccines should prevent intestinal and caecal colonization, resulting in diminished faecal shedding and should be effective against systemic infection, preventing vertical transmission and egg contamination (28). A lot of experimental vaccines against salmonellosis have been produced for chickens, and a variety of commercial vaccines are also available in the market (29). Most of these commercial vaccines do not belong to the same serogroup as *S. Kentucky* and thus confers little or no protection against this serotype. In order to reduce the spread of the circulating virulent strain of *S. Kentucky* in birds and its subsequent transmission to man, this study proposes to produce and evaluate the efficacy of a live attenuated and inactivated *S. Kentucky* vaccines in broiler chickens.

Materials and methods:

Study setting

The study was carried out at National Veterinary Research Institute Vom, located in Jos south Local Government Area (LGA) of Plateau State, Nigeria. Experimental vaccines were produced in the Bacterial Vaccine production division and the challenge study was undertaken in the Animal Experimental house of the Institute.

Bacteria isolate

Field isolate of *S. Kentucky* from previous study (24) was obtained from the Bacterial Research Division of the National Veterinary Research Institute (NVRI), Vom, Nigeria, which was identified and serotyped according to the Kauffmann-White Scheme at the OIE *Salmonella* Reference Laboratory,

Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy.

Experimental birds

A total of 100 apparently healthy day-old White Cornish broiler chicks were obtained from Ibadan (Zartech), Southwest Nigeria. Chicks were randomly distributed into five Pens (20 chicks per pen; two pens for attenuation study and three pens for vaccinal study). Their wings were tagged according to their study group. The procedures used for animal care, housing and experimentation were in accordance with the Ahmadu Bello University (ABU) Committee on Animal Use and Care and ethical clearance from NVRI, Vom, Nigeria.

Culture and isolation of faecal *Salmonella* from the chicks to ascertain their carriage of *Salmonella* spp were done according to the ISO 6579:2002 (30). Cloacal swabs were taken and pre-enriched in buffered peptone water (Oxoid, UK) in a 1:10 sample to broth ratio and incubated at 37°C for 18 hours. 0.1ml of the pre-enriched broth was inoculated into Rappaport-Vassiliadis (RV) (Oxoid, UK) enrichment medium and incubated for 18 hours at 37°C. Samples from the enrichment medium were then plated onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK). The plates were incubated at 37°C for 18 hours, and chicks positive for *Salmonella* spp were excluded (24).

Attenuation of *Salmonella Kentucky* using 5-bromouracil

The methodology of Hertman et al., (31) was adopted with modifications. Stock culture of field isolate of *S. Kentucky* were produced by picking a discrete colony of *S. Kentucky* from XLD agar (Oxoid, UK) plates and inoculating into 10ml Nutrient Broth (NB) (Oxoid, UK), incubated at 37°C for 24 hours. The NB culture containing *S. Kentucky* were further inoculated separately into five NB (Oxoid, UK) (1ml stock culture to 10ml NB) in a graduated 50ml bottle, and incubated at 37°C for 18 hours. The cultures were harvested by centrifugation at 1,000 rpm for 10 minutes. The sediments were washed and re-suspended in 10ml citrate buffer (pH 5.5). One ml each of the culture was taken for colony count (CFU/ml) by adding to 9ml of 0.85% saline (normal saline) in a 10-fold serial dilution in 10 test tubes, with aliquots (0.05ml) from each of the last five dilutions spread onto XLD agar (Oxoid, UK).

Varying amounts of 5-bromouracil (Sigma-Aldrich, Germany) at the following final concentrations in citrate buffer; 400µg/ml,

600µg/ml, 800µg/ml, 1,000µg/ml and 1,200 µg/ml were added to the bacterial suspensions (31), which were incubated in a water bath for 30 minutes at 37°C. After 30 minutes, 0.5ml each of the culture was further taken for viability and colony count by 10-fold serial dilutions of the bacteria in 0.85% saline (normal saline) and subsequent plating of 0.05 ml aliquots on XLD agar (Oxoid, UK).

The treated bacteria culture was then re-centrifuged at 10,000 rpm for 10 minutes and sediment washed twice with 10ml phosphate buffer (pH 7.2). The washed sediment was re-suspended in 10ml fresh NB (Oxoid, UK) and incubated for 18 hours at 37°C in a flask. At the varying concentrations of 5-bromouracil (5-BU), the percentage live bacterial count was determined. Only cultures with less than 20% bacterial live count after exposure to 5-BU were taken for further isolation. Bacterial colonies surviving 5-BU treatment were picked up by sterile Pasteur loops, purified by streaking on XLD agar (Oxoid, United Kingdom) plate surface twice and re-isolation of single colonies following incubation at 37°C for 24 hours (31).

Virulence attenuation test

Infective dose of 5-BU attenuated and non-attenuated *S. Kentucky* stock was prepared to an approximate stock dilution of 1×10^9 CFU/ml bacteria suspension in sterile NB (Oxoid, UK). Two groups of twenty 14-day-old broiler chicks were infected separately by oral administration by gavage of 1ml of these broth cultures and observed for clinical signs such as profuse white diarrhoea, depression, somnolence and mortality, and gross morphological lesions such as enlargement and necrotic foci in the liver (32,33).

Re-isolation and quantification of *Salmonella* from the liver was done by first homogenizing 1g of liver sample in 10ml of 2% buffered peptone water (Oxoid, UK), and inoculating 1ml of this pre-enriched broth into Rappaport-Vassiliadis (Oxoid, UK), followed by incubation for 18 hours at 37°C. Colony count by 10 fold serial dilution of the bacteria in 0.85% saline (normal saline) was done with subsequent plating of 0.05ml aliquots on XLD agar (Oxoid, UK) plates. The number in CFU/ml transformed to \log_{10} and percentage re-isolation of *S. Kentucky* from liver on XLD agar (Oxoid, UK) was calculated (34,35,36) using the formula; Percentage (%) Re-isolation = $\log_{10}N/9.0 \times 100$, where $\log_{10}N = \log_{10}$ CFU/ml, following Re-isolation, $9.0 = \log_{10}$ (1×10^9 CFU/ml) (represents infective dose and denominator).

Preparation of *Salmonella Kentucky* vaccine stock

Nutrient agar slants were first prepared in Roux flask by suspending 28g of Nutrient agar powder (Oxoid, UK) in 1 litre distilled water. The suspension was brought to boil to dissolve completely, and 250ml dispensed into each Roux flask and sterilized by autoclaving at 121°C for 15 minutes. The Roux flasks were slanted on the bench in sterile room at 25°C to solidify. Aseptically, 10ml of stock cultures of 5-BU attenuated and non-attenuated *S. Kentucky* were inoculated onto the Nutrient agar slant in Roux flasks and incubated at 37°C for 48 hours separately.

The colonies were harvested with sterile normal saline and glass beads by gentle rocking. Purity was carried out on each Roux flask culture by streaking onto Blood agar (BA) plates, and incubating plates at 37°C for 24 hours while Roux flask cultures were stored at 4°C. The 5-BU attenuated and non-attenuated cultures were pooled separately and bacterial suspension adjusted to contain 10^7 CFU/ml using colony count technique (32,36) for vaccine preparation. The purity of the pooled cultures was checked by plating on XLD (Oxoid, UK) and Blood agar plates (32).

Preparation of vaccine stabilizers

Fifteen grams of gelatin (Oxoid, UK) was dissolved in 100ml of deionized water and sterilized by autoclaving at 121°C for 15 minutes. 50g Lactalbumin hydrolysate (LAH) (Oxoid, UK), 100g sucrose (Sigma-Aldrich, Germany), 1.16g potassium dihydrogen orthophosphate (KH_2PO_4) (J.T. Baker, USA) and 7.66g sodium phosphate dibasic (Na_2HPO_4) (J.T. Baker, USA) were dissolved into 1 litre of deionized water and autoclaved at 121°C for 15 minutes. Normal saline was prepared by dissolving 8.5g in 1 litre of distilled water and autoclaved at 121°C for 15 minutes. Purity check was undertaken after 24 hours of incubating all preparations by streaking onto BA plates and incubating at 37°C for 24 hours (32).

Preparation of live attenuated vaccine

Aseptically, the 5-BU attenuated *S. Kentucky* concentrate at approximately 10^7 CFU/ml bacterial suspension was thoroughly mixed with stabilizers at a ratio of 5:4:1 (50% *S. Kentucky* concentrate: 40% LAH: 10% Gelatin). The vaccine was dispensed into sterile vials at 2.5ml per vial and lyophilized at National Veterinary Research Institute, Vom where production of the experimental vaccine was undertaken (32).

Preparation of inactivated vaccine

The non-attenuated *S. Kentucky* concentrate (3 litres) at approximately 10^7 CFU/ml bacterial suspensions was dispensed into sterile 10 litres round bottom flask, 18ml of 37% formaldehyde stabilized solution (Sigma-Aldrich, Germany) was added and thoroughly mixed before plating on Blood agar plate and incubating at 37°C for 24 hours, to ascertain inactivation. Following confirmation of bacterial inactivation, Glycerin saponin saline (GSS) [which has been prepared by mixing 1000ml of glycerin (Schutz, Indonesia) with 100g of saponin (Sigma-Aldrich, Germany) and 8.5g of NaCl (Oxoid, UK) in 1000ml of deionized water and autoclaved at 121°C for 15 mins in graduated 5000ml Schott-Duran bottle] was added to the killed bacteria suspension at a ratio of 8:2 (8 parts of inactivated bacteria suspension: 2 parts of GSS) and the vaccine was dispensed into sterile 200ml bottle (32).

Purity test on live attenuated and inactivated vaccines

Five vials of the lyophilized live attenuated vaccine were sampled randomly and reconstituted with 2.5ml sterile normal saline. Using a Pasteur wire loop, purity check was carried out by streaking each vial separately on paired BA and Sabouraud's Dextrose agar (SDA) plates. BA plates were incubated aerobically at 37°C for 24 hours and anaerobically in jar using CO₂ gas packs (CO₂Gen Oxoid, UK) at 37°C for 24 hours while the SDA plates were incubated at 25°C for 7 days. Colony count was undertaken by a 10-fold serial dilution in normal saline and aliquot (0.05ml) of the last 5 dilutions were cultured on BA plates in duplicate pairs, and incubated at 37°C for 24 hours.

Sterility (inactivation) check on the inactivated vaccine was carried out by streaking a Pasteur wire loop full on BA plate of the prepared vaccine, incubated at 37°C for 24 hours and Gram stain undertaken for the inactivated vaccine (32).

Experimental design

Three groups, each with 20 day-old broiler chicks, were reared in a deep litter system in separate pens; group 1 received 0.5ml of the prepared live attenuated *S. Kentucky* vaccine subcutaneously at day 11 of age and a repeat at day 25 of age; group 2 received 1.0ml of the prepared inactivated *S. Kentucky* vaccine subcutaneously at day 11 of age and a repeat at day 25 of age; group 3 served as the control (unvaccinated) and were inoculated with 0.5ml sterile normal saline

subcutaneously at day 11 of age and a repeat at day 25 of age (32).

The three groups were challenged on day 32 of age by oral gavage of 1ml each of *S. Kentucky* field isolate in NB (Oxoid, UK) containing approximately 5×10^7 CFU/ml bacteria suspension (34). This was prepared by first suspending six and half grams of NB powder in 500ml distilled water, mixing and dispensing into Schott Duran bottle, and sterilized by autoclaving at 121°C for 15 minutes. Discrete colonies of *S. Kentucky* on XLD agar were then inoculated onto NB and incubated at 37°C for 24 hours. Purity of the culture was checked by plating on XLD agar (Oxoid, UK) and Blood agar plates (32) and colony count was titred to bring to an approximate stock dilution of 5×10^7 CFU/ml bacteria suspension (36).

Assessment of vaccine potency

For the assessment of vaccine potency, serum samples of experimental birds were collected on day 7, day 14, day 21, day 28, day 35, day 42, day 49 and day 56 of age and stored at -20°C. Seroconversion of the experimental broiler chickens to *S. Kentucky* was measured in the serum using Chicken IgY Enzyme Linked Immunosorbent Assay (ELISA) kit (Wuhan Fine Biotech Co., Ltd) according to the manufacturer's directive. One µL of serum sample was diluted in 99µl of Standard Dilution Buffer and quantification of titre was done by measuring absorbance at 450 nm wavelength in an ELISA machine (RT-2100C; Rayto Microplate Reader). The ELISA was run at one time for all the serum samples to minimize variability.

Faecal shedding

Quantitative *Salmonella* shed in faeces at baseline and following experimental vaccine administrations were determined by taking cloacal swabs from vaccinated and non-vaccinated birds at day 7, day 14, day 21, day 28 and day 31 before challenge with field *S. Kentucky* isolate. Cloacal samples were then taken on days 35, 42, 49 and 56 after the challenge. Sample swabs were used to collect multiple faecal samples repeatedly from the broiler chickens to a stomacher bag, and 1g of the faecal sample was then diluted in 10ml of 0.85% saline (normal saline). Further, 10-fold serial dilution of the sample in normal saline in 10 test tubes was carried out and 0.05ml aliquots of each of the first five dilutions were spread onto XLD agar plates (Oxoid, UK). After 18 to 24 hours of incubation at 37°C, *Salmonella* colonies (black) on each plate were

counted (35). The prevalence of infection and percentage vaccinal efficacy were calculated using the method of Thrusfield 2007 (38) with the formula; Percentage (%) vaccinal efficacy = $C-T/C \times 100$, where C=prevalence of infection in unvaccinated animals (unvaccinated animals are defined as 'exposed' to the risk factor), and T = prevalence of infection in vaccinated animals.

Data analysis

All data obtained from the study were entered and analysed with the aid of GraphPad Prism (Version 5.01, Graphpad software Inc.). P value < 0.05 was considered statistically significant. The data for the mean quantitative CFU/ml faecal *Salmonellae* shed and the mean quantitative serum antibody (IgY) titre for the vaccinated groups versus the control/unvaccinated group over time was analysed using a two-way analysis of variance (ANOVA).

The mean antibody (IgY) titres between the live vaccine group and inactivated vaccine group were compared using paired sample t-Test before and after immunization with the prepared vaccines. The correlation between the mean quantitative serum antibody IgY (\log_{10} ng/mL) titre and the mean quantitative faecal *Salmonella* (\log_{10} CFU/ml) shed was determined by Pearson correlation test (r^2 value). Results were presented in tables, figures and charts.

Results:

Results of virulence test

Profuse white diarrhoea, depression, hurdling, anorexia and somnolence were observed in broilers challenged with the non-

attenuated stock culture. There was also depression, anorexia, somnolence and hurdling observed in broiler chickens challenged with the 5-BU attenuated stock, however white diarrhoea was absent as faeces were pasty brown.

Post mortem observable changes three days after challenge was a slightly enlarged and congested liver in broiler chickens challenged with the non-attenuated stock, with percentage hepatic re-isolation of *S. Kentucky* of 62.2%. In broiler chickens challenged with 5-BU attenuated stock, post mortem examination revealed a normal liver and a percentage hepatic re-isolation of 30%.

Vaccinal efficacy against *Salmonella Kentucky* experimental infection

Infection rates in the unvaccinated (exposed) birds on sampling days post-challenge were consistently higher than in the vaccinated birds and this was statistically significant ($p < 0.05$), however between the vaccinated groups, there was no statistically significant difference.

On day 35 of age of birds, i.e. 3 days post challenge, 53.9% of birds were protected from infection by the live vaccine and 34.4% of birds were protected from infection by the inactivated vaccine. Vaccinal efficacy increased to 100% in birds immunized with live vaccine and 82.8% in birds immunized with the inactivated vaccine on day 56 (Table 1).

Table 1: Prevalence of infection and Percentage (%) vaccinal efficacy against *Salmonella Kentucky* in broiler chickens after challenge

Age of birds in days/prevalence of infection (percentage vaccinal efficacy)				
Group	Day 35	Day 42	Day 49	Day 56
Live vaccine*	6/18 (53.86)	6/18 (60.02)	5/18 (68.73)	0/18 (100)
Inactivated vaccine*	9/19 (34.35)	7/19 (36.85)	6/19 (64.45)	2/19 (82.82)
Unvaccinated (control)	13/18	15/18	16/18	11/18

*No statistically significant difference ($p > 0.05$) between the vaccinated groups

Mean quantitative faecal *Salmonellae* shed on sampling days post challenge

On all sampling days prior to challenge, faecal *Salmonella* shedding was unnoticed in cloacal swab buttressing apparent safety of the experimental vaccines but on days 35, 42, 49 and 56 of age, there were statistically significant differences ($p < 0.001$) when the vaccinated groups were compared against each other and against the control. Faecal *Salmonella* counts in cloacal swabs of birds immunized with experimental vaccines on day 56 of age were lower than the unvaccinated group, with 0 CFU/ml for the live attenuated vaccine, 3.5×10^2 CFU/ml for the inactivated vaccine, and 1.33×10^5 CFU/ml for

the control group ($p < 0.05$). This represented 100% inhibition by the live vaccine and 49.6% inhibition by the inactivated vaccine when compared to the control (Table 2).

Antibody (IgY) titre in serum

The antibody (IgY) titre of vaccinated birds was significantly higher than those of the unvaccinated birds post vaccination ($p < 0.001$). There was no significant difference in IgY titre levels between the two vaccinated groups on sampling days post vaccination except on day 28 of age post-booster vaccination, when IgY titre in the live vaccine group was significantly higher than the inactivated vaccine group ($p < 0.05$) (Table 3).

Table 2: Mean number of *Salmonella* Kentucky count in CFU/ml re-isolated in faeces after challenge

Age of birds in days/number of CFU per ml				
Group	Day 35	Day 42	Day 49	Day 56
Live vaccine	8.80×10^3	1.28×10^3	3.50×10^2	0
Inactivated vaccine	9.00×10^3	2.43×10^3	4.75×10^2	3.50×10^2
Unvaccinated (control)	4.33×10^5	2.24×10^6	1.34×10^7	1.33×10^5

$p < 0.001$ when vaccinated groups were compared to control

Table 3: ELISA result showing the mean number of antibody (IgY) titre (ng/ml) in serum of experimental birds

Age of birds in days/antibody titre								
Group	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
Live vaccine	2.71	4.44	10.51*	19.85*a	22.15*	23.10*	22.02*	20.56*
Inactivated vaccine	2.44	4.46	11.47*	18.23*	20.81*	22.15*	20.70*	19.12*
Unvaccinated (control)	2.14	3.85	3.06	3.61	8.39	11.51	8.57	8.64

Serum dilution (1:100) thus actual value in 'ng/ml' is multiplying figure in each cell by 100

* = $p < 0.001$ when vaccinated groups values was compared to the unvaccinated group

a = $p < 0.05$ when live vaccine group was compared to inactivated vaccine group

Paired sample t-Test results between live vaccine group and killed vaccine group; p value=0.05

Correlation coefficient between Live vaccine and Inactivated vaccine groups = 0.997

Correlation of antibody (IgY) titre to faecal *Salmonella* shed in vaccinated chickens

The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal *Salmonella* shed in broiler chickens immunized with the live attenuated vaccine was 0.8519. This represents a strong positive correlation

(Fig 1). At day 35 and 42 of age, antibody titre rose while faecal *Salmonella* shed declined. At day 49 and day 56 of age, there was commensurate decline in serum antibody (IgY) titre and faecal *Salmonella* shed (Fig 1).

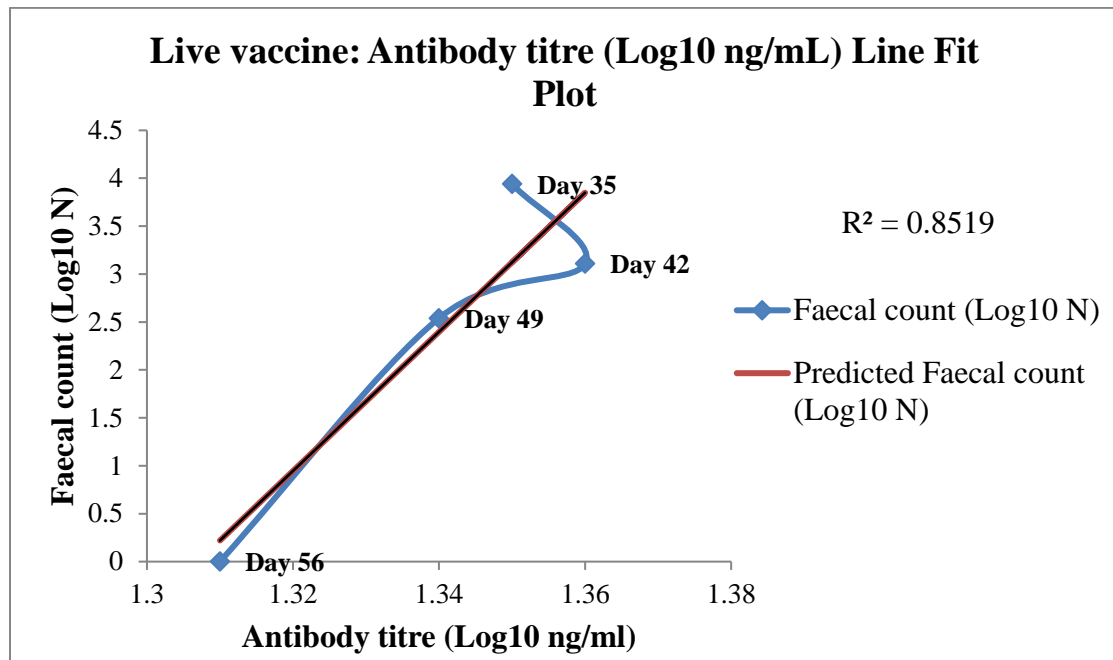


Fig 1: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal *Salmonellae* shed in broiler chickens immunized with live vaccine; R^2 (0.8519): The correlation coefficient represents a strong positive relationship; p value = 0.333

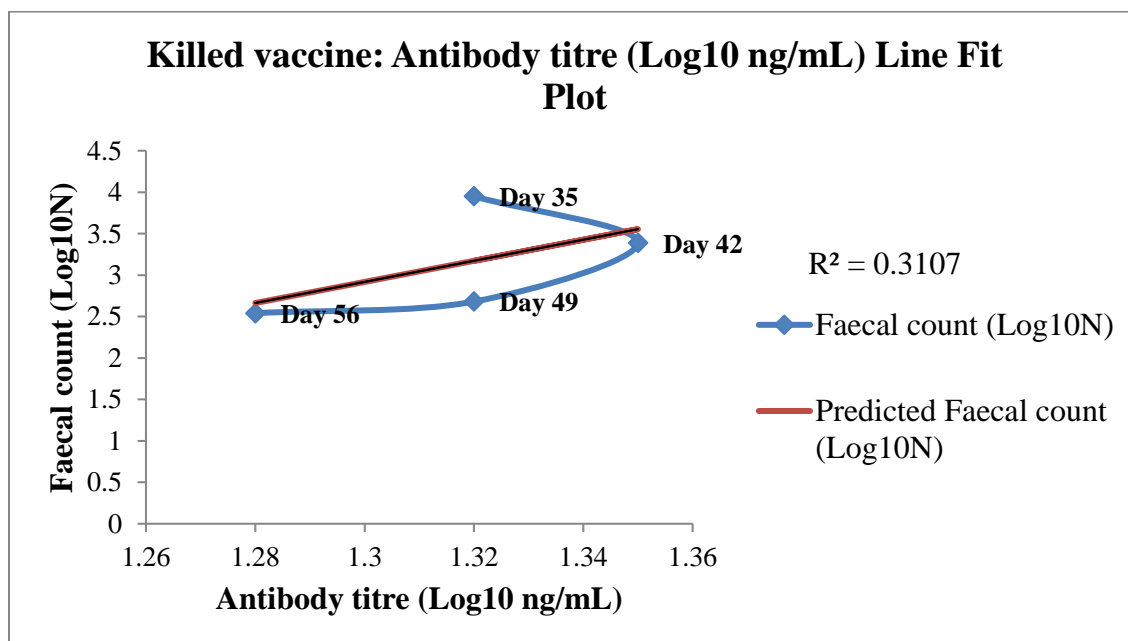


Fig 2: Correlation of the effect of mean Antibody (IgY) titre on the mean faecal *Salmonellae* shed in broiler chickens immunized with inactivated vaccine; R^2 (0.3107): The correlation coefficient represents weak positive relationship; p value = 0.417

The correlation coefficient R^2 for the mean serum antibody (IgY) titre to faecal *Salmonella* shed in broiler chickens immunized with the inactivated vaccine was 0.3107. This represents a weak positive correlation (Fig 2). All sampling days post challenge (day 35, 42, 49 and 56 of age) were not commensurate. At day 35 and 42 of age, the mean serum antibody (IgY) titre rose while faecal *Salmonella* shed declined but on day 49 and 56 of age, serum antibody (IgY) titre and faecal *Salmonella* shed declined (Fig 2).

Discussion:

The live attenuated and inactivated experimental vaccines prepared in this study significantly reduced faecal *S. Kentucky* shed in broiler chickens immunized, when compared to the unvaccinated broiler chickens. This finding is similar to those observed by Clifton-Hadley et al., (39), Papezova et al., (40), Nourhan et al., (41) and Groves et al., (42), who reported that vaccine preparations containing either live attenuated or inactivated *Salmonella* spp. can reduce faecal shed of the bacteria. It has been observed that vaccine preparations containing live organisms in comparison to inactivated ones provide a superior protection in preventing intestinal colonization and environmental contamination by *Salmonella* spp., evident by a higher decline in faecal *Salmonella* shed (39, 40, 43, 44).

In the present study, faecal shedding of *Salmonellae* between the vaccinated groups did not differ significantly from day 35 to day 42 of age post challenge, but there was statistically significant difference on day 56 of age with broiler chickens immunized with the live attenuated vaccine observed to have superior protection at this day. This superior protection has been attributed to the capacity of live attenuated *Salmonella* vaccines to stimulate the secretion of mediators (cytokines, interleukins and interferons) and inducing a T helper 1 (Th1) response cell-mediated immunity, which does not occur with inactivated products. Additionally, CD8+ T cells play a crucial role in immune protection against *Salmonella*, and these cells (CD8+ T) response are exclusive after vaccination using live attenuated vaccines (45- 48).

The result of the mean serum antibody (IgY) titre in the present study showed that the immunized broiler chickens produced and sustained a superior IgY titre from day 21 of age following first immunization to day 56 of age when compared to the unvaccinated broilers. These findings were similar to those

reported by Babu et al., (49) and Tizard (50). The superior seroconversion in immunized broiler chickens was attributed to the effect of the prepared vaccines. On day 42 of age, antibody (IgY) titre in serum peaked in all the groups, reaching 2310 ng/mL in broiler chickens immunized with the live vaccine preparation, 2215 ng/mL in the inactivated vaccine group and 1151 ng/mL in the unvaccinated group. There was also a rapid rise in antibody (IgY) titre in the broiler chickens on day 35 of age across all groups, which may be related to an increase mobilization of antibody (IgY) due to the introduction of live bacteria, similar to what was observed by Nourhan et al., (41).

A strong positive correlation of 0.997 in antibody (IgY) titre between the vaccinated groups was observed in this study and mean paring was significantly effective ($p=0.05$). This finding showed that neither the live attenuated preparations nor inactivated preparation was superior in eliciting serum antibody (IgY) titre, as immune response judge by antibody (IgY) titre produced were closely matched throughout the sampling days. This was in contrast to reports by Penha Filho and Berchieri (51) who reported a higher level of immunoglobulin levels in birds immunized with an inactivated vaccine than a live vaccine preparation. Papezova et al., (40) also reported a higher immunoglobulin titre in birds vaccinated with inactivated preparation than a live preparation. The result of our study however is similar to Babu et al., (49) who reported that live vaccines have been shown to be more effective in increasing lymphocyte proliferation in response to *Salmonella* antigens in laying hens. Several factors however may cause these variations in humoral immune response elicited in birds (52), as the level of protection depends on the challenge strain/components of the vaccine, route of administration, infection dose, age of birds and species/line of birds. Consequently, it has been difficult to compare strictly the efficacy of the vaccine preparations currently available (52,53). Papezova et al., (40) prepared an inactivated vaccine enriched by SPI-1 or SPI-2 proteins, which are proteins central to *Salmonella* virulence that may be responsible for the marked difference.

This present study made use of saponin as an adjuvant, which has been shown to have great characteristic as an immuno-adjuvant by Barbosa (54) while other studies made use of aluminium gels and salts as adjuvant. Adjuvants are defined as groups of structurally heterogeneous compounds that enhance or modulate the immunogenicity of

the associated antigens. Despite the recognition of many different types of adjuvants, however, little is known about their mode of action. The events triggered by these immuno-modulators appear to come from one or the combination of several of the following effects; depot effect, effect on antigen presenting cells (APC), nonspecific immuno-stimulating effect and particulated carrier systems (53). There are few and isolated information that explains how vaccines work and the immune response depending on the antigen and route of vaccination (44). Overall, in our study, immunized birds produced and sustained high serum antibody (IgY) levels that were able to provide protection against challenge by *S. Kentucky* proven by the significant decline in the cumulative amount of *Salmonellae* shed in faeces of immunized birds when compared to the unvaccinated birds.

Conclusion:

Our study demonstrated the interplay between vaccination regimens which include immunization with either 5-BU attenuated live *S. Kentucky* vaccine or 0.6% formalin inactivated *S. Kentucky* vaccine, and the developed antibody (IgY) titre reduced intestinal colonization and interfered with the pattern and shedding of *S. Kentucky* in faeces of broiler chickens. There was 100% vaccinal efficacy and inhibition of faecal shedding of *S. Kentucky* by the live attenuated vaccine as well as 82.8% vaccinal efficacy and 49.6% inhibition of faecal shedding of *S. Kentucky* by the inactivated vaccine preparation in broiler chickens used for the experiment.

Conflict of interest:

Authors declared no conflict of interest

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Original Article

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Active tuberculosis among adult HIV-infected patients accessing antiretroviral therapy in a tertiary health facility in Lafia, northcentral Nigeria

*¹Audu, E. S., ²Adiukwu, C., ³Bello, S., ⁴Abdulmajid, S., ⁴Anyuabaga, B., ⁵Ashuku, Y. A., and ⁶Anazodo, M.

¹Department of Medical Microbiology/Special Treatment Clinic, Dalhatu Araf Specialist Hospital, Lafia

²Department of Internal Medicine, Dalhatu Araf Specialist Hospital, Lafia

³Department of Paediatrics/Special Treatment Clinic, Dalhatu Araf Specialist Hospital, Lafia

⁴Special Treatment Clinic, Dalhatu Araf Specialist Hospital, Lafia

⁵College of Medicine, Federal University, Lafia

⁶Research Unit, Dalhatu Araf Specialist Hospital, Lafia

*Correspondence to: estanamo@gmail.com

Abstract:

Background: Tuberculosis and Human Immunodeficiency Virus (HIV) co-infection is a major problem in Nigeria and other countries that are ravaged by a high burden of both diseases. The World Health Organization (WHO) reports that the risk of developing active tuberculosis (TB) among people living with HIV is 16-27 times that of HIV negative persons. Although antiretroviral therapy (ART) reduces the risk of developing TB, there are factors which predispose those on ART to TB. This study sought to determine the prevalence of TB among adults on ART in our facility and identify the predisposing factors.

Methodology: This was a retrospective study utilizing data from clinical records (folders and electronic) of adult HIV patients who are accessing ART in our facility and have been on ART for at least 6 months. A proforma was used to collect data including demographic, clinical, ART and laboratory information of the patients. The data were entered into SPSS version 23 and analyzed using descriptive statistics and bivariate analysis. Associations were tested using Chi square with 95% confidence level.

Results: A total of 457 patients were studied, aged 18-69 years (mean age 38.3± 10 years), and 72.4% females. Majority were married (81%), unemployed (53.8%), had mean baseline CD4 cell count of 267.4 ± 185 cells/mm³ and a mean duration on ART of 100.9± 39 months. Seventeen point three percent of the patients had a previous history of TB before or within 6 months of commencement of ART. Thirteen (2.8%) of the patients had active TB while on ART. Majority of those who had active TB were females (76.9%), married (76.9%), unemployed (46%), had no previous history of TB (53.8%), baseline CD4 cell count of ≤ 350 cells/mm³ and were on first line ART medication. There was however no significant statistical association of active TB with any of these factors.

Conclusion: Few patients had active TB while on ART in this study. The high frequency of TB in those who had low baseline CD4 cell count and baseline WHO stage shows the importance of early initiation of ART in people living with HIV (PLHIV). There is need for regular screening of PLHIV for TB and innovative approaches to get people with HIV to know their TB status as well as early commencement of ART.

Keywords: Human immunodeficiency virus, Active Tuberculosis, Antiretroviral therapy.

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Tuberculose active chez des patients adultes infectés par le VIH ayant accès à un traitement antirétroviral dans un établissement de santé tertiaire à Lafia, au centre-nord du Nigéria

*¹Audu, E. S., ²Adiukwu, C., ³Bello, S., ⁴Abdulmajid, S., ⁴Anyuabaga, B., ⁵Ashuku, Y. A., et ⁶Anazodo, M.

¹Département de microbiologie médicale/Clinique de traitement spécial, Hôpital spécialisé Dalhatu Araf, Lafia

²Département de médecine interne, Hôpital spécialisé Dalhatu Araf, Lafia

³Département de pédiatrie/Clinique de traitement spécial, Hôpital spécialisé Dalhatu Araf, Lafia

⁴Clinique de traitement spécial, Hôpital spécialisé Dalhatu Araf, Lafia

⁵Collège de médecine, Université fédérale, Lafia

⁶Unité de recherche, Hôpital spécialisé Dalhatu Araf, Lafia

*Correspondance à: estanamo@gmail.com

Abstrait:

Contexte: La co-infection tuberculose et virus de l'immunodéficience humaine (VIH) est un problème majeur au Nigéria et dans d'autres pays qui sont ravagés par un fardeau élevé des deux maladies. L'Organisation mondiale de la santé (OMS) signale que le risque de développer une tuberculose active (TB) chez les personnes vivant avec le VIH est de 16 à 27 fois supérieur à celui des personnes séronégatives. Bien que la thérapie antirétrovirale (TAR) réduise le risque de développer la TB, il existe des facteurs qui prédisposent les personnes sous TAR à la TB. Cette étude visait à déterminer la prévalence de la tuberculose chez les adultes sous TAR dans notre établissement et à identifier les facteurs prédisposants.

Méthodologie: Il s'agissait d'une étude rétrospective utilisant des données provenant de dossiers cliniques (dossiers et électroniques) de patients adultes atteints du VIH qui accèdent au TAR dans notre établissement et qui sont sous TAR depuis au moins 6 mois. Un formulaire a été utilisé pour recueillir des données, y compris des informations démographiques, cliniques, ART et de laboratoire des patients. Les données ont été saisies dans SPSS version 23 et analysées à l'aide de statistiques descriptives et d'une analyse bivariable. Les associations ont été testées en utilisant le chi carré avec un niveau de confiance de 95%.

Résultats: Un total de 457 patients ont été étudiés, âgés de 18 à 69 ans (âge moyen 38,3 ± 10 ans) et 72,4% de femmes. La majorité était mariée (81%), sans emploi (53,8%), avait un nombre moyen de cellules CD4 de base de 267,4 ± 185 cellules/mm³ et une durée moyenne sous TAR de 100,9 ± 39 mois. Dix-sept virgule trois pour cent des patients avaient des antécédents de tuberculose avant ou dans les 6 mois suivant le début du TAR. Treize (2,8%) des patients avaient une tuberculose active pendant le TAR. La majorité de ceux qui avaient une tuberculose active étaient des femmes (76,9%), mariées (76,9%), sans emploi (46%), sans antécédents de tuberculose (53,8%), le nombre initial de cellules CD4 ≤ 350 cellules/mm³ et étaient médicaments antirétroviraux de première ligne. Il n'y avait cependant pas d'association statistique significative de la TB active avec aucun de ces facteurs.

Conclusion: Peu de patients avaient une tuberculose active pendant le TAR dans cette étude. La fréquence élevée de la tuberculose chez ceux qui avaient un faible nombre de cellules CD4 de base et un stade de base de l'OMS montre l'importance de l'initiation précoce du TAR chez les personnes vivant avec le VIH (PVVIH). Il est nécessaire de procéder à un dépistage régulier des PVVIH pour la tuberculose et à des approches innovantes pour permettre aux personnes vivant avec le VIH de connaître leur statut antituberculeux ainsi que le début précoce du TAR.

Mots-clés: virus de l'immunodéficience humaine, tuberculose active, thérapie antirétrovirale.

Introduction:

Tuberculosis (TB) remains one of today's global health challenges ranking as one of the leading infectious cause of death in the world. Tuberculosis and human immunodeficiency virus (HIV) co-infection also remain a major problem especially in sub-Saharan Africa which carries a high burden of both diseases. According to the World Health Organization (WHO), approximately 36.9 million people are living with HIV globally in 2017, 66% of them in sub-Saharan Africa (1,2). The WHO reports that the risk of developing TB is estimated to be 16-27 times greater in PLWHA than HIV negative persons (3-5). HIV has been reported to increase the incidence of TB as well as the morbidity and mortality associated with the disease (4).

The introduction of antiretroviral therapy (ART) has decreased the incidence of TB in PLWHA. However, patients on ART are still at risk of TB. Reports have associated different factors with the risk of TB in patients who are

on ART (4, 5). Such factors including previous exposure to TB and low immune status as indicated by their baseline CD4 cell count have been reported to contribute to the development of active TB in HIV patients on ART. Other factors include low socio-economic status with poverty and poor nutritional status. All these can also lead to poor adherence to anti-retroviral drugs (ARVs) which can lead to further deterioration of health in HIV patients (6).

Nigeria was reported in 2017 to have about 1.9 million people living with HIV and AIDS (PLWHA) (7, 8). In addition, Nigeria rank seventh among 30 high TB burden countries and second in Africa (3, 9, 10), and is among the top 14 high burden countries for TB, TB/HIV and multidrug resistant (MDR) TB (3, 9). The need to place all HIV infected patients on ART and to ensure they are virally suppressed through administration of appropriate ARV regimen with adherence to medication is sacrosanct as this has been reported to improve outcomes and reduce risk of developing active TB (11-13).

While studies on prevalence of TB and

HIV infections have been conducted in many other centers, no such study has been done in our facility which serves a large population across many states in northcentral Nigeria. This study therefore sought to highlight the occurrence of TB in HIV infected individuals who are already on antiretroviral therapy and factors associated with the development of the disease. The aim is to determine the prevalence of active tuberculosis and associated risk factors among adult HIV patient receiving ART in Dalhatu Araf Specialist Hospital (DASH), Lafia, northcentral Nigeria.

Materials and methods:

Study area:

The study was conducted in a tertiary health facility located in Lafia, northcentral Nigeria. Lafia is the capital of Nasarawa State which has boundaries with Plateau, Kaduna, Benue, Kogi and Taraba states as well as the Federal Capital Territory. The facility receives patients from the state and border communities from all the neighboring states.

Study design:

This was a descriptive cross sectional and retrospective study utilizing records of HIV infected patients registered for care at the ART clinic of Dalhatu Araf Specialist Hospital, Lafia.

Study population:

The study population were adult HIV patients receiving antiretroviral therapy (ART) at the ART clinic of the Dalhatu Araf Specialist Hospital, Lafia. A total of 5,000 adult patients were accessing ART at the clinic at the time of the study.

Sample size:

The sample size was determined using the Kish Lisle formula; $n = z^2pq/d^2$, where 'n' is the minimum sample size, 'z' is the standard normal deviate (1.96), a 'p' value of 50% was used, 'd' is the degree of confidence which is 5%, and 'q' is 1-p. This gives a sample size of 384. Since the study population is less than 10,000 and the sample size is more than 5% of the study population of 5,000, the Cochran correlation formula which is $N = n/1 + (n/\text{study population})$ was used to obtain the final sample size (N). This gave 352 and with a 15% attrition, the minimum calculated sample size (N) was 420, which was increased to 457.

Inclusion criteria:

All consenting adult HIV infected patients who are receiving antiretroviral therapy in the facility during the study period were included.

Exclusion criteria:

Adult HIV infected patients who are newly initiated on ART (less than 6 months on ART) and those with incomplete data were excluded from analysis.

Sampling method and duration:

The systematic random sampling was used. The sampling frame consisted of 5,000 adult patients. The patients' unique ART enrolment numbers were arranged serially and every 11th number was selected to make up the sample size. The study was carried out between April and October 2019.

Data collection:

Permission was sought from the Medical Records Department to have access to the sampled case notes (physical folders and electronic medical records (EMR)). Data were extracted from the case notes and EMR and entered in a proforma. The extracted data included Patients' age, sex, marital status, occupation, ethnicity, history of TB prior to commencing ART or within the first 6 months on ART, baseline CD4 cell count, whether patient had active tuberculosis or not from 6 months of commencement of ART, duration of patient on ART before diagnosis of active TB, patients' CD4 count at time of TB diagnosis, patients' ART adherence history and the ART regimen. Active TB was defined as patients who met the criteria for TB diagnosis including positive sputum microscopy or positive GeneXpert or radiological evidence of pulmonary TB in the presence of clinical symptoms and clinical diagnosis with or without laboratory support for extra pulmonary TB. Data on the type of TB was also recorded.

Data management and statistical analysis:

Data were entered in the Statistical Package for the Social Sciences (SPSS) version 23 and analyzed using univariate analysis to describe the study population. Bivariate analysis was done with Chi square test used to establish association of categorical variables.

Ethical consideration:

Ethical approval was obtained from the Research Ethics Committee of the Dalhatu Araf Specialist Hospital, Lafia. Information obtained were treated confidentially by de-identification of patient information before sharing of data, hard copies of the data which had patient identifier were kept in a locked cabinet accessible only by the researchers and electronic copies of the data were kept in a personal computer which was pass worded and only used by the researchers.

Results:

The records of a total of 457 patients were analyzed. The study population was aged between 18 and 69 years with a mean age of 38.3 ± 10 years; 72.4% were females while 27.6% were males. Majority of the patients were married (81%), unemployed (53.8%), had baseline WHO stage 2 and 3 (81%), on first line ART regimen (86.9%) and had good ART average adherence (84%). The mean baseline CD4 count was 267.4 ± 185 cells/mm³ and majority had baseline CD4 cell count of ≤ 350 cells/mm³. The mean duration on ART was 100.9 ± 39 months with a range of 7 to 204 months. A total of 79 (17.3%) patients had previous history of TB before or within 6 months of commencement of ART.

Patients who developed active TB while on ART were 13 (2.8%). Active TB occurred more frequently among patients who were married, female gender, unemployed, those who had a baseline WHO stage 2 and 3, and those who had no previous history of TB. However, bivariate analysis shows no statistically significant association between active TB and any of these factors (Table 2). Ninety-two percent (12/13) of those who

developed active TB had pulmonary TB while only 1 (7.7%) had extra pulmonary TB.

Discussion:

The prevalence of active TB among HIV infected patients receiving antiretroviral therapy in the current study is 2.8%. This rate is comparable to the 4% reported by Chang et al., in a study from a large HIV program in Nigeria (14). Other studies also from Nigeria and Ethiopia reported higher prevalence of TB in patients at commencement of ARVs (15-18). A prevalence of 7.7% was reported in a study from southeast Nigeria (7). Studies from other African countries also show varying prevalence rates ranging from 11% to 27.7% (16, 18, 19). The low prevalence of active TB in our study may be due to the exclusion of those who had TB at commencement and within the first 6 months of ART. Those who had TB before or within the first 6 months of ART were 17.3%, which is similar to the rates from other studies such as the one from Asia where 17% of HIV patients had active TB within the first 6 months of commencement of ART (20). Other studies from Africa and around the world which included all those with TB at commencement of ART also

Table 1: Demographic, clinical and laboratory characteristics of the study population

Variable	Category	Number	%	Mean
Age (years)				38.31
Gender	Male	126	27.6	
	Female	331	72.4	
Marital status	Single	49	10.7	
	Married	369	80.7	
	Widowed	25	5.5	
	Divorced/seperated	14	3.1	
Occupation	Unemployed	246	53.8	
	Civil servants	77	16.8	
	Farmer	35	7.7	
	Trader	32	7.0	
	Artisans	21	4.6	
	Others	20	4.4	
Baseline WHO stage	Stage 1	71	15.5	
	Stage 2	207	45.3	
	Stage 3	165	36.1	
	Stage 4	14	3.1	
Baseline CD4 cell count (cells/mm3)				267.4
Duration on ART (months)				39.6
Average adherence to ART	Good (>95%)	386	84.5	
	Fair (85-95%)	46	10.1	
	Poor (<85%)	25	5.5	
Previous history of TB	Yes	81	17.7	
	No	354	77.5	
	Don't know	22	4.8	
Active TB while on ART	Yes	13	2.8	
	No	444	97.2	

Table 2: Frequency distribution of active TB patients by demographic and clinical characteristics

Variable	Active TB cases	Yes n (%)	No n (%)	p value
Gender	Male	3 (23.1)	123 (27.6)	0.71
	Female	10 (76.9)	321 (72.4)	
Marital status	Single	2 (15.4)	47	0.84
	Married	10 (76.9)	359	
	Widowed	1 (7.7)	24	
Occupation	Divorced/seperated	0	14	0.67
	Unemployed	6 (46)	240	
	Civil servants	2 (15.4)	75	
	Farmer	0	35	
	Trader	1 (7.7)	31	
	Artisans	1 (7.7)	20	
	Business	2 (15.4)	24	
	Others	1 (7.7)	19	
Baseline who stage	Stage 1	0	71	0.17
	Stage 2	5 (38.5)	202	
	Stage 3	8 (61.5)	157	
	Stage 4	0	14	
Baseline CD4 cell count (cells/mm ³)	1-200	7 (53.8)	178	0.26
	201-350	6 (46.2)	166	
	351-500	0	0	
	501-1500	57	49	
ART regimen	First line	9 (69.2)	382	0.06
	Second line	4 (30.8)	56	
Total duration on ART (months)	7-12	0	2	0.35
	13-24	0	3	
	25-60	1 (7.7)	92	
	61-120	3 (23.1)	167	
	121-205	9 (69.2)	180	
Average adherence to ART.	Good (>95%)	12 (92.3)	374	0.64
	Fair (85-95%)	1 (7.7)	45	
	Poor (<85%)	0	25	
Previous history of TB	Yes	5 (38.5)	74	0.11
	No	7 (53.5)	344	
	Don't know	1 (7.7)	20	

had higher prevalence rates between 12%-34% (18, 19, 21-23). These findings show that the longer the patients are on ART, the less likely they are to develop active TB. This further buttress the need for early initiation of ART in HIV infected patients as a means to controlling the TB epidemic and reducing the morbidity and mortality associated with HIV in Nigeria and other countries with high burden of TB and HIV (6-8).

Although there was no significant association between gender and development of active TB in this study, more females than males had active TB. This finding is similar to other studies from Ethiopia that reported more females than males developing active TB (19-22). However, our finding is in contrast with the report from Tanzania where the male gender was found to be more frequently associated with active TB among HIV patients (16). Our findings may not be unconnected to the fact that

majority of the study participants (72%) were females and this is in line with many previous studies involving HIV infected patients which shows they are dominated by the female gender (1-4). The finding of more married patients having active TB in this study is in contrast with a study that reported single (unmarried) patients to be more likely to develop active TB while on ART (19, 21). This finding may be due to the majority of our study population being married (81%). The finding of more unemployed patients having active TB is also similar to studies that reported a higher TB prevalence in HIV population from low social class (17).

While our finding that active TB is more likely to occur in HIV patients with baseline WHO stage 2 or 3, those with baseline CD4 cell count less than 350 cells/mm³ and those on first line Tenofovir based regimen, is similar to findings of other studies (14, 16, 17, 21, 22). It is worth

noting that we found no significant association between these factors and the development of active TB after 6 months of being on ART. Our study found that 69.2% of those who developed active TB had been on ART for more than 10 years and 92.3% had been on ART for more than 5 years. Most of these patients were initiated on ART late when their CD4 cell count was less than 350 cells/mm³ in line with guidelines at that time.

This finding substantiates those from other studies that early initiation of ART can reduce the prevalence of TB in those living with HIV (14, 18, 19). Those who have remained on the same ART regimen for 5 – 10 years are likely to develop HIV drug resistance with possible risk of developing TB. This again is a further indication that early ART initiation and proper monitoring for development of drug resistance and appropriate change of ART regimen when there is resistance will go a long way in reducing the prevalence of TB in HIV patients.

Conclusions:

Our study has established that people living with HIV who are on antiretroviral therapy can develop active tuberculosis in the course of their treatment. None of the factors examined was significantly associated. The morbidity and mortality associated with TB in HIV patients presents the health workers the challenge of proper and regular screening for TB especially in those with low baseline CD4 cell counts and those presenting in late stages. We recommend early initiation of ART as well as proper routine monitoring of the immune status of all people living with HIV as well as routine monitoring for ART resistance in those who have been on ART for more than 5 to 10 years.

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Conflicts of interest:

No conflict of interest is declared

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Original Article

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Assessment of the performance of six *in vitro* diagnostic kits for qualitative detection of hepatitis B virus surface antigen (HBsAg) in human serum or plasma in Lomé, Togo

*^{1,2}Salou, M., ¹Ehlan, A., ²Dossim, S., ¹Ali-Edje, K., ¹Ouro-Medeli, A., ¹Douffan, M.,
and ^{1,3}Dagnra, A.

¹National Reference Center for HIV and STIs (CNR HIV/STI)

²Department of Pharmaceutical Sciences, Faculty of Health Sciences, University of Lomé, Togo

³Department of Basic Sciences, Faculty of Health Sciences, University of Lomé, Togo

*Correspondence to: mounerous@gmail.com, msalou@univ-lome.tg

Abstract:

Background: Several *in vitro* diagnostic (IVD) test kits for hepatitis B surface antigen (HBsAg) are commercially available. The question is whether they are performing well for both screening and diagnosis or not? Thus, this study aimed to evaluate the performance of six commercially available HBsAg detection kits in Togo.

Methods: This study was conducted at the National Reference Center for HIV/STI testing in Lomé (CNR-VIH/IST), Togo. Reference sera used for the assessment were collected from blood donors and patients with history of hepatitis B viral (HBV) infection between 2008 and 2014, and includes 200 non-reactive HBsAg and 150 reactive HBsAg sera that were confirmed with a reference method which consisted of the combination of an ELISA, a RDT, and a molecular test. Four ELISA kits (EKOLab ELISA-HBsAg; HEPALISA ULTRA; HEPALISA; Murex AghBs Version 3) and two RDTs kits (ACON AghBs and OnSite HBsAg Rapid Test-Cassette) were then evaluated using these serum samples. The EPI-INFO software version 7.2 was used to determine the 95% confidence interval and performed statistical analysis.

Results: Reference serum samples were collected from the population with 65.0% under 40 years of age and 61.2% males. The sensitivity of the 4 ELISA tests compared to the reference method was 100%. Apart from the HEPALISA test with a specificity of 100.0%, the specificity of the other three ELISA tests (Murex HBsAg version 3, HEPALISA ULTRA and EKOLab ELISA-HBsAg) were 98.4%, 97.3% and 91.8% respectively. For the RDTs, the sensitivity of ACON HBsAg and OnSite HBsAg Rapid Test-Cassette was 70.0% and 95.6% respectively while the specificity was 100.0% for both.

Conclusion: The ELISA tests evaluated were more sensitive than the RDTs, and HEPALISA test was the most efficient. Of the two RDTs, the OnSite HBsAg Rapid Test-Cassette was more sensitive. Our findings highlight the need for onsite verification of *in vitro* diagnostic kits for qualitative detection of hepatitis B surface antigen before their routine use in Togo.

Keywords: HBV, HBsAg, Performance, IVD test

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Évaluation des performances de six kits de diagnostic *in vitro* pour la détection qualitative de l'antigène de surface du virus de l'hépatite B (HBsAg) dans le sérum ou le plasma humain à Lomé, Togo

*^{1,2}Salou, M., ¹Ehlan, A., ²Dossim, S., ¹Ali-Edje, K., ¹Ouro-Medeli, A., ¹Douffan, M.,
et ^{1,3}Dagnra, A.

¹Centre National de référence pour le VIH et les IST (CNR VIH/IST)

²Département des sciences pharmaceutiques, Faculté des sciences de la santé, université de Lomé, Togo

³Département des sciences fondamentales, Faculté des sciences de la santé, université de Lomé, Togo

*Correspondance à: mounerous@gmail.com, msalou@univ-lome.tg

Résumé:

Contexte: Plusieurs kits de test de diagnostic *in vitro* (DIV) pour l'antigène de surface de l'hépatite B (HBsAg) sont disponibles dans le commerce. La question est de savoir s'ils fonctionnent bien pour le dépistage et le diagnostic ou non? Ainsi, cette étude visait à évaluer les performances de six kits de détection de HBsAg disponibles dans le commerce au Togo.

Méthodes: Cette étude a été réalisée au Centre national de référence pour le dépistage du VIH/IST à Lomé (CNR-VIH/IST), Togo. Les sérums de référence utilisés pour l'évaluation ont été collectés auprès de donneurs de sang et de patients ayant des antécédents d'infection virale par le virus de l'hépatite B (VHB) entre 2008 et 2014, et comprennent 200 sérums HBsAg non réactifs et 150 sérums réactifs HBsAg qui ont été confirmés avec une méthode de référence consistant en la combinaison d'un ELISA, d'un TDR et d'un test moléculaire. Quatre kits ELISA (EKOLab ELISA-HBsAg; HEPALISA ULTRA; HEPALISA; Murex AgHBs Version 3) et deux kits TDR (ACON AgHBs et OnSite HBsAg Rapid Test-Cassette) ont ensuite été évalués à l'aide de ces échantillons de sérum. Le logiciel EPI-INFO version 7.2 a été utilisé pour déterminer l'intervalle de confiance à 95% et a effectué une analyse statistique.

Résultats: Des échantillons de sérum de référence ont été prélevés dans la population dont 65,0% ont moins de 40 ans et 61,2% d'hommes. La sensibilité des 4 tests ELISA par rapport à la méthode de référence était de 100%. Hormis le test HEPALISA avec une spécificité de 100,0%, la spécificité des trois autres tests ELISA (Murex HBsAg version 3, HEPALISA ULTRA et EKOLab ELISA-HBsAg) était respectivement de 98,4%, 97,3% et 91,8%. Pour les TDR, la sensibilité de la cassette de test rapide ACON HBsAg et OnSite HBsAg était respectivement de 70,0% et 95,6% tandis que la spécificité était de 100,0% pour les deux.

Conclusion: Les tests ELISA évalués étaient plus sensibles que les TDR et le test HEPALISA était le plus efficace. Sur les deux TDR, la cassette de test rapide OnSite HBsAg était plus sensible. Nos résultats soulignent la nécessité d'une vérification sur site des kits de diagnostic *in vitro* pour la détection qualitative de l'antigène de surface de l'hépatite B avant leur utilisation en routine au Togo.

Mots-clés: VHB, AgHBs, Performance, diagnostic *in vitro*

Introduction:

Hepatitis B virus (HBV) is a DNA virus that causes acute and chronic hepatitis in humans. It is estimated that two billion people worldwide have contracted HBV and 240 million are chronic carriers of the virus. Every year, about 600,000 people die from late-onset HBV infection. HBV infection is hyper endemic in sub-Saharan Africa and Asia and is thought to be the major etiological factor in more than 75% of patients with chronic liver disease (1-3). It remains the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC), two major causes of mortality worldwide (4).

Most of HBV-infected persons remain asymptomatic for long periods but are at risk of progressive liver disease and can transmit the virus to other susceptible individuals. Early identification of infection is thus important. The primary marker for screening and laboratory diagnosis of HBV infection is hepatitis B surface antigen (HBsAg), a component of the virus envelope that is also found in the blood in large amount as non-infectious subviral particles. This small envelope protein is the first viral marker to appear after HBV infection, and can be found in the blood before the onset of symptoms or abnormalities of blood chemistry. Thus, HBsAg test has been at the forefront of blood screening for HBV infection (5).

Immunoassays for detection of HBsAg required the use of highly sensitive and specific test reagents. In recent years, there has been an increase in the frequency of HBV which HBsAg antigenic determinants have

undergone one or more mutations as a result of natural or therapeutic pressure induced by therapy or vaccination. These mutations modify the structure of the epitope with possible consequence of non-recognition of the mutated antigens by the antibodies used in the HBsAg screening reagents (6,7). Therefore, the objective of this study is to conduct an on-site assessment of the performance of 6 diagnostic test kits manufactured for HBsAg detection in humans.

Materials and Method:

Study setting

The study was conducted at the National Reference Center for HIV/STI (CNR-VIH/IST) located at the Sylvanus Olympio University Teaching Hospital (CHU SO), Lomé, Togo. One of the activities of this laboratory required by the Ministry of Health, is the evaluation of all HIV, viral hepatitis and STIs diagnostic test kits that can be used in the country for diagnosis or screening of donor's blood for transfusion.

Subjects and sample collection

Five ml of venous blood was obtained in EDTA Vacutainer tubes from each donor received at the National Blood Transfusion Center and in dry Vacutainer tubes from patients with history of HBV infection at the CHU SO, Lomé, Togo. The blood sample was centrifugated at 3000rpm for 5 minutes, then 1-2ml serum or plasma obtained after centrifugation were stored at -20°C in CNR-VIH/IST.

Detection of HBsAg by the IVD test kits and reference method

On these samples, we first prepared our reference panel by using a combination of an ELISA test kit (Monolisa HBsAg ULTRA™, BIO-RAD, France) and RDT kit (Determine HBsAg, Abbott™, Japan) in line with the WHO recommendation (8), and confirmed negative samples by a molecular test. Thus, on all plasma negative with the two tests, HBV DNA viral load was carried out on 500µL of plasma with the m2000rt Abbott Real Time HBV DNA assay (Abbott, USA), according to the manufacturer's protocol. We considered as true positive (TP) any sample that gave a positive result on both tests, and true negative (TN) as any sample that tested negative in both tests and in the molecular test.

The HBsAg was then detected from the serum using four different enzyme linked immunosorbent assay (ELISA) kits and two rapid diagnostic test kits. The four ELISA tests were EKOlabs ELISA-HBs Ag (EKOlabs, Russia), HEPALISA ULTRA (Laboratoire J. Mitra & Co., India), HEPALISA (Laboratoire J. Mitra & Co., India), and Murex AgHBs Version 3 (Murex Biotech Limited., UK), and the two RDTs were

ACON AgHBs (ACON Laboratories, NC., USA) and OnSite HBsAg Rapid Test-Cassette (CTK Biotech, Inc., USA) (Table 1). The tests were performed in accordance with each manufacturer's protocol.

Statistical analysis

The EPI-INFO software version 7.2 was used to determine the 95% confidence interval (CI) of each value. The performance of each test was obtained by determining the sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). The sensitivity was defined as the ability of the test kit to truly identify persons infected [$Se = TP / (TP + FN) \times 100$], specificity as the ability of the test kit to truly identify persons not infected [$Sp = TN / (TN + FP) \times 100$], PPV as the ability of the assay to correctly identify actual infected persons among all who tested positive with the particular kit used [$PPV = TP / (TP + FP) \times 100$], while NPV was defined as the ability of the assay to correctly identify actual non-infected persons among those who tested negative with the kit used [$NPV = TN / (TN + FN) \times 100$].

Table 1: Characteristics of hepatitis B surface antigen *in vitro* diagnostic test kits

S/N	Test	Class	Manufacturer	Storage (°C)	Sample	Test Volume (microliter)
1	EKOlabs ELISA-HBs Ag	ELISA	EKOlabs, I Budennogo Str., Elektrogorsk, Russia, 145530	2 – 8	Serum/Plasma	100
2	HEPALISA ULTRA	ELISA	Laboratoire J. Mitra & Co. Pvt. Ltd. A 180-181, Okhla Ind. Area, Ph-1, New Delhi-110 020-INDIA	2 – 8	Serum/Plasma	100
3	HEPALISA	ELISA	Laboratoire J. Mitra & Co. Pvt. Ltd. A 180-181, Okhla Ind. Area, Ph-1, New Delhi-110 020-INDIA	2 – 8	Serum/Plasma	100
4	Murex AgHBs Version 3	ELISA	Murex Biotech Limited. Central Road, Temple Hill, Dartford DA1 5LR UK	2 – 8	Serum/Plasma	75
5	ACON AgHBs	RDT	ACON Laboratories, Inc. 4108 Sorrento Valley Boulevard, San Diego, CA 92121, USA	2 – 30	Serum/Plasma	50
6	OnSite HBsAg Rapid Test-Cassette	RDT	CTK Biotech, Inc. 10110 Mesa Rim Road, San Diego, CA 92121, USA	2 – 30	Serum/Plasma/ Whole blood	45-55 40-50

Results:

The panel of reference samples used to evaluate these HBsAg test kits was gradually established from 2008 to 2014. It consisted of 350 samples, including 200 non-reactive HBsAg samples and 150 reactive HBsAg samples. All these samples were collected from the population with 65% under 40 years of age and 61.2% males. The sample size assessed ranged from 100 to 274 depending on the number of tests available per kit (Table 2).

The sensitivity of the 4 ELISA test kits was 100% as there were no false negative reactions with any of them. Apart from the HEPALISA test which gave no false positive results, the others 3 ELISA tests gave false positive results with 15 of 182 negative sera for the EKOlal ELISA-HBsAg test, 3 of 183 negative sera for HEPALISA ULTRA and 3 of 150 negative sera for Murex AgHBs Version 3 testing positive, giving specificity of 100.0%, 98.4%, 97.3% and 91.8% respectively. The PPV of these ELISA test kits ranged from 85.5% to 100.0% and the NPV was 100.0% for all of them.

In comparison, the sensitivity of the RDT kits were 70.0% for ACON HBsAg and

95.6% for OnSite HBsAg Rapid Test-Cassette, while both kits gave a specificity value of 100.0%. The PPV for the 2 kits was 100.0% while the NPV were 76.9% and 95.7% respectively for ACON HBsAg and OnSite HBsAg Rapid Test-Cassette (Table 2).

Discussion:

We conducted a field evaluation to assess the performance of 6 IVD test kits for hepatitis B virus infection screening. These tests detect the surface antigen (HBsAg) of the hepatitis B virus. The screening for viral hepatitis B is a dire need in our environment. While a lot have been done to provide quality and reliable screening for HIV infection with a well-defined algorithm, this is not yet the case for viral hepatitis, including hepatitis B.

Data from an unpublished study showed that about 10 RDTs without WHO prequalification are available in Togo and used at different levels of healthcare services. One of CNR-VIH/IST missions is to ensure the quality and performance of test kits used for the diagnosis of HIV, hepatitis B and C, and syphilis in Togo. This involves evaluating the on-site performance of the proposed test kits.

Table 2: Performance of hepatitis B surface antigen *in vitro* diagnostic test kits

Test		Year of evaluation	Number of samples	True positive	False positive	False negative	True negative	Sensitivity (%) (CI)	Specificity (%) (CI)	PPV (%) (CI)	NPV (%) (CI)
ELISA	HEPALISA ULTRA	2014	184	71	3	0	110	100 (93.6 – 100)	97.3 (91.9 – 99.3)	95.9 (87.8 – 98.9)	100 (95.8 – 100)
	HEPALISA	2014	182	68	0	0	114	100 (93.3 – 100)	100 (95.9 – 100)	100 (93.3 – 100)	100 (95.9 – 100)
	EKOlab ELISA-HBs Ag	2015	273	91	15	0	167	100 (95 – 100)	91.8 (86.5 – 95.2)	85.8 (77.4 – 91.6)	100 (97.2 – 100)
	Murex AgHBs Version 3	2015	274	124	03	0	147	100 (96.3 – 100)	98 (93.8 – 99.5)	97.6 (92.7– 99.4)	100 (96.8 – 100)
RDT	OnSite HBsAg Rapid Test-Cassette	2015	181	87	0	4	90	95.6 (88.5 - 98.6)	100 (94.9 – 100)	100 (94.7 – 100)	95.7 (88.8 – 98.6)
	ACON AgHBs	2008	100	35	0	15	50	70 (55.2-81.7)	100 (91.1 – 100)	100 (87.7 – 100)	76.9 (64.5 – 86.1)

CI = Confidence Interval; ELISA = Enzyme Linked Immuno-Sorbent Assay; RDT = Rapid Diagnostic Test; PPV = Positive Predictive Value; NPV = Negative Predictive Value

The major challenge is to choose the right reference test i. e. the gold standard. For our purpose, we first defined our reference panel based on combination of an ELISA test (Monolisa HBsAg ULTRA™, BIO-RAD, France) and RDT (Determine HBsAg, Abbott™, Japan) in line with WHO recommendation (8). In addition, we confirmed negative samples by molecular tools. Our assessment showed all the four ELISA tests to be more sensitive (100.0% sensitivity) than the two RDTs, and amongst these four, HEPALISA kit along with the two RDTs had specificity of 100.0%. These findings indicate clearly that one of these ELISA test kits can be chosen for HBV screening of blood donors in Togo. In terms of performance, our findings demonstrated the efficiency of the ELISA kits in the order HEPALISA>Murex AgHBs Version 3>HEPALISA ULTRA>EKOLab ELISA-HBs-Ag, and of the RDTs in the order OnSite HBsAg Rapid Test-Cassette>ACON AgHBs.

Although the ELISA tests appeared to be superior, they are expensive and time consuming to perform. Despite the significant burden of disease due to HBV and HIV co-infections, and the advances and opportunities for treatment, the majority of people infected with HBV remain undiagnosed and unaware of their infection (9). It is estimated that less than 5% of people with chronic hepatitis B or C viral infection know their status (8,9), and in low-and-middle-income settings, this is even lower (<1%). This is also particularly poor in key populations such as people who inject drugs, prisoners, sex workers and men who have sex with men, for whom access to care and treatment are already challenging (10).

Our goal is to increase accessibility to hepatitis B testing by implementing HBV screening in community, primary care and district hospital in Togo. If we want to increase access to HBV testing, we need accurate, practical and affordable assays that may be used at service delivery of hepatitis B testing or near to point of care (11). Although, many brands of RDTs claim to possess these abilities, the ACON AgHBs we evaluated here showed lack of sensitivity. The consequence will be misidentification of HBV infection cases. Although the RDT assays had low sensitivity, their high specificity indicates that they may be useful for screening in regions endemic for HBV such as Togo (12). Moreover, OnSite HBsAg Rapid Test-Cassette can be used on whole blood and be stored at laboratory temperature like the ACON AgHBs. Some studies conducted in lower-middle-income countries such as Cameroon (13) and Nigeria (14), identified another type of RDT kit to be more sensitive.

For all the six test kits, the PPV was greater than 85.0% and 100.0% for the 2 RDTs, which somewhat further support their use in endemic region where people who test negative could complete their screening by either an EIA or PCR technique when these tools are available, to increase accuracy of screening. In Togo, previous studies have reported HBV prevalence ranging approximately between 9% to 19% (15-17). Thus, in Togo HBV RDT could be used in key populations at care services and in high risk population such as those in the age range 20-39 years old in Lomé (17) and on students (18) during screening campaigns. However, for blood transfusion, only ELISA assays or RDT kits with high performance as regard sensitivity or specificity should be used for HBsAg screening for blood transfusion (19-22).

One limitation to this study is the fact that these evaluations were not done at the same period, and sample sizes for the different kits were also not the same, situations that could have effect on comparisons of performance between the test kits we assessed.

Conclusion:

From this study, the ELISA test kits showed better performance than the RDTs. A good assay for an infectious agent such as HBV from a diagnostic point of view is one with a high positive predictive value and high specificity (23). The most effective test kit in this study is HEPALISA and the most effective RDT is OnSite HBsAg Rapid Test-Cassette. Our findings highlight the need of onsite assessment and verification of HBV diagnosis test kits before their routine use in the country.

We recommend that assays including rapid test kits used for HBsAg screening should be validated by the CNR/VIH before use for routine screening in Togo. It is crucial to expand hepatitis testing services, especially in community-based settings, where there are challenges with cost, transport and venipuncture requirements. The RDTs with best accuracy in this study can be used as an alternative screening tool for HBV infection at the community or primary healthcare level, more so that they are simple to perform by minimally trained community workers.

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Original Article

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Laboratory survey of extended spectrum beta-lactamase producing Enterobacteriaceae from selected tertiary hospitals in south-eastern Nigeria

*¹Ugah, U. I., and ²Udeani, T. K.

¹Department of Microbiology, Faculty of Science, Alex Ekwueme Federal University, Ndufu-Alike

²Department of Medical Laboratory Science, Faculty of Health Sciences and Technology,
University of Nigeria, Enugu Campus

*Correspondence to: uchenna.ugah@funai.edu.ng; +2347062154353

Abstract:

Background: Extended spectrum beta-lactamases are produced by Gram-negative bacteria and most strains producing them belong to the family Enterobacteriaceae. The greatest challenge with ESBL-producing Enterobacteriaceae is their propensity to acquire multidrug resistance traits. This study aimed at determining the prevalence of ESBL-producing Enterobacteriaceae among selected tertiary hospitals in south-eastern Nigeria.

Methods: A total of 400 Enterobacteriaceae isolates were obtained from patients attending five selected tertiary hospitals and were identified to species level by Gram staining and conventional biochemical tests. Screening for ESBL production was determined by the Kirby-Bauer disk diffusion method using 30µg disk of ceftioxime, cefuroxime, cefpodoxime, ceftazidime, and aztreonam while confirmatory test was done using combination disk test based on the 2016 CLSI guidelines.

Results: The prevalence of ESBL production among Enterobacteriaceae isolates from selected hospitals in southeast Nigeria is 61.5% (246 of 400). Among the isolates obtained, the highest prevalence was observed in *Klebsiella oxytoca* (100%) while the least prevalence was seen in *Morganella morganii* (50.0%). *Escherichia coli* and *Klebsiella pneumoniae* had rates of 61.8% and 62.3% respectively. Among the States of the south-east Nigeria, selected hospital in Ebonyi had a prevalence of 83.5%, Abia 63.6%, Anambra 61.5%, Enugu 51.7% and Imo 36.5%. The prevalence of ESBL-producing Enterobacteriaceae differ significantly between the States ($p=0.000$).

Conclusion: ESBL-producing Enterobacteriaceae strains have been isolated from different participants, from the selected tertiary hospitals in south-eastern Nigeria. Therefore, we report a high prevalence of ESBL-producing Enterobacteriaceae in south-eastern Nigeria.

Keywords: ESBL, Enterobacteriaceae, resistant strains, southeast Nigeria

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Enquête en laboratoire sur les entérobactéries productrices de bêta-lactamases à spectre étendu de certains hôpitaux tertiaires du sud-est du Nigéria

*¹Ugah, U. I., et ²Udeani, T. K.

¹Département de microbiologie, Faculté des sciences, Université fédérale Alex Ekwueme, Ndufu-Alike

²Département des sciences de laboratoire médical, Faculté des sciences et technologies de la santé,
Université du Nigéria, Enugu Campus

*Correspondance à: uchenna.ugah@funai.edu.ng; +2347062154353

Abstrait:

Contexte: Les bêta-lactamases à spectre étendu sont produites par des bactéries à Gram négatif et la plupart des souches qui les produisent appartiennent à la famille des entérobactéries. Le plus grand défi des entérobactéries

productrices de BLSE est leur propension à acquérir des traits de résistance multidrogue. Cette étude visait à déterminer la prévalence des entérobactéries productrices de BLSE parmi certains hôpitaux tertiaires du sud-est du Nigéria.

Méthodes: Un total de 400 isolats d'Enterobacteriaceae ont été obtenus de patients fréquentant cinq hôpitaux tertiaires sélectionnés et ont été identifiés au niveau de l'espèce par coloration de Gram et tests biochimiques conventionnels. Le dépistage de la production de BLSE a été déterminé par les méthodes de diffusion sur disque de Kirby-Bauer en utilisant un disque de 30 µg de ceftriaxone, cefuroxime, cefpodoxime, ceftazidime et aztreonam tandis que le test de confirmation a été effectué en utilisant un test de disque combiné basé sur les lignes directrices de 2017 du CLSI.

Résultats: la prévalence de la production de BLSE parmi les isolats d'Enterobacteriaceae provenant d'hôpitaux sélectionnés dans le sud-est du Nigeria est de 61,5% (246 sur 400). Parmi les isolats obtenus, la prévalence la plus élevée a été observée chez *Klebsiella oxytoca* (100%) tandis que la prévalence la plus faible a été observée chez *Morganella morganii* (50,0%). *Escherichia coli* et *Klebsiella pneumoniae* avaient des taux de 61,8% et 62,3% respectivement. Parmi les États du sud-est du Nigéria, certains hôpitaux d'Ebonyi avaient une prévalence de 83,5%, Abia 63,6%, Anambra 61,5%, Enugu 51,7% et Imo 36,5%. La prévalence des entérobactéries productrices de BLSE diffère considérablement d'un État à l'autre ($p=0,000$).

Conclusion: des souches d'entérobactéries productrices de BLSE ont été isolées de différents participants, dans les hôpitaux tertiaires sélectionnés du sud-est du Nigéria. Par conséquent, nous signalons une prévalence élevée d'entérobactéries productrices de BLSE dans le sud-est du Nigéria.

Mots-clés: BLSE, Enterobacteriaceae, souches résistantes, sud-est du Nigeria

Introduction:

Some bacteria produce beta-lactamase enzymes which renders β -lactam antimicrobial agents inactive by hydrolyzing the beta-lactam rings thereby conferring resistance against β -lactam antibiotics. However, bacteria which produce beta-lactamases usually express resistance to other antimicrobial agents and as such frustrate therapeutic interventions (1). Extended spectrum beta-lactamases (ESBLs) are a group of enzymes that possess the ability to deactivate the beta-lactam rings of penicillins, first, second and third generation cephalosporins, and aztreonam, but they are inhibited by clavulanic acid (2). There are several molecular variants of ESBLs designated as TEM-1, TEM-2, SHV, OXA, CTX-M and PER amongst others but the most prevalent types are TEM and SHV enzymes (3).

ESBLs are chromosomally or plasmid-mediated. When plasmid-mediated, they are encoded on large plasmids that also carry genes which mediate resistance to other antimicrobial agents such as tetracyclines, aminoglycosides, chloramphenicol, trimethoprim and sulphonamides. Therefore, organisms which produce ESBLs usually manifest resistance to multiple antibiotic classes (4), thereby posing very serious therapeutic challenges from limited treatment options, with severe, and in some cases, fatal clinical outcomes (2). Being plasmid-encoded, ESBLs can easily be transferred from one bacterium to another via horizontal gene transfer. Although this can occur within the community, it is most often observed in healthcare facilities, and is a major challenge in nosocomial infections. A number of factors create suitable conditions for their spread

within the hospital setting, and these include; poor hygienic practices in hospitals especially those in developing countries, indiscriminate antibiotics use, empirical antibiotic prescription and therapy not supported by the laboratory, absence of antimicrobial resistance surveillance programs and inadequate infection control practices (1,5).

ESBLs are produced by Gram-negative bacteria and most strains producing them belong to the family Enterobacteriaceae. The most common mechanisms of resistance to third generation cephalosporins by Enterobacteriaceae are through the production of ESBLs. These organisms are not only present within the hospital settings but have become prevalent also in the community. Furthermore, ESBLs have been isolated from commensals in human, animals and sewage. Therefore, environment may serve as reservoirs of organisms producing ESBLs (6), which have been reported to be responsible for the worldwide spread of ESBLs (7).

The emergence of ESBLs in Enterobacteriaceae compromises the efficacy of antibiotics, and infections due to these organisms are associated with high morbidity, mortality and treatment costs (3,8). By far, the greatest challenge associated with ESBL-producing Enterobacteriaceae is their propensity to acquire multidrug resistance traits. Other challenges are gross limitation of treatment options, prolonged hospital stay and ability to spread from persons both within the hospital and the community with epidemic potential, and also to become established as endemic pathogen within community (9,10).

The drugs of choice for treatment of infections caused by ESBL-producing Enterobacteriaceae are the carbapenems. However,

with these strains becoming endemic, there have been increasing dependence and widespread use of the carbapenems and at present, studies have reported the presence of resistance to carbapenems (11). Furthermore, failure of antibiotic therapy has resulted in high mortality rates in patients infected with these bacteria (12). Globally, resistance to antimicrobial agents has been identified as a menace to public health (13), and developing countries are major regions for these multidrug resistant bacteria (14).

There have been studies conducted at different times in individual State of the south-eastern region of Nigeria but none had been conducted across all the States at once. Therefore, this study aimed to determine the prevalence of ESBL-producing Enterobacteriaceae isolates obtained from selected tertiary hospitals in each of the five States that make up the south-eastern region of Nigeria.

Materials and methods:

Study area

The southeastern region of Nigeria lies within the coordinates 5°25'N and 8°05'E, and is made up of five States; Abia, Anambra, Ebonyi, Enugu and Imo. It has an area of approximately 76,000 square kilometers. According to the 2006 census, the population of the region was approximately 16,395,555. The region has three types of vegetation; mangrove swamps and tidal waterways dominate the coastal areas; tropical rainforests dominate the regions further north of the swamps while guinea savannah dominates the northernmost parts of the region.

Study design and sampling technique

This was a cross-sectional study designed and carried out in five selected tertiary hospitals in south-eastern Nigeria. Among eleven tertiary hospitals located within the region, five were selected by simple random sampling technique.

Study population

The participants were patients who presented with clinical manifestations that suggested the presence of infection(s) caused by any member of the Enterobacteriaceae, whose appropriate clinical specimens were sent to the microbiology laboratory of each hospital for microscopy, culture and sensitivity.

Ethical consideration

Ethical approval was obtained from the State Ministries of Health of Abia, Ebonyi, Enugu and Imo States, with approval numbers;

AB/MH/AD/904 /T.167, EBSMOH/ERC/061/19, MH/MSD/REC18/045, and CON/MH/MD/195/1 respectively. Informed consent was obtained from the participants or from their parents/guardians (for those below 18 years). The participants were assured that their identities will not be linked to any data. The study was conducted following strict adherence to international, national and institutional ethical guidelines and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Sample size

The sample size was calculated using the formula; $N = Z^2 PQ / d^2$ where; N=minimum sample size, Z=1.96 (Standard deviation of a normal distribution taken at 95% confidence interval which corresponds to 1.96), d=0.05 (degree of accuracy set at 0.05 for 95% confidence interval, and P=50% (expected prevalence from available literature, however, no previous epidemiological study of this nature has been conducted within the study area hence a prevalence of 50% was assumed), and Q=1-P. Therefore, $N = 1.96^2 \times 0.5(1-0.5) / 0.05^2$ gives an estimated sample size of 384, which was increased to 400 to compensate for 10% attrition.

To determine the number of samples that would be collected from each centre, the probability proportion by size was calculated. To determine this, the average number of patients received in the medical microbiology laboratory from each centre per month was obtained (Table 1). The formula used to calculate the sample size to be collected from each centre was given as "a/b x n", where a=average total patients received by each centre per month, b=total number of patients, and n=sample size.

Inclusion and exclusion criteria:

Patients who gave informed consents and in whom Enterobacteriaceae were isolated from their specimens were included in the study. Patients who gave consent but in whom Enterobacteriaceae was not isolated from their specimens were excluded from the study. Also, based on the prescription on the patients' case folder and on verbal interview, patients on combined antibiotic therapy were excluded from the study.

Specimen collection and bacteria identification:

Specimens were collected from the participants based on the requests on their laboratory request forms. Enterobacteriaceae were isolated from various specimens such as urine, sputum, cerebrospinal fluids, stool,

blood, semen, wound, high vaginal, ear, throat, urethral and eye swabs.

The identification of the isolates was performed using standard microbiological methods described by Cheesbrough (15) and Forbes et al., (16), which includes Gram reaction and conventional biochemical tests such as indole, methyl red, Voges-Proskauer, citrate utilization, oxidase, urease, triple sugar iron, and sugar fermentation reactions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done using Kirby-Bauer disk diffusion test. Mueller-Hinton agar plates were prepared following the Manufacturer's instructions. Using sterile swab sticks for each isolate, the plates were seeded with 0.5 McFarland turbidity equivalent of the bacterial suspension made on sterile normal saline, they were then allowed to stand for 20 minutes. Thereafter standard antimicrobial disks were placed on the surface of the media, each disk being well spaced from the other. The plates were subsequently incubated in ambient air at 37°C for 18 hours. Results were read and interpreted using guidelines of the Clinical and Laboratory Standards Institute (17).

Screening for ESBL-production

All Enterobacteriaceae isolates were first screened for ESBL production using Kirby-

Bauer single disk diffusion with ceftriaxone (30µg), cefuroxime (30µg), cefpodoxime (30 µg), ceftazidime (30µg) and aztreonam (30µg) (Oxoid, UK). Isolates with inhibition zones < 27 mm (ceftriaxone), < 25mm (cefuroxime), < 22 mm (cefpodoxime and ceftazidime) and < 25 mm (aztreonam) were preliminarily identified as ESBL producers and subjected to phenotypic confirmatory test.

Confirmatory test for ESBL production

Confirmatory test for ESBL production was done with combination disk test described by CLSI (17) with minor modifications, using ceftazidime disk in combination with clavulanic acid. 10 µL of a 1000 µg/10ml stock solution of clavulanic acid was added to ceftazidime disks each day of testing. A ≥5mm increase in zone diameter of the combined disk over the zone diameter of single ceftazidime disk confirmed an isolate as ESBL-producer.

Data analysis

Data were analysed with the Statistical Package for the Social Sciences (SPSS) version 20.0. Bar and pie charts were used for the presentation of some variables; descriptive analysis, frequency tables and percentages were used for univariate analysis while Chi square test was used for bivariate analysis. P value < 0.05 was considered significant for all analyses.

Table 1: Sample size calculation from the probability proportion by size

S/N	Centre	(a)	Sample size (n)
1	Federal Medical Centre, Umuahia	900	77
2	Alex Ekwueme Federal University Teaching Hospital, Abakaliki	1200	103
3	Enugu State University Teaching Hospital, Parklane	1350	116
4	Imo State University Teaching Hospital	600	52
5	Chukwuemeka Odumegwu Ojukwu University Teaching Hospital	600	52
Total (b)		4650	400

Results:

A total of 400 patient participants were enrolled in the study, among these, 192 (48%) were males while 208 (52%) were females (male: female ratio was approximately 1:1). The age group of the participants and the total number obtained from each of the State is presented in Table 2. The specimen types collected from the participants are presented in Table 3. The most frequently collected specimens were urine from 137 (34.3%), stool from 50 (12.5%), and wound swab from 50 (12.5%) while eye swab was the least frequently collected specimens from 1 (0.3%) participant.

The Enterobacteriaceae isolates obtained from the specimens and the frequency distribution is presented in Table 4. Among the isolates, *Escherichia coli* had the highest overall frequency (30.8%), followed by *Klebsiella pneumoniae* (19.3%) while *Klebsiella oxytoca* had the least frequency of 0.3%. Other organisms isolated include; *Citrobacter freundii*, *Yersinia enterocolitica*, *Salmonella enterica*, *Enterobacter aerogenes*, *Providencia stuartii*, *Shigella sonnei*, *Proteus mirabilis*, *Morganella morganii* and *Serratia marcescens*.

The prevalence of confirmed ESBL-producing Enterobacteriaceae is presented in Table 5. Among the 400 isolates, 246 (61.5%) were ESBL producers while 154 (38.5%) were non-producers. Among the ESBL producers, *E. coli* had a frequency of 61.8% (76/123) while

K. pneumoniae had a frequency of 62.3% (48/77). However, the highest frequency was observed among *K. oxytoca* (100%) followed by *S. sonnei* (77.8%), *E. aerogenes* (77.3%) and *Y. enterocolitica* (64.3%) while the least frequency was observed in *M. morganii* (50.0%). When subjected to statistical analysis, the prevalence of ESBL-producers among the isolates tested was non-significant ($p=0.721$).

When the prevalence of ESBL-producing isolates was compared within the age group of the participants, there was no significant relationship between age group distribution and the prevalence of ESBL-producing Enterobacteriaceae ($p=0.834$). However, the highest ESBL-producing organisms were isolated from participants within the age group of 70 years and above (70.0%), followed by age group 60-69 (63.6%) whereas age group 40-49 years had the least rate of ESBL-producing Enterobacteriaceae (51.4%).

When the prevalence rates of ESBL-producing Enterobacteriaceae were compared among the five States of the southeast region, Ebonyi had the highest prevalence rate (83.5%) while Imo had the least (36.5%). The prevalence rates for other States were 51.7% for Enugu, 61.5% for Anambra and 63.6% for Abia (Table 6). The prevalence rates of ESBL-producing Enterobacteriaceae differed significantly between the States ($p=0.000$).

Table 2: Demographic variables of patients from whom Enterobacteriaceae were isolated

Demographic variables	Frequency	Percentage
State		
Abia	77	19.3
Ebonyi	103	25.8
Enugu	116	29.0
Imo	52	13.0
Anambra	52	13.0
Age group (year)		
<20	71	17.8
20-29	124	31.0
30-39	46	11.5
40-49	35	8.8
50-59	28	7.0
60-69	66	16.5
70 & above	30	7.5
Gender		
Male	192	48.0
Female	208	52.0

Table 3: Frequency distribution of the specimen types obtained from selected patients

Specimen types	Frequency	Percentage
Urine	137	34.3
Stool	50	12.5
Wound swab	50	12.5
Blood	34	8.5
High vaginal swab	32	8.0
Endocervical swab	29	7.3
Semen	17	4.3
Sputum	14	3.5
Ear swab	11	2.8
Throat swab	10	2.5
Urethral swab	8	2.0
Cerebrospinal fluid	7	1.8
Eye swab	1	0.3

Table 4: Frequency distribution of the Enterobacteriaceae isolates

Isolate	Frequency	Percentage
<i>Escherichia coli</i>	123	30.8
<i>Klebsiella pneumoniae</i>	77	19.3
<i>Citrobacter freundii</i>	41	10.3
<i>Yersinia enterocolitica</i>	28	7.0
<i>Salmonella enterica</i>	27	6.8
<i>Enterobacter aerogenes</i>	22	5.5
<i>Providencia stuartii</i>	19	4.8
<i>Shigella sonnei</i>	18	4.5
<i>Proteus mirabilis</i>	17	4.3
<i>Morganella morganii</i>	14	3.5
<i>Serratia marcescens</i>	13	3.3
<i>Klebsiella oxytoca</i>	1	0.3

Table 5: Distribution of Enterobacteriaceae isolates by ESBL production

Isolate	ESBL production			χ^2	<i>p</i> -value
	Non-producer (%)	Producer (%)	Total (%)		
<i>E. coli</i>	47 (38.2)	76 (61.8)	123 (30.8)	7.918	0.721
<i>K. pneumoniae</i>	29 (37.7)	48 (62.3)	77 (19.3)		
<i>C. freundii</i>	18 (43.9)	23 (56.1)	41 (10.3)		
<i>Yersinia enterocolitica</i>	10 (35.7)	18 (64.3)	28 (7.0)		
<i>S. enterica</i>	12 (44.4)	15 (55.6)	27 (6.8)		
<i>E. aerogenes</i>	5 (22.7)	17 (77.3)	22 (5.5)		
<i>P. stuartii</i>	9 (47.4)	10 (52.6)	19 (4.8)		
<i>Shigella sonnei</i>	4 (22.2)	14 (77.8)	18 (4.5)		
<i>P. mirabilis</i>	8 (47.1)	9 (52.9)	17 (4.3)		
<i>M. morganii</i>	7 (50.0)	7 (50.0)	14 (3.5)		
<i>S. marcescens</i>	5 (38.5)	8 (61.5)	13 (3.3)		
<i>K. oxytoca</i>	0	1 (100)	1 (0.3)		
Total	154 (38.5)	246 (61.5)	400 (100)		

Table 6: Distribution of Enterobacteriaceae and ESBL production in relation to the States of southeastern Nigeria

State	ESBL production			χ^2	<i>p</i> -value
	Non-producer (%)	Producer (%)	Total (%)		
Abia	28 (36.4)	49 (63.6)	77 (19.3)	39.560	0.000
Ebonyi	17 (16.5)	86 (83.5)	103 (25.8)		
Enugu	56 (48.3)	60 (51.7)	116 (29.0)		
Imo	33 (63.5)	19 (36.5)	52 (13.0)		
Anambra	20 (38.5)	32 (61.5)	52 (13.0)		
Total	154 (38.5)	246 (61.5)	400 (100)		

Discussion:

This study is essential to raise the awareness about the magnitude of the problem associated with multidrug resistant bacteria with the aim of initiating discussions that can lead to formulation of policies or changes in the existing policies as well as the enforcement of infection control and antibiotic resistance surveillance programs in the southeast region. This is by providing current data on the prevalence of ESBL producing Enterobacteriaceae, taking into cognizance the potential for these resistant strains to spread within the hospital setting and the community, as well as to other regions both within and outside Nigeria. Our study objective is in line with those from other countries (13,14,18,19).

The prevalence of ESBLs is known to vary widely worldwide, even in regions that are closely related. Several studies conducted in different regions of Nigeria on Enterobacteriaceae have reported varying prevalence rates for ESBL-production. A rate of 9.3% was reported for Enterobacteriaceae in Kano (20) while 2.5% for *E. coli* and 5% for *K. pneumoniae* were reported in Ogun State (21). Our study reported prevalence rate of 61.5% for ESBL-production among Enterobacteriaceae isolates in selected hospitals in the south-eastern region of Nigeria. This is higher than the prevalence rates reported from other parts of Nigeria (20,21) and 36.9% rate reported in Central India (22). When compared with previously published data in Ebonyi State, there appeared to be a sustained surge in the prevalence of ESBL-producing Enterobacteriaceae isolates from 16.5% in 2008 (23), 52% among *E. coli* in 2019 (24), to 83.5% in the present study.

The prevalence rates reported from Enugu State have also varied over the years. In 2009, a rate of 11.4% was reported among Enterobacteriaceae isolates (25), and 56.6% for *K. pneumoniae* and 59.4% for *E. coli* in 2010 (26). In 2017, prevalence rates of 59.7% and 40.4% for *Klebsiella* spp and *E. coli* respectively were reported among isolates obtained from orthopaedic wounds (27). Among paediatric population in Enugu metropolis, a prevalence rate of 19.8% for confirmed ESBL-producing Enterobacteriaceae was reported from both healthy and ill participants (28). Another study reported 35% rate among *E. coli* isolates and 75.7% from hospital-acquired infection (29). The presence of ESBL-producing organisms among paediatric population is a cause for concern as it portends an establishment of the strains in Enugu

metropolis. Also, the observed increasing prevalence from 2009 to 2019 is an indication of either a non-existent measure to curb the menace of antibiotic resistance or a dilapidation of such control measures if they are available.

In Abia State, a 50.8% prevalence rate of ESBL-producers was reported for urinary isolates in Aba (30), which is slightly lower than the rate obtained from the present study. However, there are no prevalence data on isolates from Umuahia where this current study was conducted. The least prevalence rate (36.5%) reported in our study was among isolates obtained from Owerri, which is comparable to the prevalence of 26.4% for ESBL-producing *E. coli* reported in 2018 (31). However, Duru et al., (32) reported a prevalence of 17% of ESBL-producing *E. coli* and *K. pneumoniae* among asymptomatic persons in Owerri, which represents a group of individuals who may serve as reservoirs of these resistant strains. Worse still, dissemination can continue unnoticed within the community resulting in the establishment as an endemic strain within the locality.

Other studies have detected the presence of ESBL-producing Enterobacteriaceae from animal samples within the south-eastern Nigeria (33). They have also been detected among consumables both within and outside the region. In Abakaliki, a prevalence of 8% was reported from anal swabs of donkeys (34) while 33.3% rate was reported from frozen mackerel fishes sold in the markets (35). In Enugu metropolis, a prevalence of 24.2% was reported among healthy pigs (36), and in Owerri, a prevalence of 22.2% was reported among poultry (37). In other regions, Abubakar and colleagues reported a prevalence of 8.9% and 5.7% for ESBL-producing *E. coli* in chickens and retail eggs in Sokoto metropolis (38).

Conclusion:

To the best of our knowledge, this is the first study to analyse specimens for ESBL-producing Enterobacteriaceae from the entire south-eastern States. Because no study of this nature has been conducted in Umuahia, the results of this present study provide the first information on the prevalence of ESBL-producing Enterobacteriaceae in Umuahia. Generally, our study reports a high prevalence rate of 61.5% for ESBL-producing Enterobacteriaceae in south-eastern Nigeria.

Contributions of authors:

UUI conceived the study, obtained data and interpreted data; UTK designed the study and revised it critically for intellectual content. All authors agreed to the final manuscript.

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Copyright AJCEM 2020: <https://doi.org/10.4314/ajcem.v21i3.8>**Original Article****Open Access****Antifungal susceptibility and detection of mutant *ERG11* gene in vaginal *Candida* isolates in University of Uyo Teaching Hospital, Uyo, Nigeria**

Ikenyi, C. L., *Ekuma, A. E., and Atting, I. A.

Department of Medical Microbiology and Parasitology, University of Uyo, Uyo, Nigeria

*Correspondence to: agantemekuma@uniuyo.edu.ng; +2348023075572**Abstract:**

Background: *Candida* vulvovaginitis is an important cause of morbidity among women. Fluconazole and other azoles are among the commonest antifungal agents used for the treatment of this condition. Azole resistance among *Candida* species is an increasing problem, and mutations in the *ERG11* gene is the commonest cause of fluconazole resistance in *Candida*. The objectives of this study are to determine antifungal susceptibility of vaginal *Candida* isolates and detect carriage of mutant *ERG11* gene by them.

Methods: High vaginal swabs obtained from 260 participants were cultured on Sabouraud's Dextrose agar (SDA) for isolation of *Candida*, and identified by growth on CHROMagar *Candida*, germ tube and carbohydrate fermentation tests. Antifungal susceptibility to fluconazole, voriconazole, nystatin and flucytosine was determined by the Kirby Bauer disc diffusion method on supplemented Mueller Hinton agar. *ERG11* gene was detected by conventional singleplex polymerase chain reaction (PCR) assay.

Results: *Candida* was isolated from 126 of 260 (48.5%) participants, and the identified species were *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilopsis* and *Candida famata*. There were 112 (88.9%) isolates susceptible to fluconazole, 122 (96.8%) to voriconazole, 111 (88.1%) to nystatin, and 16 (6.6%) to flucytosine. The mutant *ERG11* gene was detected in all four fluconazole-resistant isolates but not from any of five randomly selected fluconazole susceptible dose dependent (SDD) isolates.

Conclusion: Azole resistance among *Candida* in this environment is associated with mutant *ERG11* gene expression.

Keywords: antifungi, fluconazole, *Candida*, *ERG11*, PCR

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Sensibilité antifongique et détection du gène *ERG11* mutant dans des isolats vaginaux de *Candida* à l'hôpital universitaire de Uyo, Uyo, Nigéria

Ikenyi, C. L., *Ekuma, A. E., et Atting, I. A.

Département de microbiologie médicale et de parasitologie, Université d'Uyo, Uyo, Nigéria

*Correspondance à: agantemekuma@uniuyo.edu.ng; +2348023075572**Abstrait:**

Contexte: La vulvovaginite à *Candida* est une cause importante de morbidité chez les femmes. Le fluconazole et d'autres azoles sont parmi les agents antifongiques les plus couramment utilisés pour le traitement de cette condition. La résistance à l'azole chez les espèces de *Candida* est un problème croissant, et les mutations du gène *ERG11* sont la cause la plus fréquente de résistance au fluconazole chez *Candida*. Les objectifs de cette étude sont de déterminer la sensibilité antifongique des isolats vaginaux de *Candida* et de détecter le transport du gène *ERG11* mutant par eux.

Méthodes: Des écouillons vaginaux élevés obtenus auprès de 260 participants ont été cultivés sur gélose Dextrose de Sabouraud (SDA) pour l'isolement de *Candida*, et identifiés par croissance sur CHROMagar *Candida*, tube germinatif et tests de fermentation des glucides. La sensibilité antifongique au fluconazole, au voriconazole, à la nystatine et à la flucytosine a été déterminée par la méthode de diffusion sur disque de Kirby Bauer sur de

la gélose Mueller Hinton complétée. Le gène *ERG11* a été détecté par un test classique de réaction en chaîne par polymérase (PCR).

Résultats: *Candida* a été isolé sur 126 des 260 participants (48,5%), et les espèces identifiées étaient *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilopsis* et *Candida famata*. Il y avait 112 (88,9%) isolats sensibles au fluconazole, 122 (96,8%) au voriconazole, 111 (88,1%) à la nystatine et 16 (6,6%) à la flucytosine. Le gène *ERG11* mutant a été détecté dans les quatre isolats résistants au fluconazole, mais pas dans aucun des cinq isolats dépendants de la dose (SDD) sensibles au fluconazole sélectionnés au hasard.

Conclusion: la résistance à l'azole chez *Candida* dans cet environnement est associée à l'expression du gène *ERG11* mutant.

Mots-clés: antifongiques, fluconazole, *Candida*, *ERG11*, PCR

Introduction:

With a global prevalence of 5-20%, *Candida* vulvovaginitis is an important cause of morbidity among women. This condition affects up to 75% of reproductive age women at least once (1). *Candida* occurs as a normal commensal of the vagina in about 48% of women. There are about 200 species of *Candida*, of which more than 20 species are associated with human infections, with only four causing vast majorities of the clinical infections, *C. albicans*, *C. glabrata*, *C. parapsilopsis* and *C. tropicalis*. While *Candida* vulvovaginitis can occur in otherwise healthy women, it is more prevalent in women who are pregnant, diabetic, taking oral contraceptives or on prolonged antibiotic treatment.

Azole resistance among *Candida* species is an increasing problem especially against the backdrop of opportunistic *Candida* infections in immunocompromised patients (2,3). Azoles, polyenes and antimetabolites such as flucytosine are among the classes of antifungals used to treat *Candida* infections. With increasing resistance among *Candida* isolates, it is important to monitor the susceptibility pattern of these organisms to antifungal agents in order to guide empirical therapy (4). Fluconazole is a triazole antifungal drug with good activity against *Candida* as well as low toxicity and is a drug of choice for *Candida* vulvovaginitis. Fluconazole acts primarily on ergosterol biosynthesis by targeting 14- α -lanosterol demethylase encoded by *ERG11* gene resulting in the inhibition of cytochrome P450-dependent conversion of lanosterol to ergosterol. The resulting ergosterol depletion interferes with the bulk functions of ergosterol as a membrane component, but more importantly, severe ergosterol depletion may also interfere with the "sparking" functions of ergosterol, affecting cell growth and proliferation. The blocking of 14 α -demethylase results in the accumulation of toxic methylated sterols leading to membrane stress (5).

While some *Candida* species such as *C. krusei* have intrinsic resistance to fluconazole, the current increase in resistance is being driven by widespread continuous use of fluconazole as prophylaxis. Mutations in the

ERG11 gene is the commonest cause of fluconazole resistance in *Candida* (6). Point mutations in *ERG11* may lead to fluconazole resistance in two ways; first, by reducing the affinity of the target to azoles, and second, by enhancing transcription of gene or chromosomal amplification (7). The aim of this study was to determine antifungal susceptibility of vaginal *Candida* isolates and to detect carriage of mutant *ERG11* gene by the isolates.

Materials and methods:

Study setting and design

This was a descriptive, cross sectional, hospital-based study of women of child-bearing age attending the Obstetrics and Gynaecology clinics of the University of Uyo Teaching Hospital, a tertiary hospital in Uyo, Akwa Ibom State, South-South Nigeria between April and May 2017.

Subject participants and sampling method

A sample size of 236 was calculated using the formula by Godden (8) and an expected prevalence of 18.9% (9). Participants were randomly selected from women aged 18 years and above attending the Obstetrics and Gynaecology clinic of the hospital. Women on menstrual period were excluded.

Data and sample collection

All eligible participants were interviewed using a 12-point semi-structured questionnaire which covered bio-data, genital hygiene, prior history of antibiotic use and medical history. A pair of high vaginal swab (HVS) samples was collected aseptically from each participant by trained personnel in the Obstetrics and Gynaecology clinics.

Using a sterile disposable speculum, a sterile cotton swab was introduced 4-5 cm into the vagina and turned gently 3-4 times, removed and returned to the swab tube. The swab was labeled appropriately with participant's name and study number and transported to the Microbiology Laboratory of the University of Uyo Teaching Hospital for analysis.

Sample processing

One HVS sample from each participant was used for microscopic examination by wet mount preparation for the presence of yeast cells and gram staining for the presence of budding cells, yeast hyphae and beaded appearance. The second swab stick was streaked on Sabouraud's dextrose agar (SDA) (Lab M, UK) supplemented with chloramphenicol to inhibit bacterial contamination. The inoculated plates were incubated aerobically at 37°C for 24 hours. All soft, moist white-cream colonies on the plates after 24 hours were stained by Gram's method and if budding yeast morphology was present, further identification was carried out.

Identification of isolates

Candida isolates were identified by culture on CHROMagar *Candida* (HK Media, China), germ tube test and carbohydrate fermentation test using glucose, sucrose, maltose and lactose. *Candida* species were identified on CHROMagar *Candida* based on the color of the colony and morphology (rough/smooth) as stated by the manufacturer. Purple-pink colonies were identified as *C. glabrata*, iron blue colonies as *C. tropicalis*, and Beige white as other species. Germ tube test as described elsewhere was used to confirm identification of *C. albicans*.

Carbohydrate fermentation test was used for complete specie identification of all the different *Candida* species isolated. The fermentation test medium was prepared as described by Mackie and McCartney (10). The test organisms were inoculated by adding one drop of 0.5 McFarland turbidity suspensions of each isolate into each tube and incubated for 48 hours at 30°C. The ability of the yeast to ferment a sugar was shown by the presence of acid indicated by change of the yellow indicator to pink colour.

Antifungal susceptibility testing

Antifungal susceptibility test was performed on Mueller Hinton agar (Oxoid, UK) supplemented with 2% glucose and methylene blue (25 ml) by the Kirby Bauer disc diffusion method. The following antifungal agents; fluconazole (25µg), nystatin (100 iu), voriconazole (1µg) and flucytosine (1µg) disks (Oxoid, UK and Abtek, UK) were used on a 90 mm plate to test the susceptibility pattern of the isolates. For fluconazole and voriconazole, the Clinical and Laboratory Standards Institute M44-A document (11) guidelines and interpretative criteria were followed. For nystatin and flucytosine, the diameter of zone of inhibition obtained were compared with the standard zones interpretative breakpoints published by Abtek biological in a leaflet included in the disk pack. The control organism was *C. albicans* ATCC 90028 strain.

DNA extraction:

DNA from all four fluconazole-resistant isolates and five randomly selected intermediately-susceptible isolates was extracted using the ZymoResearch™ (ZR) Fungal DNA Mini prep extraction kit (Inqaba, South Africa). Fungal DNA quantification was done on a Nano-drop-1000 spectrophotometer (SN 1844 ND-1000UV/VIS spectrophotometer, USA) for purity check of the extracted DNA.

Amplification and detection of mutant *ERG11* gene by singleplex PCR:

The mutant *ERG11* gene was amplified using the forward primer sequence 5'-GTTGAAACTGTCATTGATGG-3' and reverse primer sequence 5'-TCAGAACACTGAATCGAAAG-3'. A micropipette adjusted to the required volume was used to pipette 7.68 µl of the PCR water into 9 Eppendorf tubes, 0.16 µl each of the forward and reverse primers were added into each of the tubes, and 10 µl of the PCR master mix (Taq polymerase, DNTPs and MgCl₂, from Inqaba, South Africa) was added to each of the tubes and 2 µl of the extracted DNA as template. A final volume of 20 µl was obtained in each of the 9 tubes (0.5 ml PCR tube).

The tubes were placed on the micro titer tray of the thermal cycler (ABI 9700 Applied Biosystems), which had been programmed for 30 cycles to run at the following conditions; initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 43°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes. The amplified product was resolved on a 1.5% agarose gel at 120 V for 15 minutes and visualized on a UV trans-illuminator (Analytic Jena, Germany) for the expected band size of 1650 bp.

Statistical analysis

Demographic variables and frequency of occurrence of *Candida* species was analyzed using SPSS (Statistical Package for the Social Science) version 20.0. Descriptive results were expressed in frequency and percentage. Measure of association of the categorical variables was analyzed using Pearson Chi-square. Association between variables were considered statistically significant at *p*-value less than 0.05.

Ethical consideration

Ethical approval was obtained from the Ethical Review Board of the University of Uyo Teaching Hospital, Uyo where this research was conducted before the commencement of the research work. Written informed consent was obtained from all participants prior to specimen collection. No information that could directly identify participants was included and all information from participants was treated with utmost confidentiality.

Results:

Study participants

A total of 260 women participated in the study. The ages of participants ranged from 18-46 years while the mean age was 28 years. Of the 260 women, 77 (29.6%) were single, 181 (69.6%) were married, 1 (0.4%) divorced and 1 (0.4%) widowed. One hundred and twenty-nine (49.6%) participants were pregnant.

Prevalence of *Candida* among participants

Candida was isolated from 126 (48.5%) of women examined. The most frequently isolated species was *C. albicans* (77.8%), followed by *C. glabrata* (15.1%), *C.*

tropicalis (4.8%), *C. parapsilopsis* (1.6%) and *C. famata* (0.8%).

Susceptibility of *Candida* species to antifungal agents

The antifungal susceptibility of *Candida* species isolated to antifungal agents tested is shown in Table 1. Out of the 126 isolates, 112 (88.9%) were susceptible to fluconazole, while 10 (7.9%) were intermediate and 4 (3.2%) were resistant; 122 (96.8%) were susceptible to voriconazole, while 1 (0.8%) was intermediate and 3 (2.4%) were resistant; 111 (88.1%) were susceptible to nystatin, 10 (7.9%) were intermediate and 5 (4.0%) were resistant; 16 (6.6%) were susceptible to flucytosine, 30 (24.2%) were intermediate and 78 (69.2%) were resistant.

Table 1: Antifungal susceptibility pattern of *Candida* species isolated (n=126)

Antifungal agents	FCA			VOR			NYST			FLU		
Species	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)
<i>C. albicans</i> (n=95)	87 (91.6)	4 (4.2)	4 (4.2)	94 (98.9)	1 (1.1)	0	84 (88.4)	7 (7.4)	4 (4.2)	13 (13.8)	22 (23.4)	59 (62.8)
<i>C. glabrata</i> (n=19)	15 (78.9)	4 (21.1)	0	8 (88.9)	0	1 (5.3)	18 (94.7)	0	1 (5.3)	3 (16.7)	3 (16.7)	12 (66.7)
<i>C. tropicalis</i> (n=9)	7 (77.8)	2 (22.2)	0	18 (94.7)	0	1 (11.1)	6 (66.7)	3 (33.3)	0	0	3 (33.3)	6 (66.7)
<i>C. famata</i> (n=1)	1 (100)	0	0	1 (100)	0	0	0 (0)	1 (100)	0	0	1 (100)	0
<i>C. parapsilopsis</i> (n=2)	2 (100)	0	0	2 (100)	0	0	1 (50)	1 (50)	0	0	1 (50.0)	1 (50.0)
Total	112	10	4	123	1	2	109	12	5	16	30	78

Key: FCA=Fluconazole, VOR=Voriconazole, NYST=Nystatin, FLU=Flucytosine, S=Sensitive, R=Resistant, SDD= susceptible dose dependent

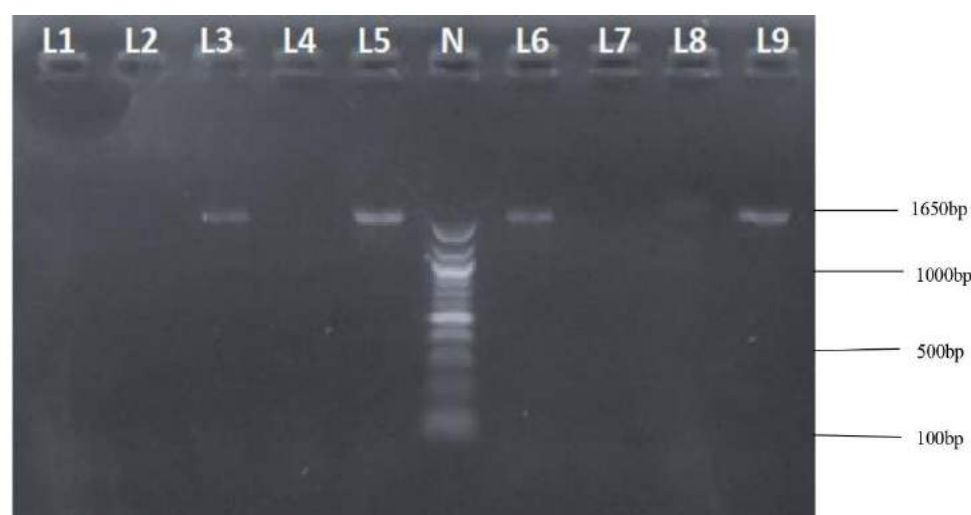


Fig 1: Gel electrophoresis picture of amplified mutated *ERG11* gene from 4 fluconazole-resistant and 5 selected intermediate-dose isolates

Lanes 3, 5, 6 & 9 show the amplified *ERG11* mutated gene bands (1650bp), lane N shows a 1000bp quick-load molecular ladder

Discussion:

Vaginal discharge is one of the most frequent gynaecological problems encountered in females especially during their reproductive age and most women regard any secretion from the vagina as abnormal discharge. The first task of primary health care providers is to ascertain whether this discharge is pathological or physiological (12). The presence of *Candida* in the vagina does not necessarily indicate infection, but colonization of the vagina usually precedes infection. Colonization rate of *Candida* species is believed to vary with geographical location, studied population and, probably, microbiological sampling methods (13).

The prevalence of vaginal *Candida* in this study was 48.5%. This prevalence is comparable to 40.8% and 42.7% obtained from similar studies in Zaria (14) and by Feyi and Amadi in Abia (15), Nigeria respectively. Higher prevalence of 70% and 64.7% have been reported in similar studies by Oyewole et al., (16) in Minna and Lennox et al., (17) in Calabar respectively. The disparity could be due to differences in populations studied and also due to the fact that the study included only participants with symptoms of vaginitis. Lower prevalence rates have been reported from similar studies by Anorlu et al., (18) who reported 22.1% in Lagos and Usanga et al., (19) who reported 21.5% in Calabar.

We found that *C. albicans* (77.8%) was the most frequently isolated specie from the study participants, which is similar to what have also been reported from Ogun and Kano States, Nigeria (20,21). The frequency of isolation of non-albicans *Candida* (NAC) species reported in this study are; *C. glabrata* 15.1%, *C. tropicalis* 4.8%, *C. parapsilopsis* 1.6% and *C. famata* 0.8%. NAC species are becoming increasingly more prevalent as reported in previous studies (16,22,23). *Candida* colonization was highest in age groups 18-24 and 25-31 years. This probably could be due to ovarian activity as well as sexual activity which peak in women at 20-30 years of age (24). During this period, the ovary produces adequate amount of estrogen, which favours *Candida* growth by maintaining the acidic pH in the vagina and enhancing the yeast adherence to vaginal epithelial cells (25). The age distribution of *Candida* colonization observed in this study is similar with the findings of Onuorah et al., (26) in Awka, southeastern Nigeria, which reported highest prevalence in the age group 20-30 years. Similarly, studies from Jos and Kano, Northern Nigeria have reported highest prevalence in the age group 21-30 years (22,27). Menza et al., (28) reported a prevalence rate of 60% within the age group 26-35 years in Kenya.

Antifungal susceptibility testing of *Candida* is recognized as a useful aid in optimizing the treatment of *Candida* infections as emergence of resistant strains continuously threatens therapy (29). In this study, vaginal *Candida* isolates showed highest antifungal susceptibility rates to voriconazole, followed by fluconazole and nystatin, and the least susceptibility to flucytosine. This observation is similar to the findings of Efunshile et al., in Lagos (30). *Candida albicans* has been reported in Nigeria (31) and other parts of the world (32) as the specie with the highest susceptibility to voriconazole. The susceptibility of *Candida* species in this study to nystatin is similar to what has been reported in studies from Younde, Cameroon (33) and Kampala, Uganda (34). Other studies have reported up to 100% resistance of *Candida* to nystatin in Yola, Nigeria (35) and 80% in southwest Cameroon (36). The antifungal susceptibility testing in this study showed high resistance rate to flucytosine among all *Candida* species isolated. This is unsurprising as secondary resistance to flucytosine frequently develops especially when used as monotherapy. Similar high rates have been observed in other studies (37,38). Contrariwise, Efunshile et al., in Lagos, Nigeria (30) reported 100% susceptibility of *Candida* to flucytosine.

In this study, PCR amplified mutant *ERG11* gene in all the 4 fluconazole-resistant isolates of *C. albicans* and not in the 5 selected intermediate isolates. Among the molecular mechanisms for azole resistance in *Candida*, the *ERG11* gene plays a prominent role. The *ERG11* gene codes for the enzyme lanosterol demethylase which is the target for azole antifungal agents including fluconazole (29, 39). Resistance to azole may result from overexpression of *ERG11* gene independently or in combination with other resistance mutations. Overexpression of *ERG11* gene has been noted in *C. albicans*, *C. parapsilosis* and *C. tropicalis*, and studies have shown that this overexpression often involves *Upc2p*, a zinc cluster transcription factor, that is induced upon ergosterol depletion (40). Point mutations in the coding regions of the *ERG11* gene which lead to amino acid substitutions may also impact susceptibility to fluconazole. Over 140 point mutations have been described for *C. albicans* most of which fall into distinct "hot spot" regions within the protein amino acids 105-165, 266-287, and 405-488 (40,41). There appears however to be less variability in the *ERG11* sequence in the NAC species (6).

Resistance to fluconazole in this study was found in 4.2% of *C. albicans* isolates but not in the NAC species. Similar resistance rates have been reported elsewhere in Nigeria and also in the United States (24,42,43). Resistance rates of up to 9.5% has been

reported in Lagos, Nigeria (44) and 11.3% in Xijing, China (38). Relatively high cost of fluconazole in Nigeria leading to reduced usage may be responsible for this low rate of resistance (29).

Our study was limited by inability to carry out full sequencing of *ERG11* gene to determine the actual mutations prevalent in this environment. This should be the aim of future studies. However, it shows the rising incidence of fluconazole resistance among vaginal *Candida* species which could lead to treatment failure where azoles are first line treatment options. In conclusion, we report fluconazole resistance among vaginal *Candida* which is related to mutation in the *ERG11* gene, with consequences for antifungal treatment of vaginal candidiasis in this environment.

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Original Article

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Qualitative evaluation of the antimicrobial efficacy of UV sterilization chambers employed by barbershops in Benin City, Nigeria

*¹Adebiyi, K. S., ¹Emeka-Ifebi, A., ²Ogbonnaya, M. J., and ¹Isiekwene, A. C.

¹Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria

²Department of Microbiology, Faculty of Sciences, Alex Ekwueme Federal University, Abakaliki, Nigeria

*Correspondence to: adebiyisalem@gmail.com; +2348138084808

Abstract:

Background: Barbershops where men and boys' hair are cut or shaved, have been implicated in the transmission of pathogens. With this growing concern, barbers are now acquiring and employing UV sterilization chambers to re-assure customers of the safety of their instrument. This study investigated the qualitative efficacy of the UV sterilization chambers employed by selected barbers in Benin City, Nigeria.

Methods: Swab samples of instruments (clippers, combs and brushes) were collected from 30 barbershops randomly selected from 6 Local Government Areas (LGAs) of Benin City before and after exposure to UV sterilization chambers employed by each barbershop. Standard microbiology techniques were employed to culture and identify the microbial (bacteria and fungi) isolates.

Results: A total of 15 genera of microorganisms (8 bacteria and 7 fungi) were identified. Three bacterial genera (*Staphylococcus* sp., *Bacillus* sp. and *Pseudomonas* sp.) and 5 fungi genera (*Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., *Trichophyton* sp. and *Penicillium* sp) were isolated from sampled instruments at the barbershops in all 6 LGAs. Evaluation of efficacy of the UV sterilization chambers showed that all microbial isolates survived exposure time of 1 min. Antimicrobial efficacy of the UV chamber increases with longer duration (time) of exposure and decreases with the age of UV chambers, with chamber of 5-6 years old being least efficacious.

Conclusion: This study confirms the presence of myriads of microorganisms including pathogenic strains on instruments used in barbershops within Benin City. It is recommended that exposure of 60 mins is the ideal duration for UV sterilization chambers used in barbershops and barbers in Benin City should endeavor to replace their UV chambers after continual use for a period of 3 years.

Keywords: microbes, antimicrobial efficacy, UV sterilization, barbershop, Benin City,

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Évaluation qualitative de l'efficacité antimicrobienne des chambres de stérilisation UV utilisées par les salons de coiffure à Benin City, Nigéria

*¹Adebiyi, K. S., ¹Emeka-Ifebi, A., ²Ogbonnaya, M. J., et ¹Isiekwene, A. C.

¹Département de microbiologie, Faculté des sciences de la vie, Université du Bénin, Benin City, Nigéria

²Département de microbiologie, Faculté des sciences, Université fédérale Alex Ekwueme, Abakaliki, Nigéria

*Correspondant à: adebiyisalem@gmail.com; +2348138084808

Abstrait:

Contexte: les salons de coiffure où les cheveux des hommes et des garçons sont coupés ou rasés ont été impliqués dans la transmission d'agents pathogènes. Avec cette préoccupation croissante, les barbiers acquièrent et utilisent maintenant des chambres de stérilisation UV pour rassurer les clients sur la sécurité de leur instrument. Cette étude a examiné l'efficacité qualitative des chambres de stérilisation UV utilisées par des barbiers sélectionnés à Benin City,

au Nigeria.

Méthodes: Des échantillons d'écouvillons d'instruments (tondeuses, peignes et brosses) ont été collectés dans 30 salons de coiffure sélectionnés au hasard dans 6 zones de gouvernement local (LGA) de Benin City avant et après exposition aux chambres de stérilisation UV utilisées par chaque salon de coiffure. Des techniques de microbiologie standard ont été utilisées pour cultiver et identifier les isolats microbiens (bactéries et champignons).

Résultats: Au total, 15 genres de micro-organismes (8 bactéries et 7 champignons) ont été identifiés. Trois genres bactériens (*Staphylococcus* sp., *Bacillus* sp. et *Pseudomonas* sp.) et 5 genres fongiques (*Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., *Trichophyton* sp. et *Penicillium* sp) ont été isolés à partir d'instruments échantillonnés dans les salons de coiffure dans tous les 6 LGA. L'évaluation de l'efficacité des chambres de stérilisation UV a montré que tous les isolats microbiens ont survécu à un temps d'exposition de 1 min. L'efficacité antimicrobienne de la chambre UV augmente avec une durée (temps) d'exposition plus longue et diminue avec l'âge des chambres UV, la chambre de 5-6 ans étant la moins efficace.

Conclusion: Cette étude confirme la présence de myriades de micro-organismes, y compris des souches pathogènes sur les instruments utilisés dans les salons de coiffure à Benin City. Il est recommandé que l'exposition de 60 minutes soit la durée idéale pour les chambres de stérilisation UV utilisées dans les salons de coiffure et les barbiers de Benin City devraient s'efforcer de remplacer leurs chambres UV après une utilisation continue pendant une période de 3 ans.

Mots-clés: microbes, efficacité antimicrobienne, stérilisation aux UV, salon de coiffure, Benin City,

Introduction:

The hairs of men are usually uncovered and directly exposed to several environmental particles such as dusts. These particles serve as means of transportation for bioaerosols (1). The hairs also get in contact with various stationary fomites housing millions of pathogenic microbes (2). These pathogens are directly or indirectly transmitted from one person to another in barbershops (3). With this, a myriad of pathogens has been isolated from various barbershops. With the ever-increasing need for men to cut their hairs to size and style, barbershops have been implicated in the increasing cross-contamination and infection of persons using the barbershops services (4).

Exposure to pathogenic strains of fungi and bacteria can lead to common diseases on the hair, skin, and respiratory tracts such as rhinitis, asthma and pneumonia (5). The structure of the human hair makes it act like an air-filter. The spaces between the hair lines forms a perfect trap for all forms of microbes and the hair scrap provides a suitable landing and proliferation ground (6). Infection may occur during hair dressing, cutting and styling while employing clippers, scissors, razors, hairpins and combs through broken skin resulting from accidental injury (7).

With the current growing public awareness and concern for the potential microbial dangers in barbershops, barbers are now widely employing the use of both branded and unbranded UV sterilization chambers in disinfecting their equipment and tools with the hope of re-assuring customers safety and satisfaction (8). However, these barbers do not have basic knowledge on how to optimally use these chambers and lack the skills required to ascertain their potency or efficacy. This study therefore aimed to investigate the types of

possible pathogens associated with their equipment, the qualitative efficacy of their UV sterilization chambers on the identified pathogens, and efficacy of their UV sterilization chambers based on the age of the chamber used.

Materials and method:

Study setting

This study was conducted in Benin City located in south-south geopolitical zone of Nigeria. Benin City is the capital of Edo State, with geographic coordinates of latitudes 06° 06' N, 06° 30' N and longitudes 005° 30' E, 005° 45' E. There are six Local Government Areas (LGAs) within the City; Oredo, Uhunmwonde, Ikpoba-Okha, Egor, Ovia South-West and Ovia North-East, and all the LGAs were involved in the study.

Selection of barbershops and sample collection

A total of thirty (30) barbershops were selected from the six LGAs with five shops randomly selected for sampling from each of the LGA. The barbers instrument (clippers, combs and brushes) were aseptically swabbed over 1 cm (length) by 1 cm (breadth) area using sterile swab sticks moistened with peptone water (9).

Swab samples were collected in pairs, with one pair for bacteria and the other for fungi before and after sterilization. Swab samples collected before exposure to UV sterilization chamber served as baseline. Swab samples were then collected after 1 min, 5 mins, 10 mins, 20 mins, 30 mins and 60 mins sterilization duration.

All swab samples were labelled appropriately and transported immediately to the Microbiology Departmental Laboratory, University of Benin, for culture isolation and identification of bacteria and fungi using standard microbiology procedures (10).

Culture and isolation of microorganisms

Swabs for bacterial isolation were inoculated on Nutrient agar containing 250 mg nystatin (to inhibit the growth of fungi) and incubated aerobically at 37°C for 24 hours. Similarly, swabs for fungi isolation were inoculated on Notman agar containing 250 mg chloramphenicol (to inhibit the growth of bacteria) and incubated at laboratory room temperature for 5 - 7 days (10).

Identification of isolates

Colonial growths on primary isolation media were sub-cultured on Nutrient agar for bacteria and Potato Dextrose agar for fungi to obtain pure cultures. A variety of selective and differential microbial media such as CHROM-agar, Czapek agar and inhibitory mold agar (IMA) for fungi, and mannitol salt agar (MSA), MacConkey agar and Eosin methylene blue (EMB) for bacteria, were used to further purify the isolates before being stored on Nutrient agar slants and kept at 4°C pending identification.

Bacteria isolates were identified to genus level using morphological, cultural and biochemical characteristics with the Bergey's Manual of Determinative Bacteriology (11). Fungi isolates were identified using the procedure described by De-hoop in Atlas of Clinical Fungi (12).

Information on UV sterilization practice

Information on the age of UV sterilization chambers (mean wavelength of sampled chambers was 254 nm with average wattage of 10W and intensity of 760 $\mu\text{W}/\text{cm}^2$) and the average time duration for sterilization of instrument was obtained by oral interview from

each barber. The age range of the sterilization chambers were categorized as 1-2 years, 3-4 years and 5-6 years while the duration of sterilization was set at 1 min, 5 mins, 10 mins, 20 mins, 30 mins and 60 mins.

Data entry and statistical analysis

Data were presented in frequency distribution tables and analysed using GraphPad Instat package (GraphPad Software Inc., San Diego). Comparison of the frequency of isolation of microorganisms in the barbershops between the LGAs was done using Chi square test and $p < 0.05$ was considered significant.

Results:

In this study, a total of 15 microbial genera were isolated from instruments of 30 selected barbershops in the 6 LGAs sampled in Benin City. Among the microbes were 8 bacteria and 7 fungi genera (Table 1). The distribution pattern of the isolates from each LGA before sterilization is shown in Table 2. Among the bacteria isolates; *Staphylococcus* sp., *Bacillus* sp. and *Pseudomonas* sp. were recovered from instruments swabbed at the barbershops in all the LGAs (100%).

Similarly, *Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., *Trichophyton* sp. and *Penicillium* sp. were recovered from barbershops in all the LGAs (100%). Barbershops in Egor LGA had the highest frequency of microbial isolates of 86.7% (13 of 15 genera isolated) while barbershops in Ovia-Southwest LGA had the least frequency of microbial isolates of 60% (9 of 15 genera isolated) from their instruments ($\chi^2 = 3.6000$; $p = 0.6083$).

Table 1: Identified microbial genera from instruments of selected barbershops in Benin City, Nigeria

Isolates ID	Bacteria genera	Isolate ID	Fungi genera
BtA	<i>Staphylococcus</i> sp.	FgA	<i>Aspergillus</i> sp.
BtB	<i>Streptococcus</i> sp.	FgB	<i>Mucor</i> sp.
BtC	<i>Micrococcus</i> sp.	FgC	<i>Rhizopus</i> sp.
BtD	<i>Corynebacterium</i> sp.	FgD	<i>Trichophyton</i> sp.
BtE	<i>Bacillus</i> sp.	FgE	<i>Penicillium</i> sp.
BtF	<i>Enterococcus</i> sp.	FgF	<i>Cladosporium</i> sp.
BtG	<i>Pseudomonas</i> sp.	FgG	<i>Candida</i> sp.
BtH	<i>Proteus</i> sp.		

ID = Identification

Table 2: Distribution of microbial isolates from barbershops in six LGAs of Benin City, Nigeria

Isolates	Oredo	Uhunmwonde	Local Government Area Ikpoba-Okha	Egor	Ovia South- West	Ovia North-East	Frequency of isolates (%)
Bacteria							
<i>Staphylococcus</i> sp.	+	+	+	+	+	+	100
<i>Streptococcus</i> sp.	+	+	-	+	-	-	50
<i>Micrococcus</i> sp.	-	+	+	-	+	-	50
<i>Corynebacterium</i> sp.	+	-	+	-	-	-	33.3
<i>Bacillus</i> sp.	+	+	+	+	+	+	100
<i>Enterococcus</i> sp.	-	+	-	+	-	-	33.3
<i>Pseudomonas</i> sp.	+	-	+	+	+	+	100
<i>Proteus</i> sp.	-	+	-	+	-	-	33.3
Fungi							
<i>Aspergillus</i> sp.	+	+	+	+	+	+	100
<i>Mucor</i> sp.	+	+	+	+	+	+	100
<i>Rhizopus</i> sp.	+	+	+	+	+	+	100
<i>Trichophyton</i> sp.	+	+	+	+	+	+	100
<i>Penicillium</i> sp.	+	+	+	+	+	+	100
<i>Cladosporium</i> sp.	-	-	+	+	-	+	50
<i>Candida</i> sp.	-	+	-	+	-	+	50
Frequency per LGA (%)	66.7	80	73.3	86.7	60	66.7	$\chi^2=3.6000$ $p=0.6083^{ns}$

χ^2 = Chi square; ns = not significant; LGA=Local Government Area

Table 3: Survival rate of microbial isolates from exposure to UV Chamber of 1-2 years of age

Before exposure to UV			After exposure to UV				
Isolates		1min	5mins	10mins	20mins	30mins	60mins
Bacteria							
<i>Staphylococcus</i> sp.	+	+	-	-	-	-	-
<i>Streptococcus</i> sp.	+	+	-	-	-	-	-
<i>Micrococcus</i> sp.	+	+	-	-	-	-	-
<i>Corynebacterium</i> sp.	+	+	-	-	-	-	-
<i>Bacillus</i> sp.	+	+	+	-	-	-	-
<i>Enterococcus</i> sp.	+	+	-	-	-	-	-
<i>Pseudomonas</i> sp.	+	+	-	-	-	-	-
<i>Proteus</i> sp.	+	+	-	-	-	-	-
Bacterial Survival rate (%)		100	12.5	0	0	0	0
Fungi							
<i>Aspergillus</i> sp.	+	+	+	-	-	-	-
<i>Mucor</i> sp.	+	+	+	-	-	-	-
<i>Rhizopus</i> sp.	+	+	-	-	-	-	-
<i>Trichophyton</i> sp.	+	+	-	-	-	-	-
<i>Penicillium</i> sp.	+	+	+	-	-	-	-
<i>Cladosporium</i> sp.	+	+	+	-	-	-	-
<i>Candida</i> sp.	+	+	-	-	-	-	-
Fungi Survival rate (%)		100	57.1	0	0	0	0
Total Isolates Survival rate (%)		100	33.3	0	0	0	0

Table 3 shows the survival rate of microbial isolates after exposure to UV Chamber of 1-2 years of age. All the microbial isolates survived exposure after 1 minute and grew to produce colonies on the agar plate. However, only *Bacillus* sp. survived 5 mins exposure time (12.5%) among all the bacteria isolates. Moreover, *Aspergillus* sp., *Mucor* sp., *Penicillium* sp. and *Cladosporium* sp. survived (57.1%) the 5 mins exposure time for fungi. The total isolate survival rate at 5 mins exposure was 33.3%, and 0% at 10, 20, 30, and 60 mins exposure time.

Table 4 shows the survival rate of isolates after exposure to UV Chamber of 3-4 years of age. All the isolates survived 1 min exposure time. However, 75% of the *Staphylo-*

coccus sp., *Streptococcus* sp., *Micrococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Proteus* sp. survived 5 mins exposure time among all the bacteria isolates while 57.1% of *Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Trichophyton* sp., *Clado- sporium* sp. and *Candida* sp. survived 5 mins exposure time among the fungi. Similarly, 62.5%% of *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Pseudomonas* sp. and *Proteus* sp. survived 10 mins exposure time among all the bacteria isolates while 71.4% of *Aspergillus* sp., *Mucor* sp., *Penicillium* sp. and *Candida* sp. survived 10 mins exposure among the fungi. The total microbial isolates survival rate at 5 mins exposure was 33.3%, 10 mins 66.7% and 0% at 20 mins, 30 mins and 60 mins exposure time.

Table 4: Survival rate of microbial isolates from exposure to UV Chamber of 3-4 years of age

Before exposure to UV		After exposure to UV					
Isolates		1min	5mins	10mins	20mins	30mins	60mins
Bacteria							
<i>Staphylococcus</i> sp.	+	+	+	+	-	-	-
<i>Streptococcus</i> sp.	+	+	+	+	-	-	-
<i>Micrococcus</i> sp.	+	+	+	-	-	-	-
<i>Corynebacterium</i> sp.	+	+	-	-	-	-	-
<i>Bacillus</i> sp.	+	+	+	+	-	-	-
<i>Enterococcus</i> sp.	+	+	-	-	-	-	-
<i>Pseudomonas</i> sp.	+	+	+	+	-	-	-
<i>Proteus</i> sp.	+	+	+	+	-	-	-
Bacterial Survival rate (%)		100	75.0	62.5	0	0	0
Fungi							
<i>Aspergillus</i> sp.	+	+	+	+	-	-	-
<i>Mucor</i> sp.	+	+	+	+	-	-	-
<i>Rhizopus</i> sp.	+	+	-	+	-	-	-
<i>Trichophyton</i> sp.	+	+	+	-	-	-	-
<i>Penicillium</i> sp.	+	+	+	+	-	-	-
<i>Cladosporium</i> sp.	+	+	+	-	-	-	-
<i>Candida</i> sp.	+	+	+	+	-	-	-
Fungi Survival rate (%)		100	85.7	71.4	0	0	0
Total Isolates Survival rate (%)		100	80.0	66.7	0	0	0

Table 5: Survival rate of microbial isolates from exposure to UV Chamber of 5-6 years of age

Before exposure to UV		After exposure to UV					
Isolates		1min	5mins	10mins	20mins	30mins	60mins
Bacteria							
<i>Staphylococcus</i> sp.	+	+	+	+	+	-	-
<i>Streptococcus</i> sp.	+	+	+	+	-	-	-
<i>Micrococcus</i> sp.	+	+	+	-	-	-	-
<i>Corynebacterium</i> sp.	+	+	+	-	-	-	-
<i>Bacillus</i> sp.	+	+	+	+	+	+	-
<i>Enterococcus</i> sp.	+	+	-	-	-	-	-
<i>Pseudomonas</i> sp.	+	+	+	-	-	-	-
<i>Proteus</i> sp.	+	+	+	+	-	-	-
Bacterial Survival rate (%)		100	87.5	50	25	3.1	0
Fungi							
<i>Aspergillus</i> sp.	+	+	+	+	+	-	-
<i>Mucor</i> sp.	+	+	+	+	+	+	-
<i>Rhizopus</i> sp.	+	+	+	+	-	-	-
<i>Trichophyton</i> sp.	+	+	+	-	-	-	-
<i>Penicillium</i> sp.	+	+	+	+	+	+	-
<i>Cladosporium</i> sp.	+	+	+	+	-	-	-
<i>Candida</i> sp.	+	+	+	+	+	-	-
Fungi Survival rate (%)		100	100	85.7	57.1	28.3	0
Total Isolates Survival rate (%)		100	93	66.7	33.3	20.0	0

Table 5 shows the survival rate of isolates after exposure to UV Chamber of 5-6 years of age. All the isolates survived 1 min exposure time. However, 85.7% of the *Staphylococcus* sp., *Streptococcus* sp., *Micrococcus* sp., *Corynebacterium* sp., *Bacillus* sp., *Pseudomonas* sp. and *Proteus* sp. survived 5 mins exposure time among all the bacteria isolates, while all fungi isolates (100%) survived the 5 mins exposure time. Similarly, 50% of the *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp. and *Proteus* sp. survived 10 mins exposure time among all the bacteria isolates while 85.7% of *Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Candida* sp., *Penicillium* sp., *Cladosporium* sp. and *Candida* sp. survived 10 mins exposure time among the fungi. At 20 mins exposure time, only 25% of *Staphylococcus* sp. and *Bacillus* sp. among the bacteria isolates survived while 57.1% of *Aspergillus* sp., *Mucor*

sp., *Penicillium* sp. and *Candida* sp. survived 20 mins exposure time among the fungi. At 30 mins exposure time, only 3.1% of *Bacillus* sp. among the bacteria isolates survived while 28.3% of *Mucor* sp. and *Penicillium* sp. survived 30 mins exposure time among the fungi. The total microbial isolates survival at 5 mins exposure was 93%, 10 mins 66.7%, 20 mins 33.3%, 30 mins 20.0% and 0% at 60 mins.

Discussion:

In this study, a total of 15 microbial genera (8 bacteria and 7 fungi) were isolated, some of which are of public health importance (7). The high level of microbial variability isolated from instruments used at the sampled barbershops to some extent may be due to open (uncovered) nature of male hair and direct exposure to dust particles, other environmental

elements, and life style. The spaces between the hair lines forms a perfect trap for all forms of microbes and the hair scrap provides a suitable landing and proliferation ground for microbes (6).

The bacterial (*Staphylococcus* sp., *Bacillus* sp. and *Pseudomonas* sp.) and fungi isolates (*Aspergillus* sp., *Mucor* sp., *Rhizopus* sp., *Trichophyton* sp. and *Penicillium* sp.) were isolated from barbering instruments across all LGAs (100%), implying that these isolates are indigenous microbes in Benin City (6,10,11,12). Egor LGA had the highest frequency of isolates recovery (86.7%), which could be attributed to the relatively poor state of hygiene practices observed at the barbershops within this locality.

Evaluation of the UV sterilization chambers revealed that all isolates survived exposure time of 1 minute as previously reported by Berrin (13) and Katara et al., (14). This is because the isolates were able to repair their altered nucleic acids resulting from the brief exposure to ultraviolet radiation (15,16). Our result therefore debunks the belief by some barbers in Benin City who presumes that brisk exposure of their clippers, combs and other items to UV light is enough to kill all microbial contaminants irrespective of the duration of exposure. Furthermore, our evaluation study shows that the older the UV chamber is, the less effective its antimicrobial efficacy, which is in consonance with the reports of Sowah and Ahiabor (8), and Mackey et al., (17). *Bacillus* sp., *Mucor* sp. and *Penicillium* sp. survived exposure duration of 30 mins in the UV chambers that were 5-6 years old, as previously reported (14,18). This study also shows that the longer the duration of exposure, the more effective the sterilization efficacy which agrees with findings of previous studies (8,14). At 60 mins, no visible microbial growth was observed, signifying highest level of disinfection/sterilization, which indicate that 60 mins is ideal time for UV sterilization of barbering instruments (19).

It was generally observed in this study that fungi were more adapted to the UV sterilization chambers than bacteria as previously established (13), with *Bacillus* sp., being the most formidable bacteria isolate (20), while *Mucor* sp. and *Penicillium* sp. were the most formidable fungi isolates (21,22). It was also observed that *Enterococcus* sp. and *Trichophyton* sp. were the most effectively eliminated bacteria and fungi isolates respectively.

Conclusion:

This study confirms the presence of myriads of microbial organisms including patho-

genic strains on instruments used in barbershops within Benin City. It is recommended that exposure of 60 mins is the ideal duration for UV sterilization chambers used in barbershops, and barbers in Benin City should endeavor to replace their UV chambers after continual use for a period of 3 years.

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Conflict of interest:

No conflict of interest is declared.

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Original Article

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Antibiogram of *Pseudomonas* isolates and potential public health impact of an abattoir effluent in Benin City, Nigeria

*Oadjajare, E. E. O., and Ebowemen, M. J.

Environmental, Public Health and Bioresource Microbiology Research Group (EPHBIOMREG),
Department of Biological Sciences, Benson Idahosa University, P.M.B. 1100 Benin City, Edo State, Nigeria*Correspondence to: eodjajare@biu.edu.ng

Abstract:

Background: Bacteria from abattoir wastes are often linked to livestock carcasses previously exposed to continuous antimicrobial use and misuse; thereby creating opportunity for community spread of multidrug resistant (MDR) strains such as *Pseudomonas* spp. The aim of this study was to investigate the antibiogram of *Pseudomonas* isolates and bacteriological quality of an abattoir effluent in lieu of its potential public health impact.

Methodology: Water samples were collected weekly for six weeks from discharge point (DP) of the abattoir effluent, effluent receiving canal confluence point (CP), and 500 m upstream (US) and 500 m downstream (DS) from points where CP made contact with the Ikpoba River, Benin City, Nigeria. Bacteria spp. were isolated, enumerated (heterotrophic bacterial plate, coliform, *Escherichia coli* and *Pseudomonas* counts) and identified using standard microbiological techniques. Identity of *Pseudomonas* isolates was confirmed by PCR while antibiogram of selected isolates was evaluated and interpreted according to the disk diffusion method of the Clinical and Laboratory Standards Institute (CLSI).

Results: Heterotrophic bacteria plate counts (HPC) varied from $1.1 \times 10^3 \pm 0.28$ CFU/ml to $1.95 \times 10^6 \pm 0.48$ CFU/ml; total coliform counts ranged between 0.0 and $1.2 \times 10^6 \pm 0.28$ CFU/ml while mean *E. coli* count varied from 0.0 to $4.9 \times 10^5 \pm 0.49$ CFU/ml, and *Pseudomonas* counts were between 0.0 to 1.4×10^3 CFU/ml. The selected strains of *Pseudomonas* spp (n=50) showed resistance to oxacillin (100%), vancomycin (52%), tetracycline (50%), gentamycin (26%) and ceftriaxone (20%), while they were sensitive to ceftazidime (82%), ofloxacin (80%) and amikacin (74%). MDR phenotype was observed in 9 (18%) of the test isolates.

Conclusion: The study revealed that untreated abattoir effluent was a considerable source of MDR *Pseudomonas* spp. among other bacteriological pollutants (e.g. HPC, coliform and *E. coli*) that could compromise the quality of the receiving river in lieu of public health concerns of riverside communities that depend on this vital water resource for their subsistence.

Keywords: *Pseudomonas*; MDR; antibiogram; abattoir effluent; public health

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Antibiogramme des isolats de *Pseudomonas* et impact potentiel sur la santé publique d'un effluent d'abattoir à Benin City, Nigeria

*Oadjajare, E. E. O., et Ebowemen, M. J.

Groupe de recherche sur l'environnement, la santé publique et la microbiologie des bioressources (EPHBIOMREG),
Département des sciences biologiques, Université Benson Idahosa, P.M.B. 1100 Benin City, Edo State, Nigeria*Correspondance à: eodjajare@biu.edu.ng

Abstrait:

Contexte: Les bactéries provenant des déchets d'abattoir sont souvent liées à des carcasses de bétail précédemment exposées à une utilisation et une mauvaise utilisation antimicrobiennes; créant ainsi une opportunité de propagation communautaire de souches multirésistantes (MDR) telles que *Pseudomonas* spp. Le but de cette étude était d'étudier

l'antibiogramme des isolats de *Pseudomonas* et la qualité bactériologique d'un effluent d'abattoir au lieu de son impact potentiel sur la santé publique.

Méthodologie: Des échantillons d'eau ont été prélevés chaque semaine pendant six semaines à partir du point de rejet (DP) de l'effluent de l'abattoir, du point de confluence du canal de réception des effluents (CP) et à 500 m en amont (US) et 500 m en aval (DS) des points où le CP a établi le contact avec la rivière Ikpoba, Benin City, Nigeria. Bacteria spp. ont été isolés, dénombrés (plaques bactériennes hétérotrophes, coliformes, dénombrements d'*Escherichia coli* et de *Pseudomonas*) et identifiés à l'aide de techniques microbiologiques standard. L'identité des isolats de *Pseudomonas* a été confirmée par PCR tandis que l'antibiogramme des isolats sélectionnés a été évalué et interprété selon la méthode de diffusion sur disque du Clinical and Laboratory Standards Institute (CLSI).

Résultats: le nombre de plaques de bactéries hétérotrophes (HPC) variait de $1,1 \times 10^3 \pm 0,28$ UFC/ml à $1,95 \times 10^6 \pm 0,48$ UFC/ml; le nombre total de coliformes variait entre 0,0 et $1,2 \times 10^6 \pm 0,28$ UFC/ml tandis que le nombre moyen d'*E. coli* variait de 0,0 à $4,9 \times 10^5 \pm 0,49$ UFC/ml, et le nombre de *Pseudomonas* était compris entre 0,0 et $1,4 \times 10^3$ UFC/ml. Les souches sélectionnées de *Pseudomonas* spp (n=50) ont montré une résistance à l'oxacilline (100%), à la vancomycine (52%), à la tétracycline (50%), à la gentamycine (26%) et à la ceftriaxone (20%), alors qu'elles étaient sensibles à la ceftazidime (82%), l'ofloxacine (80%) et l'amikacine (74%). Le phénotype MDR a été observé dans 9 (18%) des isolats testés.

Conclusion: L'étude a révélé que les effluents d'abattoir non traités étaient une source considérable de MDR *Pseudomonas* spp. entre autres polluants bactériologiques (par exemple HPC, coliformes et *E. coli*) qui pourraient compromettre la qualité de la rivière réceptrice au lieu des préoccupations de santé publique des communautés riveraines qui dépendent de cette ressource vitale en eau pour leur subsistance.

Mots-clés: *Pseudomonas*; MDR; antibiogramme; effluent d'abattoir; santé publique

Introduction:

The meat processing industry consumes 29% of the total freshwater used by the agricultural sector worldwide (1). This creates an opportunity for the generation of large amounts of wastewater that are ultimately discharged into the environment. Wastes from such facilities have been reported to be of public health concern, especially in developing countries such as Nigeria, where abattoir effluents are directly disposed into streams and rivers with little or no treatment (2). Incidentally, many of these streams and rivers also serve as water resource for domestic, agricultural, recreational, as well as drinking purposes for communities downstream (3).

Contamination of rivers by abattoir wastes could constitute significant environmental and health hazards, promoting growth of disease-causing organisms including *Pseudomonas* spp. among others (4,5). Auwalu et al., (6) reported a significant nexus between abattoir effluents and diseases such as typhoid fever, diarrhoea illnesses, respiratory diseases, cough, foot and mouth diseases and dengue, affecting abattoir neighboring communities in Malaysia while Bello and Oyedemi (7) associated diseases such as pneumonia, diarrhoea, typhoid fever, asthma, wool sorter disease, and respiratory and chest diseases, with abattoir activities in Ogbomoso, Nigeria.

The genus *Pseudomonas* comprises Gram-negative, motile, aerobic Gamma-proteobacteria grouped into the family Pseudomonadaceae (8). There are 191 validly described species in this genus; many of which demonstrate a great deal of metabolic diversity and

consequently colonize a wide range of ecological niches including soil, water, humans, plant and animal surfaces, cosmetics, medical products and instruments, and foods of animal and vegetal origins (9,10). *Pseudomonas* spp. are opportunistic pathogens with intrinsic antimicrobial resistance due to low outer membrane permeability as well as extensive efflux pump system (11). The United States Center for Disease Prevention and Control (CDC) reported *Pseudomonas* species as one of the top 18 antibiotic-resistant pathogens that pose serious threats in the US (12). The document further observed that more than 6700 multidrug resistant (MDR) *Pseudomonas* infections with 440 deaths per year were reported in 2019 (12). *Pseudomonas* has been incriminated in infection of the urinary tract, blood stream, pneumonia, pharyngitis, and many other medical conditions with remarkably high morbidity and mortality (13).

Abattoir effluents are particularly pivotal in the spread of MDR pathogens in the community. Many isolates from this environment emanate from livestock carcasses previously exposed to continuous antimicrobial use and misuse for therapeutic and/or prophylactic purposes, as well as growth promoters. This creates ample opportunity for such MDR strains to be transferred via the food chain to humans and spread in the population (5). The spread of MDR pseudomonads from different sources to humans and the environment indicates the frequent dissemination of resistance genes by horizontal gene transfer (HGT), since many of these genes are located in plasmids, integrons,

or transposons (10).

Odjajdare et al., (14) reported a significant correlation between wastewater effluent quality and that of receiving waters, indicating that release of effluents containing MDR *Pseudomonas* spp into the environment could potentially compromise the public health of persons who depend on such resource for sundry uses. Beside the higher rate of fatality among patients with resistant bacteria related infections, the occurrence of MDR infection mandates longer hospitalization and therapies with complex and expensive treatment modalities especially in low-and middle-income countries like Nigeria. According to the Organization for Economic Cooperation and Development (OECD), 2.4 million deaths due to MDR related infections are expected in Europe, North America, and Australia, with an associated cost of up to US\$3.5 billion per year in the next 30 years (12). These impact and cost estimates are expected to be even worse in Africa and other developing countries where there is severe shortage of infrastructure and healthcare delivery facilities.

Antibiotic resistant pseudomonads in abattoir have been previously reported in Nigeria (15,16,17), thus highlighting a growing public health concern over possible outbreak of difficult to treat community acquired *Pseudomonas* infections. It is therefore imperative that surveillance of resistance patterns of *Pseudomonas* strains from the environment are

regularly and continuously conducted to generate data that would help clinicians choose correct empirical treatment in lieu of public health preservation. Thus, the aim of this study was to investigate the antibiotic resistance profile and public health impact of *Pseudomonas* species isolated from an abattoir effluent that empties into the Ikpoba River, Benin City, Nigeria.

Material and Methods:

Study site and sampling

The study was conducted in an abattoir that introduces its effluent into the Ikpoba River, Benin City, Nigeria. Fig 1 shows the sampling sites as derived from Google map using a Global Positioning System (GPS). Samples were collected weekly for six weeks from four (4) points within the abattoir catchment including abattoir effluent discharge point (point source; DP), effluent receiving canal (confluent point; CP), and 500 m upstream (upstream; US) and 500 m downstream (downstream; DS) from the point where CP makes contact with the Ikpoba River.

The samples were collected in 1-liter capacity sterile plastic containers in duplicates and transported in cooler boxes containing ice packs to the Microbiology Laboratory of Benson Idahosa University for further analysis. All samples were analyzed within 24 hours of sample collection.

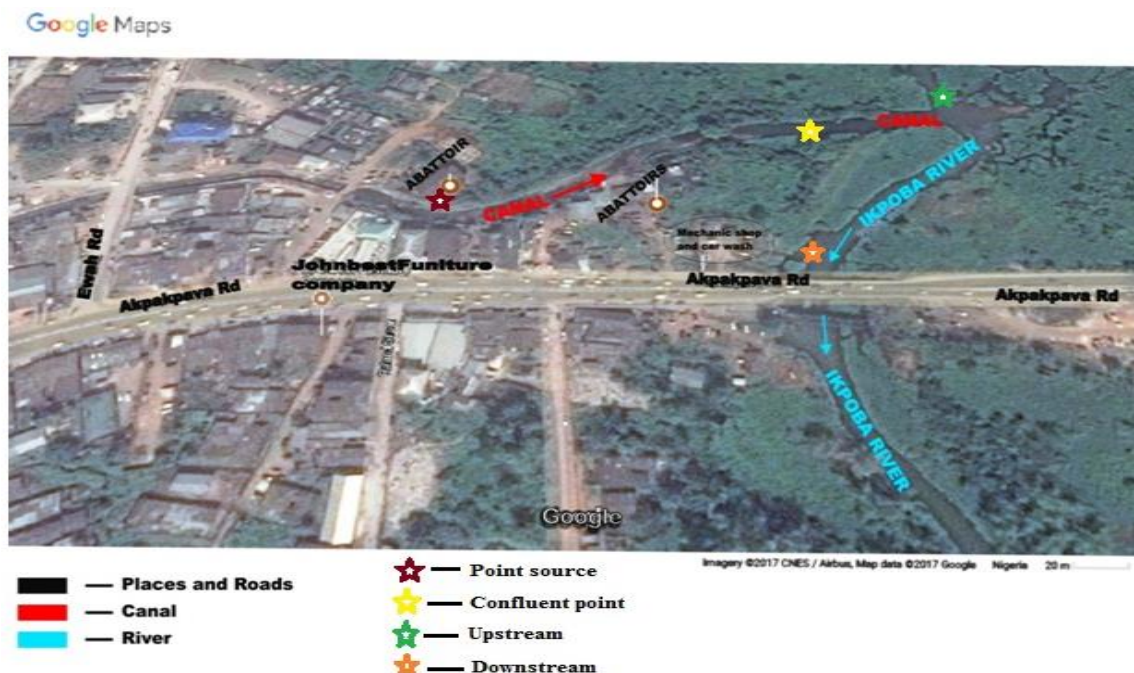


Fig 1: Images of the abattoir catchment as derived from Google map using a Global Positioning System (GPS).

www.googlewmaps.com

Microbiological analysis

Each water sample was serially diluted and used to inoculate sterile nutrient agar, MacConkey agar, EMB agar and Cetrimide agar (containing 10% glycerol) plates following standard spread plate technique. All plates were incubated at 37°C for 24-48 hrs. After incubation, total (heterotrophic) bacterial counts (TBC) otherwise known as heterotrophic plate counts (HPC), total coliform counts, *E. coli* and *Pseudomonas* counts were recorded from the respective agar plates. Coliforms on MacConkey agar appeared as pink colonies; *E. coli* on EMB agar appeared as green colonies with metallic-sheen; while *Pseudomonas* spp. colonies present with green pigmentation on Cetrimide agar.

Pseudomonas isolates were phenotypically identified using colonial, cultural, morphological (Gram staining, motility) and biochemical (catalase and oxidase) characteristics (18). Presumptive *Pseudomonas* isolates were then sub-cultured onto sterile nutrient agar plates and subsequently stored on nutrient agar slants at 4°C in the refrigerator until needed for further analysis.

Antibiotic susceptibility testing of *Pseudomonas* isolates

The antibiotic susceptibility test against 8 selected antibiotics was performed and interpreted based with the disk diffusion method of the Clinical and Laboratory Standards Institute (19) using 24 hours culture of *Pseudomonas* isolates (n=50) on Mueller Hinton agar (HIMEDIA®, India, REF-M173) plates. The paper antibiotic disks (Mast Diagnostics, Merseyside, UK) used were amikacin (30µg), ceftazidime (30µg), ceftriaxone (30µg), gentamicin (10µg), ofloxacin (5µg), oxacillin (1µg), tetracycline (30µg), and vancomycin (30µg).

Molecular confirmation of *Pseudomonas* isolates

DNA extraction

DNA extraction from pure cultures of phenotypically identified *Pseudomonas* isolates (n=50) was carried out as described by Odjadjare and Igbiosa (3) with slight modifications. Single colonies of presumptive *Pseudomonas* isolates grown overnight at 37°C on nutrient agar plates were picked with sterile wire loop, suspended in 200µl of sterile nuclease free water (Life Science Biotechnology, Solon, South Africa) and the cells lysed by heating on a heating-block (Fisher Scientific, Model: 2052-1CEFS, California, U.S.A) for 15 mins at 100°C.

The cell debris was removed by centrifugation at 11,000×g for 2 mins using a MiniSpin micro-centrifuge (Model: Mini-14K, TOMOS Life Science Group, Shanghai, China). The cell lysates (5µl) were used as DNA template in the PCR assays immediately after extraction.

PCR amplification of target genes

PCR was performed in a GenePro Thermal Cycler (Model: TC-E-96G Hangzhou Bioer Technology Co., China LTD). The PCR master mix (Inqaba Biotech, South Africa) for a typical 20µl reaction included; 2×Master mix (10µl), nuclease free water (3.0µl), Primer F (5pmol/u) (1.0µl), Primer R (5pmol/u) (1.0µl) and DNA template (5.0µl). The primers used were; *Pseudomonas* genus 16S rRNA forward (5'-GACGGGTGAGTAATGCCTA-3') and 16S rRNA reverse (5'-CACTGGTGTTCCTTCCTATA-3') primers (20).

The PCR conditions for amplification were as follows; initial denaturation at 95°C for 5 mins., followed by 10 cycles of denaturation at 94°C for 15 sec, annealing at 53°C for 30 sec and 72°C for 45 second. This was repeated for another 25 cycles with exception of the 72°C elongation step, which was increased by 5 sec at every cycle; and a final extension step of 72°C for 10 min. Amplicon size of the gene was 617 bp (20). The amplified PCR products were held at 4°C on completion of the reaction until it is used for further analysis.

Agarose gel electrophoresis

The PCR products were resolved by electrophoresis in 1.8% agarose gel in 1× TAE buffer (4.85g of Tris, 0.37g of EDTA and 1.64g of sodium acetate in 1000 ml of distilled water) stained with ethidium bromide (5µl/100ml). Five microlitre (5µl) of the PCR products was mixed with 5µl loading dye and loaded onto sample wells. Electrophoresis was run at 100 V for 30 to 45 mins. The resolved bands were visualized under UV transilluminator and photographed (3).

Statistical analysis

Calculation of means and standard deviation was done using Microsoft Excel Office 2013. Comparisons of means were analysed statistically, using the one-way analysis of variance (ANOVA) and Pearson chi-square. Relationships were tested using the Pearson correlation index. All statistical analyses were performed using SPSS 23.0 software. Values were deemed to be statistically significant at 95% confidence interval (i.e. $p < 0.05$).

Results:

Bacteria load estimation and identification

The total heterotrophic bacterial counts (TBC) during this study ranged between $1.1 \times 10^3 \pm 0.28$ CFU/ml and $1.95 \times 10^6 \pm 0.48$ CFU/ml (Table 1). The highest bacterial count was observed in samples collected at CP in week 6, while the lowest was observed in week 4 at US. Bacterial counts at DP and CP were relatively higher than those recorded at US and DS; however, the observed difference was not statistically significant across sampled points. There was significant correlation between TBC and coliform counts ($p < 0.01$) and TBC versus *Pseudomonas* count ($p < 0.05$). However, there was no significant correlation between TBC and

E. coli count.

Table 2 shows the total coliform counts during the study. The highest coliform count was observed in the sixth week at CP ($1.2 \times 10^6 \pm 0.28$ CFU/ml) while the lowest (0.0) were observed in weeks 2 and 3 at US. There was no significant correlation between coliform count and other bacterial counts (e.g. *E. coli* and *Pseudomonas* counts), except with TBC as earlier indicated. The mean *E. coli* count during the study ranged from 0.0 in US and DS (weeks 2 to 4) to $4.9 \times 10^5 \pm 0.49$ CFU/ml in CP (week 6) (Table 3). *E. coli* count did not vary significantly with sampling point and time. There was also no significant correlation between *E. coli* and other bacterial counts (including TBC, coliform and *Pseudomonas* counts).

Table 1: Mean total bacterial counts of test samples (CFU/ml) of abattoir effluents in Benin City, Nigeria

Sampled points	Sampling Weeks					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Point source (DP)	$1.98 \times 10^5 \pm 0.14^a$	$2.85 \times 10^5 \pm 0.7$	$1.97 \times 10^5 \pm 0.19$	$1.19 \times 10^5 \pm 0.2$	$1.79 \times 10^5 \pm 0.15$	$1.67 \times 10^6 \pm 0.64$
Confluent point (CP)	$1.16 \times 10^5 \pm 0.24$	$1.71 \times 10^6 \pm 0.14$	$3.5 \times 10^4 \pm 0.2$	$2.3 \times 10^5 \pm 0.5$	$2.88 \times 10^5 \pm 0.19$	$1.95 \times 10^6 \pm 0.48$
Upstream (US)	$4.05 \times 10^5 \pm 0.35$	$2.75 \times 10^5 \pm 0.4$	$2.6 \times 10^5 \pm 0.3$	$1.1 \times 10^3 \pm 0.28$	$2.68 \times 10^4 \pm 0.36$	$1.63 \times 10^4 \pm 0.49$
Downstream (DS)	$5.2 \times 10^4 \pm 0.14$	$4.9 \times 10^4 \pm 0.21$	$5.6 \times 10^4 \pm 0.35$	$2.7 \times 10^4 \pm 0.14$	$8.4 \times 10^4 \pm 0.2$	$7.1 \times 10^3 \pm 0.28$

^avalues represent mean \pm standard deviation

Table 2: Mean total coliform counts of test samples (CFU/ml) of abattoir effluents in Benin City, Nigeria

Sampled points	Sampling weeks					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Point source (DP)	$3.25 \times 10^4 \pm 0.19^a$	$7.15 \times 10^5 \pm 0.12$	$5.15 \times 10^5 \pm 0.35$	$2.8 \times 10^5 \pm 0.85$	$6.0 \times 10^4 \pm 0.89$	$1.56 \times 10^4 \pm 0.28$
Confluent point (CP)	$3.5 \times 10^4 \pm 0.42$	$9.25 \times 10^5 \pm 0.7$	$8.1 \times 10^5 \pm 0.28$	$8.65 \times 10^5 \pm 0.35$	$2.7 \times 10^5 \pm 0.49$	$1.2 \times 10^6 \pm 0.28$
Upstream (US)	$2.9 \times 10^4 \pm 0.14$	$99.5 \times 10^2 \pm 0.71$	$3.7 \times 10^4 \pm 0.1$	$1.3 \times 10^3 \pm 0.1$	$7.65 \times 10^3 \pm 0.35$	$1.15 \times 10^4 \pm 0.31$
Downstream (DS)	$3.1 \times 10^4 \pm 0.14$	0 \pm 0	0 \pm 0	$7.5 \times 10^3 \pm 0.4$	$5.7 \times 10^4 \pm 0.42$	$1.39 \times 10^5 \pm 0.14$

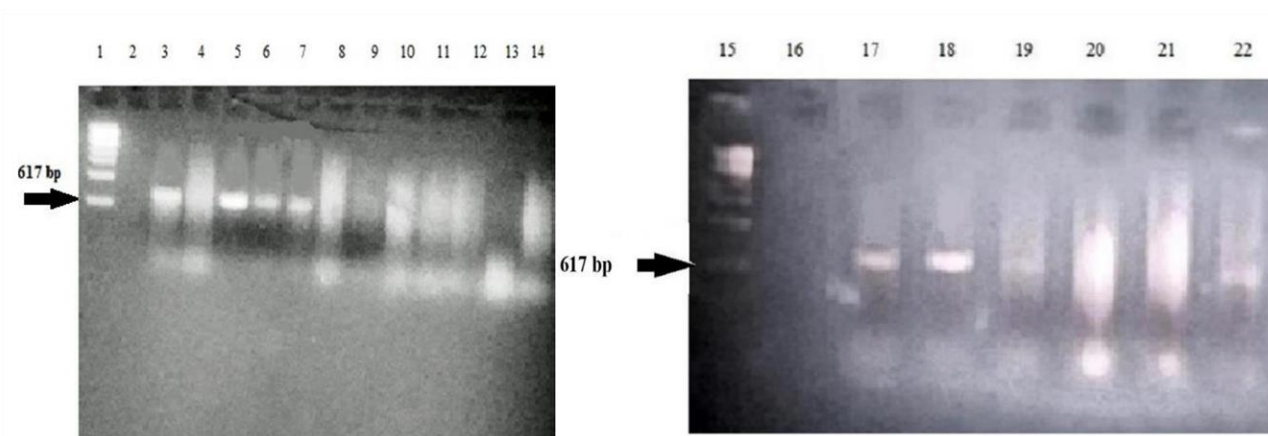
^avalues represent mean \pm standard deviation

Table 3: Mean *Escherichia coli* counts of test samples (CFU/ml) of abattoir effluents in Benin City, Nigeria

Sampled points	Sampling weeks					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Point source (DP)	$2.0 \times 10^4 \pm 0.7^a$	$8.0 \times 10^3 \pm 0.11$	$6.0 \times 10^3 \pm 0.85$	$1.14 \times 10^4 \pm 0.22$	$2.9 \times 10^4 \pm 0.56$	$1.9 \times 10^5 \pm 0.28$
Confluent point (CP)	$5.5 \times 10^3 \pm 0.71$	$1.3 \times 10^4 \pm 0.14$	$1.4 \times 10^4 \pm 0.14$	$7.4 \times 10^3 \pm 0.78$	$2.8 \times 10^4 \pm 0.49$	$4.9 \times 10^5 \pm 0.49$
Upstream (US)	$1.8 \times 10^4 \pm 0.42$	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0
Downstream (DS)	$3.1 \times 10^4 \pm 0.28$	0 \pm 0	0 \pm 0	0 \pm 0	$5.1 \times 10^4 \pm 0.53$	$1.13 \times 10^4 \pm 0.12$

^avalues represent mean \pm standard deviationTable 4: Mean *Pseudomonas* counts of test samples (CFU/ml) of abattoir effluents in Benin City, Nigeria

Sampled Points	Sampling Weeks					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Point source (DP)	$1.0 \times 10^2 \pm 10^a$	$3.3 \times 10^2 \pm 18$	0 \pm 0	$4.1 \times 10^2 \pm 14$	$1.4 \times 10^3 \pm 65$	$5.5 \times 10^2 \pm 25$
Confluent point (CP)	$3.25 \times 10^2 \pm 85$	$6.0 \times 10^2 \pm 10$	0 \pm 0	$1.8 \times 10^2 \pm 65$	$7.0 \times 10^2 \pm 30$	$6.0 \times 10^2 \pm 0$
Upstream (US)	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	$2.0 \times 10^0 \pm 0.7$
Downstream (DS)	0 \pm 0	0 \pm 0	0 \pm 0	$6.5 \times 10^1 \pm 5$	$2.5 \times 10^1 \pm 0.5$	0 \pm 0

^avalues represent mean \pm standard deviationFig 2: Gel electrophoresis picture of the PCR amplicons of 16S rRNA gene specific for representative *Pseudomonas* isolates. Lanes 1 and 15 - 1 kb DNA ladder; Lane 2 and 16 - negative control; Lanes 3, 5, 6, 7, 17 and 18 - positive PCR amplicons

Presumptive *Pseudomonas* counts on cetrimide agar are shown in Table 4. The highest *Pseudomonas* count (1.4×10^3 CFU/ml) was observed at DP in week 5 while the lowest (0.0) were recorded at US in weeks 1 to 6 and DS in weeks 1,2,3 and 6. *Pseudomonas* count for CP varied significantly ($p < 0.05$) with counts from US and DS. However, *Pseudomonas* count did

not vary significantly with sampling time. *Pseudomonas* count did not also correlate with other bacterial counts except for TBC as earlier mentioned. Fifty (50) presumptive *Pseudomonas* strains isolated and selected based on cultural, morphological and biochemical characteristics were confirmed by genus specific PCR (Fig 2).

Table 5: Antibiotic susceptibility profile of *Pseudomonas* spp isolated from abattoir effluents in Benin City, Nigeria

Antibiotic class	Antibiotics	Antibiogram (n=50)		
		Resistant (%)	Intermediate (%)	Sensitive (%)
Aminoglycoside	Amikacin	0	23 (26)	37 (74)
	Gentamicin	13 (26)	17 (34)	20 (40)
Cephem	Ceftazidime	1 (2)	8 (16)	41 (82)
	Ceftriaxone	10 (20)	38 (76)	2 (4)
Fluoroquinolone	Ofloxacin	4 (8)	6 (12)	40 (80)
Penicillin	Oxacillin	50 (100)	0	0
Tetracycline	Tetracycline	25 (50)	17 (34)	8 (16)
Glycopeptide	Vancomycin	26 (52)	24 (48)	0

Table 6: Multiple drug (MDR) resistance profile of the *Pseudomonas* isolates^a

S/N	MDR Phenotype	No of isolates with MDR Phenotype
1.	OFX, GM, T, CRO	1
2.	GM, T, CRO	7
3.	OFX, T, CRO	1

^aMDR profiles presented in the table are exclusive of the non-pseudomonal antibiotics (oxacillin and vancomycin); OFX = Ofloxacin; GM = Gentamicin; T = Tetracycline; CRO = Ceftriaxone

Antibiogram of *Pseudomonas* isolates

The antibiogram of the *Pseudomonas* strains indicated that the isolates were resistant to oxacillin (100%), vancomycin (52%), tetracycline (50%), gentamicin (26%) and ceftriaxone (20%); whereas, they were sensitive to ceftazidime (82%), ofloxacin (80%) and amikacin (74%). *Pseudomonas* isolates exhibited intermediate susceptibility (76%) to ceftriaxone (Table 5).

The MDR phenotypes involving four antibiotic classes (fluoroquinolone, cephem, aminoglycoside and tetracycline) excluding resistance to the non-anti-pseudomonas antibiotics (oxacillin and vancomycin) was observed in 9 (18%) of the 50 selected isolates (Table 6).

Discussion:

There are conflicting reports in the literature with regards to the public health significance of heterotrophic bacterial load in waters. Hellard et al., (21) observed that there was no clinical correlation with elevated heterotrophic bacterial load in water. However, Payment et al., (22) associated high bacterial load with water-borne diseases, while Allen et al., (23) submitted that there is no epidemiological evidence

that higher heterotrophic bacteria density has any public health significance. The bacterial load observed in this study was similar to that reported by Atuanya et al., (24) but relatively higher than those observed by Nafarnda et al., (25) and Onuoha et al., (4). Shukri et al., (26) reported far higher bacterial load in Kalerwe abattoir effluents which empties into the Nsooba channel of Uganda.

The conflicting reports on health significance of heterotrophic plate counts (HPC) in water notwithstanding, several countries across the globe have set different allowable maximum limits for bacterial loads in water samples ranging from 20 to 1,000 CFU/ml (23). This implies that the water sources in our study (across all sampled sites) were of poor bacteriological quality with reference to heterotrophic bacterial load. The HPC index of poor water quality was further corroborated by the significant correlation observed between bacterial load and coliform ($p < 0.01$), and between bacterial load versus *Pseudomonas* densities ($p < 0.05$). The observation therefore suggests that bacterial load is an important index for measuring the possible presence of coliform and potential pathogens such as *Pseudomonas* in water samples.

Coliforms are a range of bacteria in the family Enterobacteriaceae, used as indicator of faecal pollution from warm blooded animals (27). They are important water quality index for determination of the relative risk of possible presence of microbial pathogens of faecal origin in a water sample (28). The total coliform count in this study was similar to that reported by Ezeigbo et al., (29) but relatively lower than those observed in previous studies (24, 30, 31). The coliform load observed in the abattoir effluent was generally higher than the WHO limit (<1000/100ml) for wastewater effluent indicative of risk of transmission of bacterial infections through the use of untreated wastewater (32, 33). This observation validates the poor bacteriological quality of the effluent and its potential negative public health impact.

However, contrary to the report of WHO (32), Grobrow et al., (34) observed that there is no direct correlation between numbers of any indicator and enteric pathogens, and Havelaar et al., (33) reported that many earlier classic failures of coliforms to identify waterborne pathogens have resulted in waterborne outbreaks associated with water sources prejudged to be free of faecal coliforms. These assertions were corroborated by the lack of significant correlation between coliform and *E. coli*, and coliform versus *Pseudomonas* counts as observed in this study. The observation is consistent with the opinion of Havelaar et al., (33) who stated that epidemiologic studies often fail to show relationship to microbial indicators, due to widely fluctuating ratio of pathogens to faecal indicators and their varying virulence.

The total *Pseudomonas* counts (TPC) observed in the abattoir effluent (DP) was generally higher than the zero pathogen limits envisaged by WHO (32). The presumptive *Pseudomonas* load was significantly ($p < 0.05$) higher in the effluent (DP) and CP compared to US and DS, suggesting that the abattoir effluent was a major contributor of potentially pathogenic *Pseudomonas* strains to the Ikpoba River. The *Pseudomonas* loads were similar to those reported by Odjadjare et al., (14) in municipal wastewater effluents in South Africa. However, Havelaar et al., (33) reported *Pseudomonas* density in hospital wastewater relatively higher (10^4 to 10^6 CFU/100ml) than those observed in this study.

Pseudomonas spp. are regarded as emerging waterborne pathogens. They have been incriminated in a number of waterborne outbreaks including those associated with use of recreational waters (35), showers, hot tubs and swimming pools (36). Thus, discharge of the abattoir effluent under study into the Ikpoba

river portends serious public health risks to those who depend on the river water for sundry uses. It is therefore imperative for stakeholders in the public health sector to include *Pseudomonas* in the list of potential pathogens screened for in routine surveillance of abattoir waste effluents in the interest of public health.

Notwithstanding the inherent antimicrobial resistance of *Pseudomonas* spp. to many commonly deployed antibiotics, previous reports suggested that the degree of resistance to antipseudomonal agents vary considerably (37). Consistent with the observation in this study, *Pseudomonas* spp. from environmental sources were reported to be sensitive to amikacin (38,39), ceftazidime (40), gentamycin (10,14,17) and ofloxacin (14,41,42). Ceftazidime was the most effective antibiotics recorded in this study with 82% sensitivity, followed by ofloxacin (80%), amikacin (74%) and gentamycin (40%). Conversely, previous studies have reported *Pseudomonas* resistance to amikacin (37), ceftazidime (5,10,15,16), gentamycin (5,15,40) and ofloxacin (17).

In agreement with the findings of this study, pseudomonads resistance was previously reported against oxacillin and vancomycin (11, 14), as well as tetracycline (5,42). Although Igwe et al., (43) reported 20% sensitivity of *Pseudomonas* isolated from women with UTI in a tertiary hospital in Abakaliki, Nigeria, there is a general dearth of information in the literature on pseudomonads susceptibility to oxacillin. In a similar vein, there is scarcity of information on vancomycin susceptible pseudomonads, despite Agwu et al., (44) report of 52% sensitivity to the drug among isolates from delayed foot ulcers in Ekpoma, Nigeria. Lower sensitivity of *Pseudomonas* spp. to vancomycin was also previously reported in the literature (14,45). The observations suggest that these antibiotics were generally ineffective against *Pseudomonas* spp. and therefore should not be administered in the therapeutic management of *Pseudomonas* infections.

Despite the fact that oxacillin and vancomycin are known to be non-traditional anti-pseudomonas antibiotics, they were included in this study because previous reports (14,43,44,45) have documented strains of *Pseudomonas* spp sensitive to these drugs. Hence, it was necessary to test whether these observations marked an evolving trend or were probably one-off discoveries on the antibiogram of this organism. None of the isolates in the current study were sensitive to both antibiotics. However, it will be important to further investigate the biological, molecular and physiological basis of *Pseudomonas* sensitivity to these anti-

biotics if and when they are isolated in the future. Contrary to the observation of this study, Odjadjare et al., (14) reported *Pseudomonas* isolates from municipal wastewater effluents which were generally sensitive to tetracycline.

The MDR phenotypes observed in this study were similar to those reported previously (5,40,42), indicating the MDR coverage of the fluoroquinolones, cepheims, aminoglycosides and tetracyclines. However, contrary to the observation of this study, reports elsewhere on MDR phenotypes among *Pseudomonas* spp. did not involve the fluoroquinolones and aminoglycosides (10,14). Our observation suggests that abattoir effluent is a considerable source of MDR *Pseudomonas* strains, and poses risk to public health through the dissemination of community acquired antibiotic resistant bacteria. Chika et al., (15) asserted that MDR *Pseudomonas* in the community portends danger for the healthcare sector because such pathogens could spread to the hospital environment via community-acquired infections when patients report to the hospital for medical attention and become hospitalized.

The multiple antibiotics resistance observed in this study did not come as a surprise, as food animal production often apply antibiotics for therapeutic, prophylactic and growth enhancement purposes. Such practices enhance the chances of increased transfer and spread of antibiotics resistance determinants in the environment through the discharge of abattoir wastes containing unmetabolized drugs or their metabolic intermediates (17). The findings in this study revealed that untreated abattoir effluent was a considerable source of MDR *Pseudomonas* spp. among other bacteriological pollutants (e.g. HPC, coliform and *E. coli*) that could compromise the quality of the receiving river as well as the public health of riverside communities that depends on this vital water resource for their subsistence. There is therefore an urgent need to educate stakeholders involved in the slaughter house business on the importance of effluent treatment before discharge into the environment. This will help to prevent the dissemination and spread of antibiotics resistant bacteria in the community and preserve the public health.

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Copyright AJCEM 2020: <https://doi.org/10.4314/ajcem.v21i3.11>**Short Communication****Open Access****Rifampicin resistant tuberculosis among patients attending General Hospital, Kagarko, Kaduna State, Nigeria***¹Bitet D. E., ²Kumurya, S. A., ³Joseph, L., and ⁴Bathelomow, P.^{*1}General Hospital, Zango Kataf, Ministry of Health and Human Services, Kaduna State, Nigeria²Department of Medical Laboratory Science, Faculty of Allied Health Science, Bayero University Kano, Nigeria³Quality Control Unit, Directorate of Diagnostic Services, Ministry of Health, Gombe State⁴General Hospital, Kagarko, Ministry of Health and Human Services, Kaduna State, Nigeria*Correspondence to: ezekiildogobetet@gmail.com; +2348036523901**Abstract:**

Background: The National Tuberculosis and Leprosy Control Program (NTBLCP) in collaboration with Koninklijke Nederlandse Centrale Vereniging tot bestrijding der tuberculose (KNCV) (Dutch Tuberculosis Foundation) and National Agency for Control of AIDS (NACA) installed and equipped many health centres in Kaduna State, Nigeria with modern diagnostic tools (GeneXpert) to offer molecular services for the rapid detection of *Mycobacterium tuberculosis* complex (MTBC) and rifampin resistance.

Methodology: This study analyzed routine samples from patients attending General Hospital Kagarko, from September 2016 to March 2019 with total samples of 1056 from 1056 patients. The GeneXpert machine was used for the rapid detection of *M. tuberculosis* and rifampin resistance (RIF) from all the sputum samples received in the clinical laboratory department of the hospital.

Results: A total of 182 (17.2%) samples tested positive for *M. tuberculosis* out of which 5 (2.7%) were resistant to rifampicin. Males were more frequently affected with a prevalence of 23% than females with a rate of 10.7% ($\chi^2=27.801$, $p=0.0001$). RIF was detected in 3 male and 2 female patients ($p=1.000$). The prevalence of MTB was highest in the age group 36-45 years (23%) and age group 26-35 years (20.3%) and lowest in age group 5-15 years with 10.9% ($t=0.599$, $p=0.55$).

Conclusion: There is need for the GeneXpert technology to be replicated in other health centers across the state and the country at large to reduce the burden of multi-drug resistant tuberculosis (MDR-TB) in Nigeria.

Keywords: RIF resistance, MDR-TB, GeneXpert, *Mycobacterium tuberculosis*

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Tuberculose résistante à la rifampicine chez les patients de l'hôpital général de Kagarko, État de Kaduna, Nigéria*¹Bitet D. E., ²Kumurya, S. A., ³Joseph, L., et ⁴Bathelomow, P.^{*1}Hôpital général, Zango Kataf, Ministère de la santé et des services sociaux, État de Kaduna, Nigéria²Département des sciences de laboratoire médical, Faculté des sciences de la santé connexes, Université Bayero Kano, Nigéria³Unité de contrôle de la qualité, Direction des services de diagnostic, Ministère de la santé, État de Gombe Hôpital⁴Hôpital général, Kagarko, ministère de la Santé et des Services sociaux, État de Kaduna, Nigéria*Correspondance à: ezekiildogobetet@gmail.com; +2348036523901**Abstrait:**

Contexte: Le programme national de lutte contre la tuberculose et la lèpre (NTBLCP) en collaboration avec Koninklijke Nederlandse Centrale Vereniging tot bestrijding der tuberculose (KNCV) (Fondation néerlandaise pour la lutte contre la tuberculose) et l'Agence nationale de lutte contre le sida (NACA) ont installé et équipé de nombreux

centres de santé dans l'État de Kaduna , Nigeria, avec des outils de diagnostic modernes (GeneXpert) pour offrir des services moléculaires pour la détection rapide du complexe *Mycobacterium tuberculosis* (MTBC) et la résistance à la rifampicine.

Méthodologie: Cette étude a analysé des échantillons de routine de patients fréquentant l'hôpital général de Kagarko, de septembre 2016 à mars 2019, avec des échantillons totaux de 1056 sur 1056 patients. L'appareil GeneXpert a été utilisé pour la détection rapide de *M. tuberculosis* et de la résistance à la rifampicine (RIF) pour tous les échantillons d'expectorations reçus dans le service de laboratoire clinique de l'hôpital.

Résultats: Un total de 182 échantillons (17,2%) ont été testés positifs pour *M. tuberculosis*, dont 5 (2,7%) étaient résistants à la rifampicine. Les hommes étaient plus fréquemment touchés avec une prévalence de 23% que les femmes avec un taux de 10,7% ($X^2=27,801$, $p=0,0001$). Le RIF a été détecté chez 3 hommes et 2 femmes ($p=1,000$). La prévalence du VTT était la plus élevée dans le groupe d'âge 36-45 ans (23%) et le groupe d'âge 26-35 ans (20,3%) et la plus faible dans le groupe d'âge 5-15 ans avec 10,9% ($t=0,599$, $p=0,55$).

Conclusion: Il est nécessaire que la technologie GeneXpert soit reproduite dans d'autres centres de santé de l'État et du pays dans son ensemble pour réduire le fardeau de la tuberculose multirésistante (TB-MR) au Nigéria.

Mots-clés: résistance au RIF, TB-MR, GeneXpert, *Mycobacterium tuberculosis*

Introduction:

Drug-resistant tuberculosis (DR-TB) is a tuberculous disease resulting from *Mycobacterium tuberculosis* resistant to at least one first-line anti-TB drug; isoniazid (INH) and rifampicin (RIF). Drug resistant TB could be mono-resistance in which there is resistance to only one first-line anti-TB drug or poly-resistance in which there is resistance to more than one first-line anti-TB drugs other than isoniazid and rifampicin (1,2). Multidrug resistance (MDR) is defined as resistance to at least both isoniazid and rifampicin, and extensively drug resistance (XDR) as resistance to any fluoroquinolone, and at least one of three second-line injectable drugs (capreomycin, kanamycin and amikacin) in addition to multidrug resistance (2,3). Rifampicin resistance (RR) is resistance to rifampicin, detected by phenotypic or genotypic methods, with or without resistance to other anti-TB drugs, and includes mono-resistance to rifampicin (2,4).

The World Health Organization (WHO) estimates that TB kills approximately 2 million people worldwide each year, and MDR-TB is becoming an increasing public health challenge in many parts of the world, basically due to poor patient adherence to the six-month tuberculosis treatment regimen. About 5% of all TB cases are reported to be MDR-TB (2). Resistance to rifampin is one of the primary reasons for treatment failure and fatal clinical outcome in TB patients (4,5).

Treatment of drug-resistant TB is difficult and inappropriate management can lead to life-threatening outcomes (1,6). The traditional method for diagnosis of drug resistant TB is usually by susceptibility testing involving the growth of *M. tuberculosis* on liquid or solid culture medium, which takes about 2 months. Culture methods are

expensive and time consuming, thus delay opportunity for prompt treatment of patients. With the recent development of nucleic acid amplification technologies, which are rapid, sensitive, and specific, opportunity of timely, accurate and precise diagnosis of drug resistant TB is broadened (3,7). The GeneXpert assay uses real-time polymerase chain reaction (PCR) technology to simultaneously amplify and detect *M. tuberculosis*-specific sequence of the *rpoB* gene (1,6).

Rifampicin has significant early bactericidal effect on metabolically active *M. tuberculosis* and excellent terminal removal activity on low inactive micro-organisms passing through short bursts of metabolic activity. The mechanism of action of rifampicin is inhibition of mycobacterial transcription by targeting DNA dependent RNA polymerase. Resistance to rifampicin develops from mutations within the 27 codons in the well-defined 81 base pair (bp) central region of the gene (*rpoB*) that encodes the β -subunit of RNA polymerase. More than 96% of the rifampicin-resistant strains contain a mutation in this region thus facilitating a straightforward rapid approach for detecting rifampicin resistance and/or MDR (3,8,9). Therefore, the aim of this study was to determine the prevalence of rifampicin resistance in TB patients attending General Hospital, Kagarko using the GeneXpert MTB/RIF assay.

Materials and methods:

Study setting

The study was conducted in General Hospital, Kagarko in Kagarko Local Government Area (LGA) of Kaduna State, Nigeria. The laboratory department of the hospital receives samples within the hospital covering the entire LGAs and from other health facilities in neighboring LGAs.

Study population

This study population was made of patients who presented with cough of two or more weeks duration and referred to the clinical laboratory department of the hospital for sputum examination by the GeneXpert assay between November 2016 and March 2019. One sputum sample was collected per patient according to the GeneXpert sample collection guidelines, and the total number of patients was 1056.

Ethical approval

The study was approved by the Research and Ethics Committee of the Kaduna State Ministry of Health and Human services.

Laboratory analysis by GeneXpert assay

Sample reagent was added to sputum sample in a 2:1 ratio and in a 3:1 ratio to suspended sediment of decontaminated or digested sputum specimen. The lid of the sputum container was replaced, and manually shaken vigorously 10-20 times (one back and forth movement in single shake), and incubated at room temperature for 10 minutes. This was again shaken vigorously 10-20 times (or vortex) and further incubated for 5 minutes at room temperature with samples perfectly fluidics with no visible clumps of sputum at the end of incubation.

The cartridges were labeled appropriately on the left or right side with sample ID. Two ml of the prepared sputum samples were transferred to the cartridges using transfer pipette with avoidance of solid particles and aerosols or bubbles. The lids of the cartridges were firmly closed and then inserted into the test platform located in the clean room. The test platform (Cepheid) is the integrated diagnostic device that performs sample processing and simultaneous amplification and detection of fluorescence (real-time polymerase chain reaction; rt-PCR) in a single hands-free step for the identification of *M. tuberculosis* and simultaneous rapid detection

of rifampicin (RIF) resistance in the sputum specimens. The electronic results were sent directly from the MTB/RIF test system to the central database in about 120 minutes.

Statistical analysis

Data were entered into Microsoft Access (Microsoft Corp., Redmond, WA, USA) and analyzed with Microsoft Excel. Comparison of the prevalence of MTB and rifampin resistance with respect to age group and gender of patients was done with Chi square test and $p < 0.05$ was taken as significant value.

Results:

This study included 1056 patients (568 males, 488 females) with presumptive TB whose sputum samples (one sample per patient) were received and tested at the clinical laboratory department of General Hospital Kagarko, Kaduna State between September 2016 and March 2019. A total of 182 (17.2%) sputum specimens were positive for *M. tuberculosis* with the GeneXpert MTB/RIF assay including 5 (0.5%) that were rifampicin resistant, while 874 (82.7%) were negative (Table 1).

The prevalence of TB was significantly higher in the male patients with 23% (130 of 568) than the female patients with 10.7% (52/488) ($p = 0.0001$) but the rifampicin resistance rate of 0.5% (3/568) in the male was not significantly different from 0.4% (2/488) in the female patients ($p = 1.000$) (Table 1).

The prevalence of TB in relation to the age group showed that age group 36–45 years had the highest MTB detected with 23% (43/187), followed by age group 26–35 years with 20.3% (53/261), age group 46–55 years 18.4% (27/147), age group > 55 years 14.8% (27/199), age group 16–25 years 12.8% (23/180) and age group 5–15 years 11% (9/82) ($p = 0.55$) (Table 2).

Table 1: Frequency distribution of MTB detection by GeneXpert with respect to gender of patients

Gender	No tested	MTB positive (%)	MTB Rif Resist (%)	Total (%)	χ^2	p value
Male	568	127 (22.4)	3 (0.5)	130 (23)	27.801	0.0001*
Female	488	50 (10.2)	2 (0.4)	52 (10.7)		
Total	1056	177 (16.8)	5 (2.7)	182 (17.2)		

* $p < 0.05$ – statistically significant for MTB; χ^2 = Chi square

Table 2: Frequency distribution of MTB detection by GeneXpert with respect to age group of patients

Age group (years)	No tested	MTB positive (%)	t	df	CI	p value
5 – 15	82	9 (11.0)	0.599	288.9	0.159-0.299	0.55*
16 – 25	180	23 (12.8)				
26 – 35	261	53 (20.3)				
36 – 45	187	43 (23.0)				
46 – 55	147	27 (18.4)				
> 55	199	27 (13.6)				
Total	1056	182 (17.2)				

* $p > 0.05$ – not statistically significant; CI = Confidence Interval at 95%

Discussion:

MDR TB has become a serious public health challenge globally but especially in developing countries (10). The GeneXpert MTB/RIF assay has opened up opportunity for the rapid detection of MTB and resistance to primary anti-TB drugs (11). In this study, MTB was detected by the GeneXpert in 17.2% of the 1056 sputum specimens obtained from patients with presumptive TB among whom 5 (2.7%) were rifampicin resistant MTB. Majority of the MTB patients were between the age groups 36-45 (23%) and 26-35 years (20.3%), which is generally considered the active and vulnerable age group to MTB infections. Also, the prevalence rate of TB by the GeneXpert assay in males (23%) was significantly higher than in females (10.7%) ($p=0.0001$). The difference could be related to social attitude of the males which make them more exposed and vulnerable to MTB infection than the females.

Otu et al., (12) in their study reported drug resistance rate of 42% to at least one anti-TB drug on mycobacterial culture in patients with pulmonary TB in Calabar, south-south Nigeria. They found mono-resistance rate of 7% to ethambutol and 7% to streptomycin but none to rifampicin. The difference in methods of testing for rifampicin resistance does not allow for direct comparison as we used GeneXpert MTB/RIF assay in our study which is programmed to detect only rifampicin resistance. The rifampicin resistance rate of 2.7% reported in our study is lower than the 14.7% reported by Ikuabe and Ebuanyi (1) in Yenagoa using GeneXpert MTB/RIF assay.

The low rifampicin resistance rate in our study may be related to the new history of use of rifampicin in this part of the country and Africa as a continent (12) but differences in rates may also be related to level of patients'

awareness, compliance/adherence to anti-TB drugs and geographical location. Poor management of active pulmonary TB is a known factor for emergence of drug resistant TB, which could be aided by several factors such as poor prescribing practices with insufficient treatment duration and poor drug compliance by patients (13). Other factors include poor public health awareness and financial resources, shortages in drug supplies to directly observed treatment (DOT) centres and lack of proper health education of patients on the medication (11, 13). Rifampicin resistance rate in our study was not associated with gender and age group as the rate of 0.5% in the males was not significantly different from the rate of 0.4% in the females ($p=1.000$), however, this result should be interpreted with caution because of the small number of patients with RIF in this study.

Conventional laboratory techniques such as microscopy for detection of acid-fast bacilli (AFB) in sputum samples for the diagnosis of tuberculosis are far from being sensitive. Moreover, mycobacterial cultures are time-consuming, require biosafety measures, and need trained laboratory personnel. In our study, the GeneXpert assay used have been reported to be highly sensitive and specific (over 97%) for TB diagnosis from pulmonary specimens, and generate results rapidly, therefore reducing the turn-around time for TB diagnosis (14). The GeneXpert assay also targets the region of rifampicin resistance associated *rpoB* gene by real-time polymerase chain reaction (rt-PCR) with three specific primers that simultaneously detect *M. tuberculosis* DNA and rifampicin resistance. The processes of bacterial lyses, DNA extraction, real time amplification and amplicon detection are automated, which give room for less interference and contamination.

Conclusion:

The prevalence of MDR-TB as determined by rifampicin resistance rate of 2.7% in our study emphasizes the need to make the GeneXpert MTB/RIF machines widely available to facilitate rapid detection of MDR-TB in Nigeria.

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CORRIGENDUM

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Corrigendum

Prevalence and factors associated with *Helicobacter pylori* infection among treatment naïve dyspeptic adults in University of Benin Teaching Hospital, Benin City, Nigeria

¹Odigie, A. N., ^{*2}Adewole, A. J., and ²Ekunwe, A. A.

¹Edo State Hospital Management Board, Benin City, Nigeria

²Department of Family Medicine, University of Benin Teaching Hospital, PMB 1111, Benin City, Nigeria

*Correspondence to: afolabi.adewole74@gmail.com; +2348037222755

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In their published article, the authors detected errors with the initials and address of the first author. These errors have been corrected as appropriate.

Prévalence et facteurs associés à l'infection à *Helicobacter pylori* chez les adultes dyspeptiques naïfs de traitement à l'hôpital universitaire de Bénin, Benin City, Nigéria

¹Odigie, A. N. ^{*2}Adewole, A. J., et ²Ekunwe, A. A.

¹Conseil de gestion de l'hôpital d'État d'Edo, Benin City, Nigéria

²Département de médecine familiale, hôpital universitaire de Bénin, PMB 1111, Benin City, Nigéria

*Correspondance à: afolabi.adewole74@gmail.com; +2348037222755

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Dans leur article publié, les auteurs ont détecté des erreurs avec les initiales et l'adresse du premier auteur. Ces erreurs ont été corrigées le cas échéant.



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