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Department of Medical Microbiology,
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PREVALENCE OF *ESCHERICHIA COLI* VIRULENCE GENES IN PATIENTS WITH DIARRHOEA IN OUAGADOUGOU, BURKINA FASO

Somda[1,2],N. S., Bonkoungou[2,3],O. J. I., Zongo[1], C., Kpoda[2], D. S., Tapsoba[1], F., Bassolé[3], I. H. N., Traoré[1], Y. & Savadogo[1] A.

[1]Laboratoire de Biochimie et d'Immunologie Appliquée (LABIA). UFR en Sciences de la vie et de la terre, Ecole Doctorale Sciences et Technologies, Université Ouaga I Pr Joseph Ki-Zerbo, 03 BP 7021 Ouagadougou 03, Burkina Faso. [2]Laboratoire National de Santé Publique (LNSP), 09 BP 24 Ouagadougou 09, Burkina Faso.[3]Laboratoire de Biologie Moléculaire d'Epidémiologie et de Surveillance desagents; * Transmissibles par les Aliments (LaBESTA), UFR en Sciences de la vie et de la terre, Ecole Doctorale Sciences et Technologies, Université Ouaga I Pr Joseph Ki-Zerbo, 03 BP 7021 Ouagadougou 03, Burkina Faso.

Correspondence: Prof SAVADOGO Aly, E-mail: alysavadogo@gmail.com

ABSTRACT

Objective: **Diarrhoeagenic *E. coli* (DEC) strains are important causes of diarrhoea in the developing world and, to a lesser extent, inthe developed world. In this study, we investigated the prevalence of the virulence genes specific for five major pathogroups of diarrheagenic *Escherichia coli* (DEC) in primary cultures from diarrhoeagenic patients in Burkina Faso.*Methodology:* From September 2016 to Mars 2017, a total of 211 faecal samples from diarrhoeagenic patients from urban hospitals of Ouagadou, Burkina Faso have been analysed. A 16-plex PCR was used to detect simultaneously, the five major DEC pathotypes (enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC)).**

Results: **At least one diarrhoeagenic *E. Coli* pathotype was detected in 31 samples (14.7%) in children and adults with diarrhoea. EAEC was the most common pathotype detected 9.5% (20/211), followed by EIEC2.4% (05/211) and STEC 0.5% (01/211). More than one DEC pathotype were detected in 2.4% (05/211) patients. EPEC and ETEC were not detected in single infection but in co-infection with others pathotypes.**

Conclusion: **DEC, especially enteroaggregative, may be important responsible of diarrhoeas in Burkina Faso from all ages patient.**

Key Words: Diarrhoeagenic *Escherichia coli*, 16-plex PCR, Burkina Faso, human diarrhoeas stool.

PREVALENCE DES GENES DE VIRULENCE D'*ESCHERICHIA COLI*ISOLEES DES SELLES DIARRHEIQUES CHEZ LES PATIENTS A OUAGADOUGOU, AU BURKINA FASO.

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Correspondance : Prof SAVADOGO Aly, E-mail: alysavadogo@gmail.com

RESUME

Objectifs: Les *E. coli* diarrhéiques (ECD) sont la cause des diarrhées chez les enfants comme chez les adultes dans les pays en développement. Dans cette étude nous avions pour objectif d'évaluer la présence des cinq principaux pathogroups ECD, leur association avec la diarrhée chez les enfants et les adultes au Burkina Faso.

Méthodologie: De Septembre 2016 à Mars 2017, un total de 211 échantillons de selles diarrhéiques a été recueilli chez des patients dans 2 centres de santé à Ouagadougou. La PCR 16-plex a été utilisée pour détecter la présence de *E. Coli* enteroagrégrégative (ECEA), *E. Coli* enteropathogène (ECEP), *E. Coli* enterotoxigénique (ECET), *E. Coli* entérohémorragique (ECEH) et*E. Coli* enteroinvasive (ECEI).

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Résultats: Au moins un pathovarde *E. coli* diarrhéique a été détecté dans 31 échantillons (14,7%) chez les enfants et les adultes atteints de diarrhée. ECEA était le pathhotype le plus fréquent détecté à 9,5% (20/211), suivi de ECEIà 2,4% (05/211) et en fin de ECSTà 0,5% (01/211). ECEP et ECET n'ont pas été détectées en elles seules mais elles ont été détectées en co-infection avec d'autres pathotypes.

Conclusion:ECD, en particulier *E. coli*entero-aggregatif, pourrait être le plus redouté des agents responsables des diarrhées chez les personnes de tous âges au Burkina Faso.

Mots clés : ***Escherichia coli*** **Diarrhéique, PCR 16-plex, Burkina Faso, selles diarrhéiques.**

INTRODUCTION

Diarrhoea remains a leading cause of mortality and morbidity worldwide, particularly in developing countries [1]. The aetiological agents include a wide range of viruses, bacteria and parasites [2]. Among bacterial pathogens, diarrhoeagenic*Escherichia coli* (DEC) is an important agent of endemic and epidemic diarrhoea worldwide [3]. *Escherichia coli (E. coli)* is a heterogeneous group of typically non-pathogenic gram-negative bacteria, which are a natural partof the intestinal flora of animals and humans [4, 5]. However,the pathogenic strains are associated with several diseases,including diarrhoea, urinary tract infections and meningitis[6]. Diarrhoeagenic*E. coli* (DEC) strains are important causesof diarrhoea in the developing world and, to a lesser extent, inthe developed world [3].Diarrheal illness causes much mortality worldwide, particularly in children under the age of 5 [7]and particularly in countries in sub-Saharan Africa and South Asia, where children suffer many diarrhoea-related deaths. While there are many etiological agents responsible for diarrhoea, pathogenic *E. coli* is major contributor [8, 9].Diarrheal disease morbidity in children living in underserved countries is a leading cause of mortality andmorbidity in children living in underserved countries [9]. Close to 2 millions of children below 5 years of age are estimated to die every year because of diarrheal diseases[1].*E. coli* strains involved in intestinal infections in humans are classified into six (06) pathotypes:enteropathogenic*E. coli* (EPEC), *Shiga toxin-producingE. coli* (STEC) (e.g., *enterohemorrhagicE. coli* [EHEC]), *Shigella/enteroinvasiveE. coli* (EIEC), *enteroaggregativeE. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and *enterotoxigenicE. coli* (ETEC). Globally, EPEC are most frequently causeof infantilediarrhoea.STEC is much more present from ruminant animals and in the environment. Different plants consumed by humans may be contaminated by of STEC, either by fertilization from contaminated animals, or when contaminated water is used for irrigation.EAEC were most responsible to traveller's diarrhoea. To date, only one report on DEC rotavirus detected from children with and without diarrhoea in urban and rural Burkina Faso has been published [2]. In this study, we investigated the prevalence of the virulence genes specific for five major pathogroups of DEC, theirs association with diarrhoea from both children and adults in Burkina Faso.

MATERIAL AND METHODS

Samples collection: From September 2016 to Mars 2017, 211diarrhoeas stools samples were collected in 2 health centers of Ouagadougou:Laboratoire National de Santé Pulique (LNSP) and Hopital du District de Bogodogo (HDB). These samples compound 194 to LNSP and 17to HDB. The samples were transported to the laboratory and kept at 4°C until the microbiological examination.All microbiological and molecular tests were carried out at LNSP Ouagadougou, Burkina Faso.

Cultivation samples: The stool samples were cultured onto sorbitol MacConkay agar and incubated at 37° C overnight. Bacterial mass on the sorbitol MacConkey agar plates was collected and stored for further analysis at -30°C in tubes with 1 mL brain-heart infusion broth containing 15% (volume/volume) glycerol.

16-plex PCR assay:The presence of STEC, EPEC, ETEC, EIEC and EAEC in human diarrhoeal stoolssamples was detected by 16-plex PCR for the genes *uidA, pic, bfp, invE, hlyA, elt, ent, escV, eaeA, ipaH, aggR, stx1, stx2, estIa, estIb*and *astA*. The primers and PCR conditions were as previously described [10]. The nucleotide sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used in this study are shown in **Table 1.** The following criteria for identification of *E.coli*pathogroups were used: for STEC, the presence of *stx1* and/or *stx2* and possibly *eaeA, escV, ent* and EHEC-*hly*; for EPEC the presence of *eaeA* and possibly *escV, ent* and *bfpB* (the absence of *bfpB* indicated a EPEC); for ETEC, the presence of *elt* and/or *estIa*or *estIb*; for EIEC, the presence of *invE*and *ipaH*; for EAEC, the presence of *pic* and/or *aggR*.

For DNA extraction a loop ful of bacterial growth was taken from the first streaking area of the plate. It was suspended into 250 µl of sterile water in an Eppendorf tube, boiled at 100 °C for 10 min, and centrifuged.

PCR was performed in a reaction of 20 µl containing 2.5µl 10 X PCR buffer (SolisBiodyne), 0.75µl dNTPs (10mM), 0.25 µl MgCl2 (50 mM), 0.2µl Taq DNA polymerase (5 U/µl), 0.5µl of an each

mixture of the 16 primer pairs at the concentrations listed in Table1, 12.8 µl of PCR-grade water and 2.5 µl of DNA sample was added to bring the final volume to 10µl. The cycling conditions used in the thermal cycler (Applied Biosystem, 2720 thermal cycler, Singapore) were 98 °C for 30 s, 35 cycles of 98 °C for 30 s, 63 °C for 60 s, 72 °C for 90 s with a final extension at 72°C for 10 min.

The amplified PCR products were separated by agarose gel (2% w/v) electrophoresis and visualized under UV light (Bioblock Scientific, Illkirch, CEDEX) after staining with ethidium bromide. Reference strains RHE 4283 (E 2348/69) for EPEC, FE94725 (Burkina Faso, beef) for ETEC, FE102301 (Burkina Faso, beef) for STEC, RHE 6647 (145-46-215, Statens Serum Institute [SSI], Copenhagen, Denmark) for EIEC, IH 56822 (patient isolate [11], for EAEC, and FE95562 (Burkina Faso, beef) for STEC-ETEC were included in each PCR run. All the 16-plex PCR positive results were confirmed by single PCRs.

Statistical analysis

Excel and the ANOVA tests or the chi-square test were used to determine the statistical significance of the data. A value of $p<0.05$ indicated statistical significance.

Ethical considerations

Permission to conduct this study was obtained from the hospital authorities of Burkina Faso and informed verbal consent was obtained from adults' patients and the parents/guardians of every child before taking the stool samples.

RESULTS

Clinical specimens

A total 211 diarrhoeas stools sample whose 194 to LNSP and 17 to HDB were collected during September 2016 to march 2017 from two big hospital centers in Ouagadougou, Burkina Faso. This collection concerned only children under five years to HDB and all ages to LNSP. One hundred and ten (110) stool samples (52.1%) were collected from males and 101 (47.9%) from female. Forty four percent (93/211) were children under five years, 15% (32/211) were young people to 6 years to 18 years, and40.8% (86/211) were adult patients over 19 years of age.

TABLE 1: OLIGONUCLEOTIDE PRIMERS USED FOR DETECTION OF THE VIRULENCE GENES

Pathovars	Genes	Sequences	bp	Concentration	reference
STEC, EPEC	*eae*-F	TCAATGCAGTTCCGTTATCAGTT	482	0.1	[12]
	eae-R	GTAAAGTCCGTTACCCCAACCTG		0.1	
	escV-F	ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	[13]
	escV-R	CGTCCCCTTTTACAAACTTCATCGC		0.4	
	ent-F	TGGGCTAAAAGAAGACACACTG	629	0.4	[13]
	ent-R	CAAGCATCCTGATTATCTCACC		0.4	
Typical EPEC	*bfpB*-F	GACACCTCATTGCTGAAGTCG	910	0.1	[13]
	bfpB-R	CCAGAACACCTCCGTTATGC		0.1	
STEC	*hlyEHEC*-F	TTCTGGGAAACAGTGACGCACATA	688	0.1	[10]
	hlyEHEC-R	TCACCGATCTTCTCATCCCAATG		0.1	
	stx1A-F	CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	[13]
	stx1A-R	AATGCCACGCTTCCCAGAATTG		0.2	
	stx2A-F	GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	[13]
	stx2A-R	AGCGTAAGGCTTCTGCTGTGAC		0.4	
EIEC	*ipaH*-F	GAAAACCCTCCTGGTCCATCAGG	437	0.1	[10]
	ipaH-R	GCCGGTCAGCCACCCTCTGAGAGTAC		0.1	[14]
	invE-F	CGATAGATGGCGAGAAATTATATCCCG	766	0.2	[13]
	invE-R	CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
EAEC	*aggR*-F	ACGCAGAGTTGCCTGATAAAG	400	0.2	[13]
	aggR-R	AATACAGAATCGTCAGCATCAGC		0.2	
	pic-F	AGCCGTTTCCGCAGAAGCC	1111	0.2	[13]
	pic-R	AAATGTCAGTGAACCGACGATTGG		0.2	
	astA-F	TGCCATCAACACAGTATATCCG	102	0.4	[13]
	astA-R	ACGGCTTTGTAGTCCTTCCAT		0.4	
ETEC	*LT*-F	GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	[13]
	LT-R	CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
	STIa-F	CCTCTTTTAGYCAGACARCTGAATCASTTG	157	0.4	[13]
	STIa-R	CAGGCAGGATTACAACAAAGTTCACAG		0.4	
	STI-F	TGTCTTTTTCACCTTTCGCTC	171	0.2	[13]
	STI-R	CGGTACAAGCAGGATTACAACAC		0.2	
E. coli	*uidA*-F	ATGCCAGTCCAGCGTTTTTGC	1487	0.2	[13]
	uidA-R	AAAGTGTGGGTCAATAATCAGGAAGTG		0.2	

STEC= Shiga toxin-producing *E. coli*; EPEC= enteropathogenic*E. coli*; EIEC= enteroinvasive*E. coli*; EAEC= enteroaggregative*E. coli*; ETEC= enterotoxigenic*E. coli*.

Detection of DEC Multi-plex PCR (16-plex PCR) was used to detect virulence genes carried by diarrheagenic*E. coli* and to classify the strains as STEC, EPEC, ETEC, EIEC, or EAEC. At least one diarrhoeagenic*E. coli*pathotype was detected in 31 samples (14.7%) in patientswhose 14% (27/194) at LNSP and 23.5% (04/17) at HDB.Globally DEC pathogroups were detected to 14.7% (31/211) including 11.8% (13/110) from male and 17.8% (18/101) from female.EAEC was the most common pathotype detected 9.5% (20/211), followed by EIEC2.4% (05/211) and STEC 0.5% (01/211) (**Table 2 and figure 1**). EPEC and ETEC were not detected in single infection but in co-infection with others pathotypes. One EPEC isolates was typical as EPEC isolates was only positive for *bfpA*and negative for *eae*. This EPEC was co-infection with EAEC.No atypical EPEC were detectedfor all stool samples analysed. Five DEC co-infections were detected to all stools samples analysed (**Table 2**). Considering the different ages groups, DEC pathogroups were detected in 18.9% (07/37) from children under five years, whose 8.1% (03/37) EAEC, 8.1% (03/37) EAEC and one co-infection to EAEC + ETEC (**figure 2**).One or more DEC pathogroups were detected in (6.2%) 12/194 patient more than five years all from LNSP. Therewere significant differences in DEC prevalence betweenchildren under five years and adults ($p<0.0001$).

TABLE 2: DETECTION OF DIARRHOEAGENIC *ESCHERICHIA COLI* PATHOVARS FROM CHILDREN AND ADULTS WITH DIARRHOEA IN OUAGADOUGOU, BURKINA FASO

Pathovars/aeras/sex	LNSP n=194 (%)	HDB n=17 (%)	M n=110 (%)	F n=101 (%)	Total n=211 (%)
EAEC	20 (10.3)	00	08 (7.3)	12 (11.9)	20 (9.5)
EIEC	02 (01.3)	03 (17.6)	03 (2.3)	02 (1.9)	05 (2.4)
STEC	01 (0.5)	00	00	01 (1.0)	01 (0.5)
EAEC + EIEC	01 (0.5)	00	01 (0.9)	00	01 (0.5)
EAEC + ETEC	00	01 (5.9)	00	01 (1.0)	01 (0.5)
EEAC + STEC	02 (01.0)	00	01 (1.0)	01 (0.9)	02 (0.9)
EAEC + t-EPEC	01 (0.5)	00	00	01 (1.0)	01 (0.5)
Total	27 (14.0)	04 (23.5)	13 (11.8)	18 (17.8)	31 (14.7)

STEC= Shiga toxin-producing*E. coli*; EPEC= enteropathogenic*E. coli*; EIEC= enteroinvasive*E. coli*; t-EPEC= typical-enteropathogenic*E. coli*; ETEC= enterotoxigenic*E. coli*, LNSP= Laboratoire National de Santé Publique, HDB= Hôpital de District de Bogodogo, % percentage, n= number, M= males, F= females

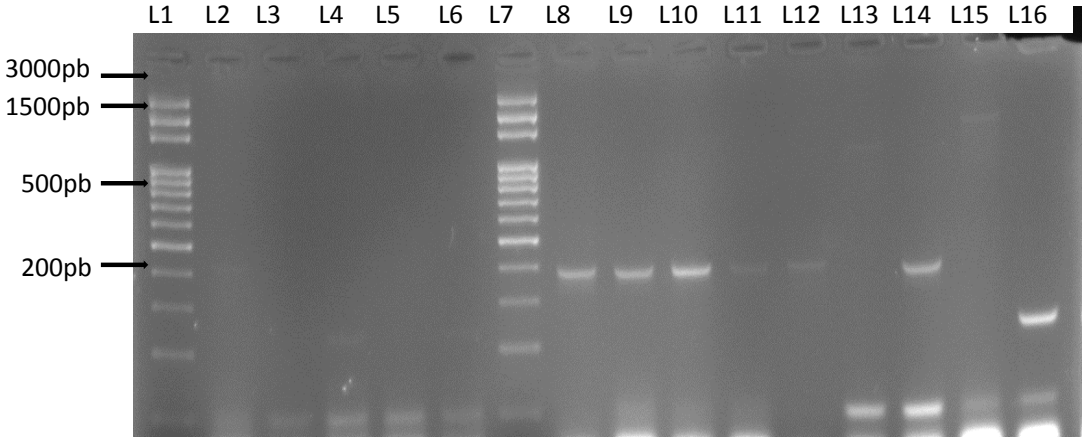

FIGURE 1:EXAMPLE OF THE 16-PLEX PCR RESULTS TO DEC ISOLATED FROM HUMAN DIARRHEAS STOOLS SAMPLES IN LNSP AND HDB.L1=marker, L2= RHE6647 (EIEC), L3=IHE56822 (EAEC), L4=FE102301 (STEC), L5=FE94725 (ETEC), L6=FE95562 (STEC-ETEC), L7=marker, L8=CpI-73, L9=Cp349, L10=Cp354, L11=Cp393, L12=Cp419, L13=Cp435, L14=CpI-99, L15=CpI-02, L16 =Cp63

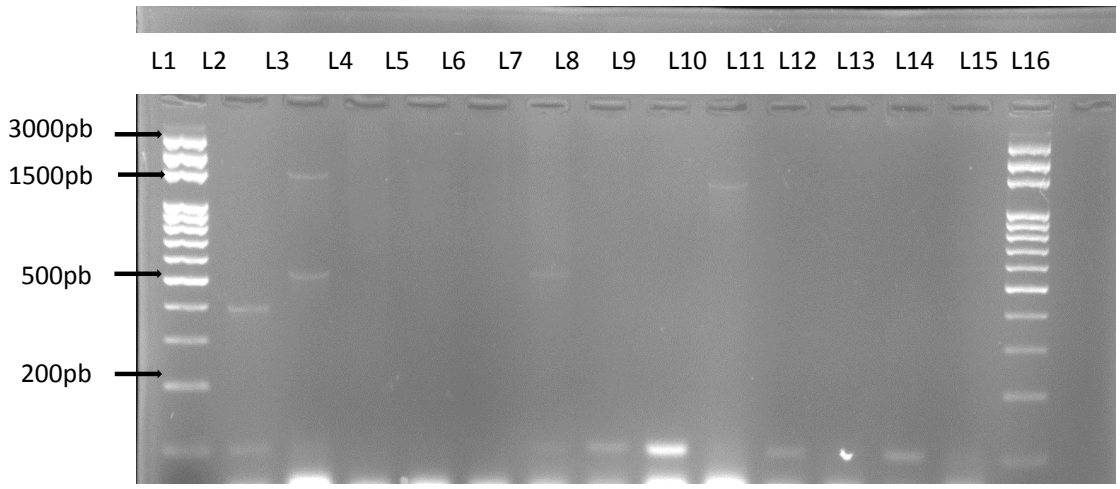

FIGURE 2: EXAMPLE OF THE 16-PLEX PCR RESULTS TO DEC ISOLATED FROM HUMAN DIARRHEAS STOOLS SAMPLES (FOLLOWING) L1=marker, L2= HDB535, L3= HDB536, L4=HDB540, L5=HDB541, L6=Cp88, L7=marker, L8=Cp91, L9=Cp95, L10=Cp96, L11=Cp111, L12=Cp113, L13=Cp120, L14=Cp121, L15=marker,L16 =Water (negative control).

DISCUSSION

Diarrhoea is an important cause of mortality and morbidity in different areas of the world (developed and developing countries) and among all age groups [15].The epidemiology of enteric pathogens that cause diarrhoeasuggests that most infections are acquired from food, water,and hand contact and many diarrheal diseases can be prevented by simple rules of personal hygiene and safer foodpreparation[15, 16].

In this study, from all stool samples analysed; DEC were detected in 14.7 %of cases, of which 8.5 % were from children under five yearsand 6.2 % from adults. These results were similar with others study's results in Africa and Brasilia [17, 18, 19], however in 2012 Bonkoungou*et al.* [2] have detected 45% of DEC from diarrheal stool samples from children under five years in urban and rural hospital centers in Burkina Faso.Our studyshowed that different pathovars DEC were more found to children under five years than adults patients.This could be explained by the fact that in children under five years old the immune system is much more fragile whereas *E. coli* leaves the flora in adults. Diarrheal diseases caused by DEC are most often linked to the hygienic conditions of the environment in which we live. Indeed, these results indicate a change in the respect of good hygiene practices in the population. Moreover, it can be said that the objectives set by the hygiene services on raising public awareness of good hygiene practices are half achieved. Nonetheless, sensitization must continuate to improve hygiene in water supplies and kitchens, and better sanitation is necessary to reduce the incidence of gastro-enteric infections, including DEC infections in Burkina Faso.Moreover, with the epidemic of Ebola in West Africa, populations have incorporated hand washing systems or systematic disinfection of the hands. That was not the case in 2012.

EAEC is an emerging pathogen associated with diarrhoea. It has been identified in travellers, children in the developing world and human immunodeficiency virusinfected patients with diarrhoea[20, 21]. In the present study, EAEC strains were the most frequently isolated pathotype of *E. Coli*confirming earlier reports of EAEC's role as a human pathogen [21, 22].This is a strong indication that this pathotype occur widely in the hospital center in Burkina Faso.Studies conducted in many countries havedemonstrated the importance of EAEC in hospital centers. This might be due to the poor hygienicpractices and very low standards of living indeveloping countries, and it implies that improvementsin sanitary conditions and water quality can beeffective control measures.

STEC are mostly associated with outbreaks and two important diseases, including haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) [23]. Cattle are perceived as their primary reservoir and are the major source of food contamination [24, 25]. In this study low frequency of STEC and EIEC (one only) was detected, but co-infections was found with EAEC. We can hypothesize that our strains (diarrheal stool samples) analysedwere not related tohaemorrhagic colitis and haemolytic uremic syndrome. Similar results were found in sub-Saharan Africa and others countries [2, 26, 27, 28].

ETEC causes a significant number of cases of childhooddiarrhoea and gastroenteritis among travellers. ETEC were also the most common agent of traveller'sdiarrhoea with food and water implicated as the modes of transmission [3]. In our study, we did not found ETEC but co-infections was found with EAEC. However Bonkoungou*et al*. in Burkina Faso showed the presence of ETEC in the children with diarrhoea. ETEC were detected on important proportion in others study conducted in sub-Saharan Africa countries [3, 5, 9, 19].

EPEC is also a very important pathogen in children with diarrhoea. EPEC infection is primarily a disease of infants younger than 2 years of age. Numerous case–control studies in many countries have found that EPEC is more frequently isolated from children with diarrhoea than from healthy controls [2, 3].However, in our study, the detection rate of EPEC strains from adults with diarrhoea was low (only one typical EPEC) and it co-infection with EAEC. In this study no EPEC was detected from children under five years.

The subjectsemployed in this study may be infected by otherpathogens other than diarrhoeagenic*E. coli*since there are different pathogens that cancausediarrhoea mostly in children, including Rotavirus,*Salmonella* spp., *Shigella*spp., *Campylobacterjejuni*, *Entamoeba histolytica,* and *Giardia lamblia*[5].

ConclusionThe multiplex PCR assay used in our study amplifying 16 virulence genes in a single reaction, is a practical and fast tool for DEC identification in routine clinical diagnostic and epidemiological studies.This study showed the diversity of *E. coli* virulence gene expression in two big hospital centers to Burkina Faso. It suggests that DEC is an importantdiarrhegenic agent and should be considered in routinestudies and surveillance for childhood acute diarrhealdisease.Hand washing with soap and good hygienic practices in the families and public places could contribute to decrease DEC infection.

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ORIGINAL ARTICLE

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THE BACTERIA PROFILES OF WOUNDS IN DIABETIC PATIENTS HOSPITALIZED IN NORTHERN KWAZULU-NATAL, SOUTH AFRICA

Mthembu[1,2], W., Penduka*[1], D., Mosa[1], R.,Shoba, B., Zobolo[2], A. and Opoku[1], A

[1]Department of Biochemistry and Microbiology; [2]Department of Botany, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, South Africa. [3]NgwelezanaHospital,Thandisiwe Road,Kuleka,Empangeni,3880, South Africa.

*** Correspondence: Wendy Mthembu, Department of Biochemistry and Microbiology, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, South Africa.mthembuwendy49@gmail.com**

ABSTRACT

Diabetic wound infections still remain a health concern such that correct identification of bacteria is essential in monitoring the spread of the infections as well as in the administration of the correct treatment. This study therefore focuses on isolating and identifying bacteria present in diabetic wounds of hospitalized patients in northern KwaZulu-Natal and assessing their distribution.The wound specimen were collected and swabbed onto selective and differential media. The bacteria identities were presumptively ascertained through biochemical characterization (Gram-stain, catalase test, oxidase test and API) and then confirmed through 16S rDNA sequencing.A total of 42 isolates were recovered from 83% of the patients sampled from the three participating hospitals (X, Y, and Z). Gram-negative bacilli from *Enterobacteriaceae*were predominant followed by *Staphylococci spp* and *Enterococcus faecalis*with 43% polymicrobial cases from hospital Z and 29% from hospital X. Distribution of some opportunistic pathogens and nosocomially-acquired pathogens were also observed across the patients with five bacterial identities distributed among hospital X and Z. The adverse effects associated with the recovered bacteria in diabetic wounds pose a serious health concern and preventive measure should be taken.

Keywords: Diabetes mellitus, wounds, bacteria, infection

LES BACTÉRIES ADULTE DES PLAIES CHEZ LES PATIENTS DIABÉTIQUES HOSPITALISÉS DANS LE NORD DU KWAZULU-NATAL, AFRIQUE DU SUD

Mthembu[1,2], W., Penduka*[1], D., Mosa[1], R.,Shoba, B., Zobolo[2], A. and Opoku[1], A

1Département de biochimie et de microbiologie ; 2Département de botanique de l'Université du Zululand, Private Bag X1001, KwaDlangezwa 3886, Afrique du Sud. NgwelezanaHospital,Thandisiwe Road, Kuleka, Empangeni, 3880, South Africa.

*** Auteur correspondant:Wendy Mthembu, Département de biochimie et de microbiologie, de l'Université du Zululand, Private Bag X1001, KwaDlangezwa 3886, Afrique du Sud.mthembuwendy49@gmail.com**

Résumé

Les infections de plaiesdiabétiquesdemeurent un problème de santé telsquel'identificationcorrecte des bactériesestessentielledans la surveillance de la propagation des infections ainsiquedansl'administration de l'untraitement correct. Cetteétudeportedoncsurl'isolation et l'identification des bactériesprésentesdans les plaiesdiabétiques de patients hospitalisésdans le Nord du KwaZulu-Natal et l'évaluation de leur distribution. Spécimen de la blessureontétérecueilliesetfrottéesur des médias et du différentiel. Les bactériesontétéidentitésprésuméesrévélée par caractérisationbiochimique (coloration de Gram, catalase, oxydaseet test test API) et ensuiteconfirmé par séquençage de l'ADNR 16s. Un total de 42 isolatsontétéretrouvésdans 83 % des patients échantillonnésdans les troishôpitaux participants (X, Y et Z). Les bacilles à Gram négatifd'Enterobacteriaceaeétaientprédominantessuivies par les staphylocoques et spp Enterococcus faecalis avec 43 % des cas de l'hôpitalpolymicrobial Z et de 29 % de l'hôpital X. La répartition de certainspathogènesopportunistes et nosocomially-pathogènesacquisontétéégalementobservésdans les patients avec cinqidentitésbactériennerépartis entre l'Hôpital X et Z. Les effetsindésirablesassociés à la récupération de bactériesdans les plaiesdiabétiquesposent un grave problème de santé et de préventiondoiventêtreprises.

Mots-clés: diabètesucré, blessures, les bactéries, les infections

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INTRODUCTION

Literature abounds with reports of bacterial flora on human skin [1, 2], this predispose patients to an increased risk of being infected by bacteria that are free-living on the skin [3]. However, the type and quantity of the microorganisms serves as an indication of the wound infection [4]. Diabetic wound infection is one of the main chronic complication of diabetes with life-threatening adverse effects in healthcare [3, 5].The increased blood glucose impairs the blood flow, leukocyte function, and chemotaxis of the neutrophils and macrophages [3, 6]. Other factors such as surgical procedures, hospitalization and prolonged antibiotic therapy may predispose patients to infection [7]. Infection is driven by the pathogenicity and virulence of the bacteria [7-9], as some bacteria become more virulent in the presence of high glucose [8]. Diabetic wound infections are normally polymicrobial [9], and this can further compromise the host cell function [4].

Accurate identification of polymicrobial bacterial species present in the wound siteis important in determining the cause and predicting the outcome of an infection [10]. Routine analysis of wound specimen normally involves the use of traditional culture methods such as selective and differential agar media to culture the anaerobic and aerobic bacteria [4]. The organisms are classified by means of similarities and differences based on their phenotypic characteristics such as cell appearance, cell shape, size, pigmentation [11-13]. Gram staining, biochemical tests (catalase and oxidase) and controlled growth conditions are required for definitive grouping of bacteria [12]. Biochemical tests demonstrate the ability of test organisms to degrade specific substrates such as carbohydrates, amino acids, and other organic molecules. Other biochemical tests involve the ability of an organism to grow in the presence of a single nutrient source [13]. The major role played by routine analysis of bacteria in wound care is the appropriate use of antimicrobial agents however, it is essential to correctly identify the microbes to help eliminate healthcare burdens [14].

It has become more difficult to identify polymicrobial bacterial species present in an infection through culture methods [10]. However, with the aid of molecular diagnostic techniques, identification has shown that most chronic wounds are polymicrobial [11]. Culture-based techniques alone often fail to identify fastidious bacteria that are important in diagnosis and they may underestimate microbial diversity [11] while culture-independent methods are able to detect bacterial species that were omitted by culture-based techniques [16]. The ability to characterize bacteria using 16S ribosomal RNA (rRNA)-based phylogenies has enabled a much faster way to identify bacteria and elucidate the role of bacterial pathogens in the development of infectious diseases [16]. The 16S rDNA sequencing surveys only a portion of the microbial genome that encodes the 16S rRNA subunit [17]. This molecular technique determines the nucleotide sequence of ribosomal RNA from various bacteria in order to assess their relative position in the evolutionary order [18], thereby grouping bacterial isolates into taxonomic and phylogenetic groups based on their genetic composition [17]. The significance of 16S rRNA is that it is present in all prokaryotic cells with conserved and variable sequence regions evolving at different rates therefore making it suitable for bacteria identification [19].

The assessment of the bacteria present in wounds is essential, it provides antibiotic therapy guide that can help manage and prevent amputations thereby improving the quality of life [20].Tothe best of our knowledge, South Africa (and indeed the Northern KwaZulu-Natal region) has been minimally represented in similar studies. It is hoped that this study will provide the necessary and essential information in this particular field.

MATERIALS AND METHODS

Specimen Collection

This study was carried out after the approval (UZREC 171110-030 PGM 2015/195) from the ethical committee of the University of Zululand was obtained. The full cooperation of the patients was duly obtained. The wound specimens of 18 hospitalized diabetic patients (diagnosed by medical doctors to be diabetic; 22% male and 78% female) were collected from three different rural-based Northern KZN hospitals in 2015-2016. Hospital X is a district healthcare facility which provides services to the rural community while Hospital Y is a district healthcare center that provides health care service to even some neighboring healthcare institutions and Hospital Z is a regional hospital, providing healthcare service that are of high safety standards and cost effective. The demographic data of patients such as age, gender, and ethnic group were recorded prior to sampling. The medical doctors were responsible for swabbing the wounds after washing them with sterile saline and sterile cotton pads. Sterile swabs were introduced at the base of the wound and then subsequently inserted in Amies transport media to maintain the specimen during transportation to the University of Zululand's biochemistry laboratory [5].

Specimen Isolation

The spread plate method described by Ørskov[21] was used to inoculate the specimen from the swabs

onto the primary media containing plates namely nutrient agar, mannitol salt agar and MacConkey agar exclusively. The plates were incubated at 37°C for 24-48hours, after which successive quadrant streak technique was used to purify the colonies. Pure colonies were kept on nutrient agar plates at 4°C and glycerol stocks at -80°C [5].

Identification of the Isolated Bacteria

Isolates were primarily identified using Gram-staining, [22] morphological characterization (colony shape, size, pigmentation)according to the methods of [13]. Standard biochemical tests such as catalase [23], oxidase [24] were carried out followed by the presumptive identification of bacteria using Analytical Profile Index (API) test kits namely; API 20 Staph, API 20 Strep, API 20E, API 20NE according to the manufacturer's instructions (Biomerieux S.A). The confirmation of the bacteria identities was done using PCR by amplifying the 16S rDNA and analyzing the sequenced products through BLAST Search (NCBI) [25]. Universal PCR primer sequences were used (table 1).

TABLE 1: 16S PRIMERS SEQUENCES

Name of Primers	Target	Sequence (5′ to 3′)
16S-27F	**16S rDNA sequence**	**AGAGTTTGATCMTGGCTCAG**
16S-1492R	**16S rDNA sequence**	**CGGTTACCTTGTTACGACTT**

Data Analysis

Variants were analysed using Graphpad prism version 6, determining the one way ANOVA, two way ANOVA, means and standard deviations.

RESULTS

Data Collection

A Total of 7 patients from hospital X, 4 patients from hospital Y and 7 patients from Hospital Z participated in the study. The classification of the patients sampled is asshown in table 2.

TABLE 2: GENERAL CLASSIFICATION OF THE PATIENTS

Variables	**Mean (%)**
Age (years)	**66.6**
Male gender	**22.2**
Female gender	**77.8**
Wound site (Lower limb)	**94.4**
(Other body parts)	**5.6**

Isolation and Presumptive identification

A total of 42 isolates were recovered from 15 (83%) patients; no isolates were obtained from the wounds of 3 (17%) patients. Fifteen, six and 21 isolates were recovered from hospital X (36%), hospital Y(14%) and hospital Z(50%) respectively. Figure 1. Shows the overall distribution pattern of the isolates from the three hospitals

The isolates classified according to their microscopic morphology during the Gram-staining (figure 2) revealed that Hospital Y had more bacilli (83%) isolates compared to the other hospitals. Cocci isolates were predominant at hospital Z (28.6%) while a cocco-bacillus was only recovered from hospital Y. Figure 3 shows how much of the Gram-positives were isolated in comparison with Gram-negatives.

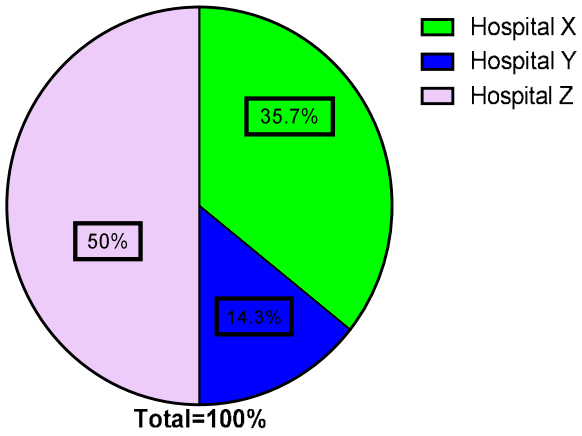
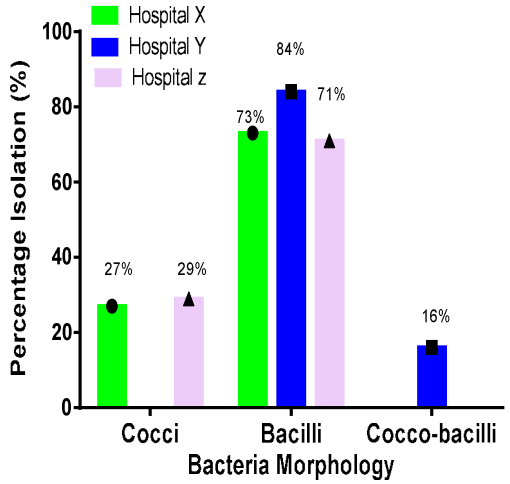


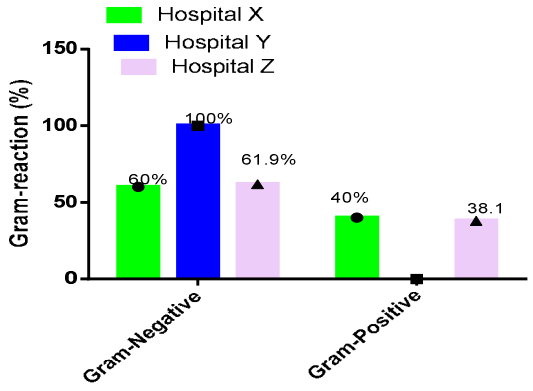
FIGURE 1: THE OVERALL ISOLATION PERCENTAGE ACROSS THE DIFFERENT HOSPITALS.



(Data was subjected to 95% Confidence interval analysis)
FIGURE 2: THE DIFFERENT BACTERIA MORPHOLOGIES ISOLATED FROM THE HOSPITALS

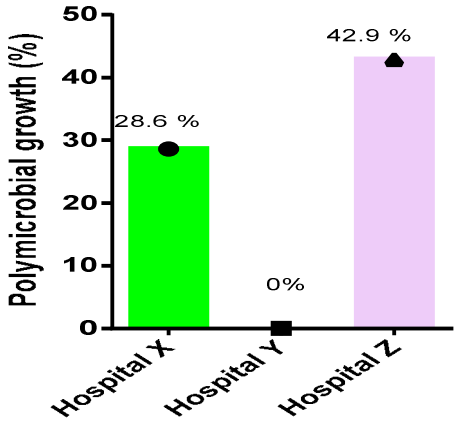
Some wounds were colonized by several types of bacteria, whereby 29% and 43% of the wounds from hospital X and hospital Z were polymicrobial respectively (more than 3 isolates recovered) as indicated in figure 4. No polymicrobial growth was evident from hospital Y patients.

The presumptive identities obtained from API were compared with the 16S rDNA results as shown in table 3 to 5, the observed differences are highlighted in blue. The observed phenotypic differences indicate the anomalies between culture-dependent techniques and 16S rDNA sequencing.

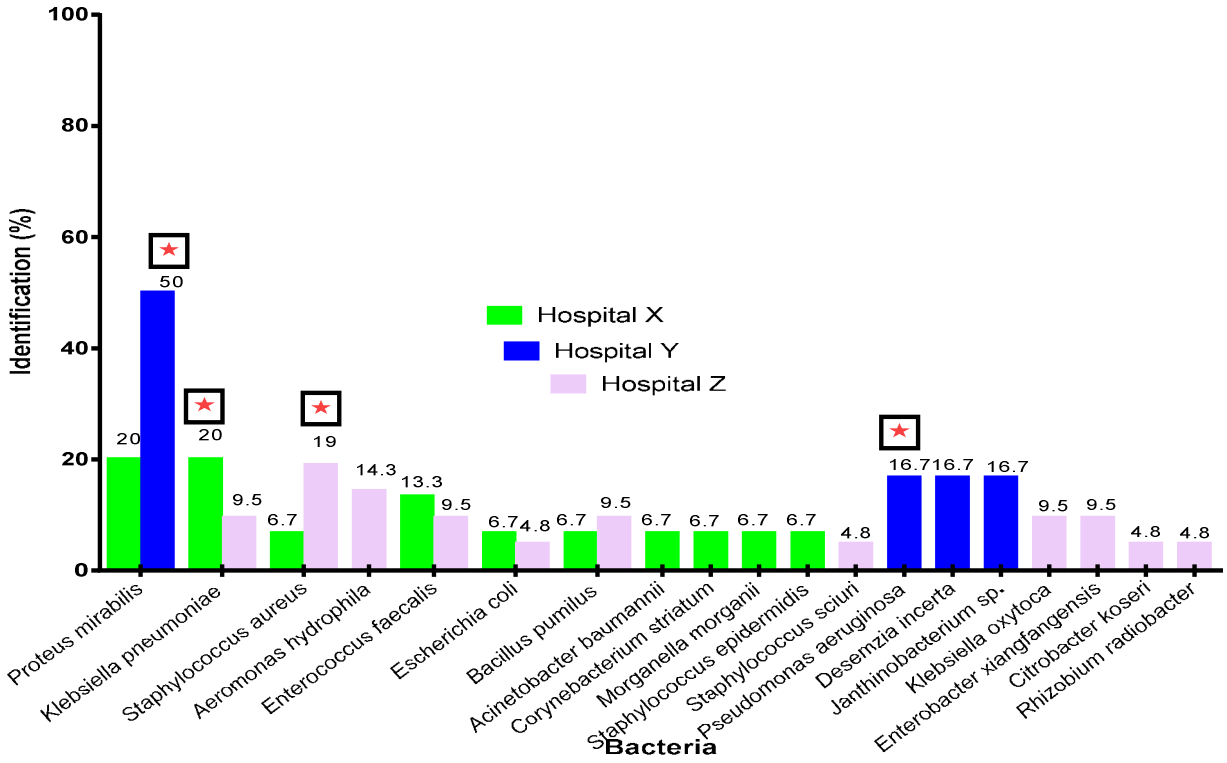


(Data shown to be significantly different through one-way Anova ****, P < 0, 0001)

FIGURE 3: THE GRAM-REACTION OF THE ISOLATES



(Data was subjected to 95% Confidence interval analysis)
FIGURE 4: THE OCCURRENCE OF POLYMICROBIAL GROWTH IN THE DIFFERENT WOUNDS OF HOSPITALIZED PATIENTS



(Data shown to be significantly different through two-way Anova **, P-value = 0.0013)

FIGURE 5: THE PREVALENCE OF BACTERIA ISOLATES RECOVERED FROM THE HOSPITALS UNDER STUDY.

TABLE 3.THE ISOLATES CHARACTERISTICS AND PRESUMPTIVE IDENTITIES FROM HOSPITAL X

Bacterial Isolate	Gram-stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification
*Pat A1	Gram+	cocci	Positive	Positive	*Micrococcus*	*Kocuria varians*
	Gram-	Bacilli	Negative			
Pat A2	Gram -	Bacilli	Negative	Positive	*Enterobacteriaceae*	*Proteus mirabilis*
*Pat B1	Gram-	Coccobacill	Positive	Positive	Non-*E*	*Sphingomonas paucimobilis*
	Gram+	Bacilli				
*Pat B2	Gram-	Bacilli	Positive	Positive	Non-*E*	*Rhizobium radiobacter*
			False Positive	Slow-Positive		
*Pat B4	Gram+	Cocci	Negative	Negative	*Enterococcus*	*Aerococcusviridans*
	Gram-	Bacilli		Slow-Positive		

*Pat B5	Gram+ Gram-	Cocci Bacilli	Positive Negative	Negative Positive	*Enterococcus*	*Globicatellasanguinis*
*Pat B6	Gram -	Bacilli	Positive False- Positive	Positive	Non-*E*	*Burkholderiacepacia*
*Pat C1	Gram -	Bacilli	Positive	Positive	Non-*E*	*Burkholderiacepacia*
*Pat D1	Gram -	Bacilli	Positive Negative	Positive	Non-*E*	*Aeromonashydrophila*
*Pat D2	Gram+	Cocci Bacilli	Negative	Negative Positive	*Enterococcus*	Aerococcus viridans1
*Pat E1	Gram-	Bacilli	Positive Negative	Positive	Non-*E*	*Ochrobactrumanthropi*
Pat F1	Gram +	Cocci	Negative	Positive	*Micrococcus/ Staphylococcus*	*Staphylococcus epidermidis*
Pat F2	Gram+	Cocci	Negative	Positive	*Micrococcus/ Staphylococcus*	*Staphylococcus xylosus*
*Pat F3	Gram +	Cocci pairs/ chains	Negative	Negative	*Enterococcus*	*Streptococcus porcinus*
*Pat F4	Gram+	Cocci cluster	Negative	Negative	*Enterococcus*	*Aerococcus viridans1*
Pat G	-	-	-	-	-	-

Key: Non-E denotes non –*Enterobacteriaceae*, * denotes some anomalies among the biochemical tests, presumptive ID and the Blast report, - denotes no growth

TABLE 4: THE ISOLATES CHARACTERISTICS AND PRESUMPTIVE IDENTITIES FROM HOSPITAL Y

Bacterial Isolate	Gram stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification
Pat A1	Gram-	Bacilli	Positive	Positive	Non-*E*	*Pseudomonas aeruginosa*
Pat A2	Gram-	Bacilli	Negative	Positive	*Enterobacteriaceae*	*Proteus mirabilis*

Pat B1	**-**	**-**	**-**	**-**	**-**	**-**
***Pat C1**	**Gram+**	**Cocci**	**Negative**	**Negative**		***Enterococcus durans***
		Coccobacilli				
***Pat C2**	**Gram+**	**Cocci**	**Negative**	**Negative**	**Non-*E***	***Enterococcus faecium***
	Gram-	**Bacilli**	**Positive**	**Positive**		
Pat D1	**Gram-**	**Bacilli**	**Negative**	**Positive**	***Enterobacteriaceae***	***Proteus mirabilis***
***Pat D2**	**Gram+**	**Cocci**	**Positive**	**Positive**		***Kocuria varians***
	Gram-	**Bacilli**	**Negative**			

Key:Non-E denotes non –*Enterobacteriaceae*, * denotes some anomalies among the biochemical tests, presumptive ID and the Blast report, - denotesno growth

TABLE 5: THE ISOLATES CHARACTERISTICS AND PRESUMPTIVE IDENTITIES FROM HOSPITAL Z

Bacterial Isolate	Gram-stain	Morphology	Oxidase test	Catalase test	Presumptive ID	API Identification
***Pat A1**	**Gram-**	**Bacilli**	**Negative**	**Positive**	***Enterobacteriaceae***	***Proteus vulgaris***
	Gram+	**Cocci**				
***Pat A2**	**Gram-**	**Bacilli**	**Positive**	**Positive**	**Non- *E***	***Vibro alginolyticus***
			Negative			
***Pat A4**	**Gram+**	**Cocci**	**Negative**	**Negative**	***Enterococcus***	***Aerococcus viridans 1***
				Positive		
***Pat A5**	**Gram+**	**Cocci**	**Negative**	**Negative**	***Enterococcus***	***Streptococcus porcinus***
***Pat B1**	**Gram-**	**Bacilli**	**Positive**	**Positive**	***Pseudomonas***	***Pseudomonas aeruginosa***
	Gram+					
***Pat B2**	**Gram-**	**Bacilli**	**Positive**	**Positive**	***Pseudomonas***	***Pseudomonas aeruginosa***
	Gram+					
Pat C1	**Gram-**	**Bacilli**	**Negative**	**Positive**	***Enterobacteriaceae***	***Citrobacter koseri***
***Pat C2**	**Gram+**	**Cocci**	**Negative**	**Positive**	***Staphylococcus***	***Staphylococcus xylosus***
	Gram-	**Bacilli**				
Pat D1	**Gram+**	**Cocci**	**Negative**	**Positive**	***Staphylococcus***	***Staphylococcus spp***

*Pat D2	Gram-	cocci Bacilli	Negative	Positive	*Enterococcus*	*Aerococcus viridans*
Pat E1	Gram+	Cocci	Negative	Positive	*Micrococcus/ Staphylococcus*	*Staphylococcus aureus*
Pat E2	Gram+	Cocci	Negative	Positive	*Staphylococcus*	*Staphylococcus aureus*
Pat E3	Gram-	Bacilli	Positive	Positive	Non-*E*	*Aeromonas hydrophila/ caviae*
*Pat E4	Gram-	Bacilli	Positive False-Positive	Negative Positive	Non-*E*	*Aeromonas hydrophila/ caviae*
Pat E5	Gram-	Bacilli	Negative	Negative	*Enterobacteriaceae*	*Klebsiellaoxytoca*
*Pat F1	Gram-	Bacilli	Positive Negative	Positive	Non-*E*	*Aeromonas hydrophila*
Pat F2	Gram+	Cocci	Negative	Positive	*Micrococcus/ Staphylococcus*	*Staphylococcus aureus*
Pat F3	Gram-	Bacilli	Positive	Positive	Non-*E*	*Rhizobium radiobacter*
Pat F4	Gram-	Bacilli	Positive	Positive	Non-*E*	*Aeromonas hydrophila*
*Pat F5	Gram-	Bacilli	Positive False-Positve	Negative	Non-*E*	*Aeromonas hydrophila*
*Pat F6	Gram-	Bacilli	Positive Negative	Positive	Non-*E*	*Aeromonas hydrophila*
Pat G 1	-	-	-	-	-	-

Key: ŋ/s denotesnot sequenced, Non-E denotes non –*Enterobacteriaceae*, * denotes some anomalies among the biochemical tests, presumptive ID and the Blast report, - denotesno growth

The Prevalence and Distribution Patterns of the Bacteria Species

The Gram-negative bacilli from *Enterobacteriaceae* such as the *Proteus mirabilis* (20%) and *Klebsiella pneumonia* (20%) were the predominant bacteria species from hospital X in comparison to hospital Z, where *Staphylococcus aureus* (19%)was mostly recovered and *Proteus mirabilis* (50%) from hospital Ywas common, as shown in figure 5. A few skin commensals such as *Corynebacterium striatum, Staphylococcus epidermidis* were also recovered. More species diversity was observed in the wounds of the patients from hospital Z, two species of *Klebsiella* were recovered (*Klebsiella pneumoniae* and *Klebsiella oxytoca*). Table 6 presents the frequency distribution of bacteria across the hospitals that participated in the study.

TABLE 6: THE DISTRIBUTION OF BACTERIA ACROSS THE DIFFERENT HOSPITALS

Bacteria Identities	Distribution (%)		
	Hospital X	Hospital Y	Hospital Z
Staphylococcus aureus	25	0	75
Enterococcus faecalis	50	0	50
Bacillus pumilus	33.3	0	66.7
Proteus mirabilis	50	50	0
Escherichia coli	50	0	50
Klebsiella pneumonia	60	0	40

A total of six species were identified to be distributed among the different hospitals; *Proteus mirabilis* between hospital X and Y while five of the identities; *Klebsiella pneumonia*, *Bacillus pumilus, Enterococcus faecalis, Escherichia coli and Staphylococcus aureus* were distributed among patients from Hospital X and Z as shown by Table 6.

DISCUSSION

Diabetic wound infections are a global challenge especially in developing countries, compromising the quality of life [20]. In this study, bacteria were recovered from the wounds of 83% of the sampled diabetic patients, indicating the high prevalence of bacteria in the wounds of diabetic patients, as in agreement with Dunyach-Remy *et al.*,[26]. The recovery of bacteria in diabetic wounds is one of the signs of infection along with clinical symptoms such as erythema, pain, tenderness and pus [9].The wounds were in the lower limbs in 94% of the cases and this in literature has been attributed to the vascular permeability that causes impaired blood supply to the peripheries during a diabetic state [27] and can result to limb amputations [7].The wounds were also noted mostly in the elderly (> 60 year) of which whose immune system is already compromised due to ageing [28] and diabetes thereby increasing the risk of bacterial infections [29]

Biochemical tests are solely based on phenotypic properties of bacteria which are shared by most species [12, 13, 17], as a result misidentification is common, which can also account for the anomalies observed in table 3- 5 whereby culture-based methods of identification (API) misinterpreted some of the results which were confirmed to be different by the 16S rDNA. In relation to this, several studies have reported that antimicrobial therapy may affect the bacterial cell wall without killing the bacteria leading to altered cell morphology thus misidentification is common especially in the Gram-stain [30, 31]. Gram-viable bacteria stain opposite from their true Gram-reaction therefore, limiting the use of the Gram-stain in bacteria identification [30]. Catalase and oxidase tests play a crucial role in enzyme-based methods of identification however, some bacteria contain enzymes different from catalase or cytochrome oxidase c that alter these particular reactions thereby giving false results [14, 17], therefore the 16S rDNA results were the considered results in this study because bacteria was accurately identified. The 16S rDNA technique is able to identify even the unculturable strains, therefore, giving a better understanding of bacteria etiology in infections [16].

The Gram-negative bacilli were most recovered from the patients' wounds in all three hospitals (figure 3), supporting what has been reported by Kamel *et al.,* [5] and Akhi *et al.,* [20] that most diabetic wounds are colonized by Gram-negative bacilli. The wounds were monomicrobial in 76% of the cases, which closely associates them with mild diabetic wound infections [4]. Polymicrobial wounds on the other hand are inclined to severe infections [20] and were noted in 24% of the cases in the study. In severe infections there is an increased risk of biofilm formations which in turn delay wound healing, due to the impaired host defense[32], decreased uptake of treatment drug by biofilms and microbial synergy between less invasive and virulent bacteria [34], leading to longer hospital stays and in extreme cases which may affect the quality of life [32].

The results of the study have shown microbial diversity in diabetic wounds, ranging from skin commensals, opportunistic pathogens, true pathogens and nosocomial-acquired microorganisms which all play a role in the wound etiology [35, 55]. Severe wound infections have been reported to be linked to

facultative anaerobic and aerobic bacteria such as *S. aureus, S epidermidis, Enterococci* spp, *Pseudomonas* spp, *Escherichia* coli [20], which were also recovered in some patients in this study. *Proteus mirabilis* being the most predominant isolate in the study is associated with both nosocomial and community acquired infections [37] and can cause infections in the different body sites [38]. It occurs in moist environments and is a common pathogen implicated in wounds and immuno-compromised hosts along with *E. coli, Enterobacter spp and Klebsiella* spp which were also recovered in the study [39-40]. Through its virulence factors such as fimbriae and flagella it can adhere onto epithelial tissue and cause infection [37].

The factors contributing to the severity of diabetic wound infection includes virulence and pathogenicity which can be attributed to some of the isolates recovered in this study such as *P. aeruginosa* and *S. aureus* that have been reported to produce virulence factors that are so destructive in the wound healing process. *P. aeruginosa* possesses virulence factors such as exoproteases, siderophores, exotoxins, hydrogen cyanide and pyocyanin that attack host defenses and impair wound healing [35] while *S. aureus* possess factors such as coagulase, catalase and clumping factors that play a role in infection mainly occurring in immuno-compromised individuals such as diabetic individuals [9]. *S. aureus* has a role in deepening and spreading infections in body tissue by damaging the host cell membranes and causing cell lysis [26], which can be also attributed to diabetic wounds.

Staphylococcus sciuri (coagulase-negative) among the recovered identities in this study, has been implicated in hospital and community acquired infections [41]. The two species of *Klebsiella* identified in this study are frequently accountable for nosocomial infection in humans and they greatly impact on immunocompromised hosts [36], emphasizing the threat that they pose on public health. Less has been reported about the virulence of *K. pneumoniae* [35], however, three distinct phylagroups (Kp I, Kp II, Kp III) have been defined and all three are implicated in human infections [36].

Conclusion

The presence of bacteria alone is not indicative of infection, however, most bacteria recovered in the study have been reported to have debilitating effects in wounds and in immunocompromised hosts, therefore their recovery alone in diabetic patients' wounds is a serious health concern, such that necessary measures should be taken to curb their spread especially in the hospital setting.

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Disclosure Policy

There is no conflict of interest regarding the publication of this paper.

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DISTRIBUTION AND TYPES OF WATER-BORNE BACTERIAL PATHOGENS IN RIVER SOKOTO, NIGERIA AND THEIR HEALTH IMPLICATION

*Raji, M.I.O. and Oyeniyi, Y.J.

Department of Pharmaceutics and Pharmaceutical Microbiology, Usmanu Danfodiyo University, Sokoto, Nigeria.

* Correspondence: Raji, M.I.O., Department of Pharmaceutics and Pharmaceutical Microbiology, UDU, Sokoto, Nigeria, E-mail: rajimuda@gmail.com; Tel.: +2348032845124.

ABSTRACT

The quality of water from River Sokoto was assessed to determine its bacterial load and types. Standard bacteriological techniques were used to perform the total heterotrophic bacteria, faecal coliform and enterococci counts of water samples collected from six sampling points on the river and distribution of bacteria in the water samples was also determined using standard procedures. The study indicated high heterotrophic bacteria, faecal coliform and enterococci counts above permissible limits for drinking and recreational waters according to World Health Organization (WHO) and United States Environmental Protection Agency (USEPA). A total of 434 bacteria organisms were isolated comprising nineteen different species. Among the *Enterobacteriaceae, Escherichia coli*, which are human pathogenic organisms, had the highest percentage (11.98%) followed by *Enterobacter aerogenes* and *Klebsiella pneumoniae subspecies pneumonia. Pseudomonas aeruginosa* constituted the majority of non-*Enterobacteriaceae* Gram negative organisms. *Staphylococcus aureus* was the highest among the Gram positive organisms followed by *Staphylococcus saprophyticus* (5.99%). Other isolates in significant numbers are *Streptococcus faecalis, Bacillus subtilis, Elizabethkingia meningoseptica* and *Aeromonas sobria*. Bacteria of aquatic habitat like *Providencia rettgeri, Raoultella ornithinolytica, Staphylococcus cohnii subspecies urealyticus* and *Staphylococcus chromogenes* that have not been isolated before in the study area were also isolated. River Sokoto predominantly contained *E. coli* which is an indication of faecal contamination and that makes it unsuitable for drinking and agricultural uses. People in the area should be encouraged to practice adequate sanitation.

Key words: River Sokoto, water quality, bacterial pathogens, *E. coli*, health implication.

RÉPARTITION ET TYPES DE BACTÉRIES PATHOGÈNES TRANSMISES PAR L'EAU DE LA RIVIÈRE À SOKOTO, NIGERIA ET LEUR IMPLICATION SUR LA SANTÉ

*Raji, M.I.O. et Oyeniyi, Y.J.

Département de pharmacie et pharmaceutique Microbiologie, Université Usmanu Danfodiyo, Sokoto, Nigeria.

* Correspondance : Raji, M.I.O., Département de pharmacie et microbiologie pharmaceutique, UDU, Sokoto, Nigeria, E-mail : rajimuda@gmail.com ; Tél. : 2348032845124.

Résumé

La qualité de l'eau du fleuve Sokoto a été évalué pour déterminer sa charge bactérienne et types. Les techniques bactériologiques standard ont été utilisés pour effectuer l'ensemble des bactéries hétérotrophes, coliformes et entérocoques chefs d'eau prélevés dans six points de prélèvement sur la rivière et la distribution des bactéries dans les échantillons d'eau a été déterminé en utilisant les procédures standard. L'étude indiquait des bactéries hétérotrophes, coliformes et entérocoques compte au-dessus des limites acceptables pour l'eau potable et des eaux récréatives selon l'Organisation mondiale de la Santé (OMS) et l'United States Environmental Protection Agency (EPA). Un total de 434 organismes ont été isolées de bactéries comprenant dix-neuf espèces différentes. Parmi les entérobactéries, *Escherichia coli*, qui sont des organismes pathogènes, présente le pourcentage le plus élevé (11,98 %) suivie de *l'Enterobacter aerogenes Klebsiella pneumoniae* et sous-espèces la pneumonie. *Pseudomonas aeruginosa* constituaient la majorité des non-*Entérobactéries* organismes Gram négatif. Staphylococcus aureus a été le plus élevé parmi les organismes Gram positif suivi de *Staphylococcus saprophyticus* (5,99 %). D'autres isolats en nombres importants Streptococcus faecalis, Bacillus subtilis, *Elizabethkingia meningoseptica* et *Aeromonas sobria* Les bactéries de l'habitat aquatique comme *Providencia rettgeri, Raoultella ornithinolytica, Staphylococcus cohnii Staphylococcus chromogenes urealyticus* sous-espèce et qui n'ont pas été isolés avant dans la zone d'étude ont aussi été isolés. River Sokoto principalement contenues *E. coli*, qui est une indication de contamination fécale et qui le rend impropre à la consommation et les utilisations agricoles. Les gens dans la région devraient être encouragés à pratiquer un assainissement adéquat.

Mots clés : Fleuve Sokoto, la qualité de l'eau, de bactéries pathogènes, E. coli, l'implication de la santé.

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INTRODUCTION

Water is an indispensable natural resource essential for the existence of all living creatures. It is required for various human daily activities such as drinking, cooking, tooth-brushing, bathing, washing utensils and also for agricultural and industrial purposes (1 ,2). However, poor water quality continues to be a leading cause of health problems especially in developing countries where it is estimated that 80% of all illnesses are linked to water and sanitation and 15% of all child deaths under the age of 5 years result from diarrhoeal diseases (3, 4). Currently, an estimated 884 million people worldwide do not use improved sources of drinking water and 2.6 billion are not provided with adequate sanitation. The majority of these are in Southern Asia (25%) and sub-saharan Africa (37%) (5). In Nigeria, increasing population and infrastructural breakdown have made municipal pipe borne water to be inadequate in quantity and quality (6). Today, less than 30% Nigerians have access to safe drinking water due to these inadequacies and most of the populations have to resort to drinking water from wells and streams especially in the rural and suburban communities. These water sources are largely untreated and might harbour waterborne and vector-borne diseases such as cholera, typhoid fever, diarrhoea, hepatitis and guineaworm (7-9). These diseases are caused by pathogenic bacteria, viruses, protozoa and other microbes which are shed in human faeces and pollute water supplies which people utilize for drinking and washing purposes. Many rivers, streams and wells worldwide are affected by faecal contamination leading to increased health risks to persons exposed to the water, degradation of recreational and drinking water quality (10-17).

Pollution of river waters with pathogenic microorganisms has been on steady increase in the recent past. The major source of microbes in water is faeces from human and other mammals (18). Entry of pathogens into rivers can occur either from a point source, non- point sources or both. Non-point source microbial pollution of rivers occurs from rainwater surface run-offs, storm sewer spillages or overflow, while point-source pollution comes from discharge of untreated or partially treated effluents from wastewater treatment plants (19). One of the most frequent types of contamination in rural communities is faecal pollution from different sources, most frequently livestock and inadequate on-site human waste disposal systems (20). Microbiological contamination, is therefore dispersed, sporadically and influenced by a range of interacting environmental factors such as the watersheds physical characteristics, climatic conditions and the activities of man like waste disposal and agricultural management practices (21). The quality of water from River Sokoto was therefore assessed to determine its bacterial load.

MATERIAL AND METHODS

Study area

The segment of River Sokoto around Kalanbaina industrial area of the metropolis where there are industries, human settlements and irrigation farming activities was used in the study. Six sampling points were chosen namely; a point 5 kilometres away from farmland (P1), a point close to farmland about 2 kilometres from P1 (P2), a point close to residents along the riverside about 4 kilometres from P2 (P3), a point on stream drainage immediately from Sokoto Cement Factory (P4), a point on the stream just about to enter the river about 3 kilometres from P4 (P5), and a point 2 kilometres away from P5 on the river (P6) (Figure 1).

Bacteriological analysis

Water samples collected from six sampling points on River Sokoto were analysed for their bacterial load. Heterotrophic bacteria, coliforms and enterococci counts; and types of bacteria were determined by serial dilution and plating of water samples on differential culture media following the method of (22). Discrete isolates from heterotrophic plate agar, kept on nutrient agar slant were subjected to Gram Staining. Gram negative bacteria and Gram positive *Staphylococcus* species were isolated and identified using identification kits – ID 32E (BioMerieux, France) and MICROBACT STAPH 12S (Oxoid Ltd, England). Other Gram positive bacteria (*Streptococcus faecalis* and *Bacillus subtilis*) were identified and characterized biochemically following the methods described in Bergey's Manual of Systematic Bacteriology (23).

RESULTS

The heterotrophic plate counts (HPC), faecal coliforms (FC) and faecal streptococci (FS) counts of water samples collected from River Sokoto for the sampling period between January and December, 2014 are presented in Table 1. The result showed HPC in tens of thousands CFU/ml, FC counts in thousands CFU/ml and FS counts in hundreds CFU/ml. As shown in the table, sampling point P1 recorded the highest heterotrophic and total coliforms counts during the sampling periods with highest count in July, 2014. On the other hand, lowest heterotrophic and coliform counts were recorded at site P4 which is the point through which effluents from Sokoto Cement Factory entered River Sokoto.

TABLE 1: HETEROTROPHIC BACTERIA AND COLIFORM COUNTS OF RIVER SOKOTO WATER AT DIFFERENT SAMPLING POINTS IN JANUARY TO DECEMBER, 2014

Month	Sampling point	HPC	FCC	FSC	
J	P1	$1.25 \pm 0.48 \times 10^5$	1200	160	
A	P2	$0.98 \pm 0.27 \times 10^5$	1800	110	
N	P3	$0.92 \pm 0.15 \times 10^5$	1100	110	
	P4	$0.18 \pm 0.78 \times 10^5$	900	100	
	P5	$1.00 \pm 0.83 \times 10^5$	1000	100	
	P6	$1.20 \pm 0.33 \times 10^5$	1500	110	
F	P1	$2.00 \pm 0.28 \times 10^5$	1800	180	
E	P2	$1.13 \pm 0.18 \times 10^5$	1600	150	
B	P3	$1.82 \pm 0.21 \times 10^5$	1600	120	
	P4	$0.19 \pm 0.26 \times 10^5$	1100	118	
	P5	$1.45 \pm 0.25 \times 10^5$	1400	120	
	P6	$1.75 \pm 0.48 \times 10^5$	1800	160	
M	P1	$3.00 \pm 0.40 \times 10^5$	2000	220	
A	P2	$2.75 \pm 0.39 \times 10^5$	1800	180	
R	P3	$1.65 \pm 0.21 \times 10^5$	1700	150	
	P4	$0.39 \pm 0.47 \times 10^5$	1500	120	
	P5	$1.20 \pm 0.45 \times 10^5$	1700	130	
	P6	$2.82 \pm 0.30 \times 10^5$	2000	200	
A	P1	$3.02 \pm 0.24 \times 10^5$	2100	242	
P	P2	$1.28 \pm 0.63 \times 10^5$	2000	200	
R	P3	$1.97 \pm 0.31 \times 10^5$	2000	200	
	P4	$0.42 \pm 0.12 \times 10^5$	1500	130	
	P5	$2.86 \pm 0.38 \times 10^5$	1800	160	
	P6	$2.82 \pm 0.33 \times 10^5$	2100	210	
M	P1	$3.45 \pm 0.53 \times 10^5$	2700	300	
A	P2	$2.91 \pm 0.44 \times 10^5$	2500	220	
Y	P3	$2.48 \pm 0.34 \times 10^5$	2500	200	
	P4	$0.95 \pm 0.99 \times 10^5$	1800	160	
	P5	$1.26 \pm 0.14 \times 10^5$	2200	160	
	P6	$3.32 \pm 0.48 \times 10^5$	2500	300	
J	P1	$5.36 \pm 0.62 \times 10^5$	3200	400	
U	P2	$3.92 \pm 0.35 \times 10^5$	3000	250	
N	P3	$3.80 \pm 0.60 \times 10^5$	2900	220	
	P4	$3.60 \pm 0.56 \times 10^5$	2000	160	
	P5	$3.68 \pm 0.28 \times 10^5$	2700	180	
	P6	$4.86 \pm 0.72 \times 10^5$	3000	310	
J	P1	$8.20 \pm 0.26 \times 10^5$	4500	800	
U	P2	$7.00 \pm 0.96 \times 10^5$	4200	260	
L	P3	$6.08 \pm 0.10 \times 10^5$	3800	230	
	P4	$3.84 \pm 0.72 \times 10^5$	2600	200	
	P5	$4.20 \pm 0.68 \times 10^5$	3500	220	
	P6	$8.05 \pm 0.22 \times 10^5$	4200	320	
A	P1	$6.02 \pm 0.72 \times 10^5$	4000	530	
U	P2	$4.89 \pm 0.30 \times 10^5$	3200	220	
G	P3	$4.20 \pm 0.18 \times 10^5$	2700	210	
	P4	$2.20 \pm 0.62 \times 10^5$	2400	160	
	P5	$3.02 \pm 0.86 \times 10^5$	2500	200	
	P6	$5.73 \pm 0.78 \times 10^5$	3300	300	
S	P1	$4.75 \pm 0.60 \times 10^5$	2600	300	
E	P2	$3.80 \pm 0.57 \times 10^5$	2300	200	
P	P3	$3.02 \pm 0.48 \times 10^5$	2200	180	
	P4	$1.96 \pm 0.56 \times 10^5$	1800	150	
	P5	$3.00 \pm 0.86 \times 10^5$	2200	180	
	P6	$4.26 \pm 0.71 \times 10^5$	2500	290	
O	P1	$3.80 \pm 0.54 \times 10^5$	2300	280	
C	P2	$2.76 \pm 0.43 \times 10^5$	1800	180	
T	P3	$2.64 \pm 0.35 \times 10^5$	1700	160	
	P4	$1.62 \pm 0.62 \times 10^5$	1500	150	
	P5	$1.94 \pm 0.74 \times 10^5$	1600	150	
	P6	$3.00 \pm 0.40 \times 10^5$	2200	280	
N	P1	$3.49 \pm 0.56 \times 10^5$	2300	250	
O	P2	$2.64 \pm 0.28 \times 10^5$	1800	180	
V	P3	$1.92 \pm 0.56 \times 10^5$	1600	140	

	P4	$0.98 \pm 0.38 \times 10^5$	1200	120	
	P5	$1.80 \pm 0.45 \times 10^5$	1500	120	
	P6	$2.75 \pm 0.39 \times 10^5$	1800	250	
D	P1	$2.75 \pm 0.87 \times 10^5$	1800	240	
E	P2	$1.32 \pm 0.16 \times 10^5$	1700	150	
C	P3	$1.20 \pm 0.94 \times 10^5$	1200	120	
	P4	$0.85 \pm 0.62 \times 10^5$	1000	100	
	P5	$1.10 \pm 0.68 \times 10^5$	1200	100	
	P6	$1.80 \pm 0.56 \times 10^5$	1800	200	

KEY: HPC = Heterotrophic plate count; FCC = Faecal Coliforms count; FSC = Faecal Streptococci count.

TABLE 2: DISTRIBUTION AND PERCENTAGE FREQUENCY OF BACTERIA ORGANISMS ISOLATED FROM WATER SAMPLES COLLECTED FROM RIVER SOKOTO

Organisms	Number Isolated	% Frequency
Enterobacteriaceae		
Klebsiella pneumoniae subspecies pneumonia	**38**	**8.76**
Klebsiella oxytoca	**13**	**3.00**
Enterobacter cloacae	**26**	**5.99**
Enterobacter aerogenes	**38**	**8.76**
Salmonella typhi	**28**	**6.45**
Shigella flexneri	**23**	**5.30**
Escherichia coli	**52**	**11.98**
Providencia rettgeri	**13**	**3.00**
Raoultella ornithinolytica	**26**	**5.99**
Non-*Enterobacteriaceae* Gr-ve org		
Elizabethkingia meningoseptica	**26**	**5.99**
Pseudomonas aeruginosa	**32**	**7.37**
Aeromonas sobria	**24**	**5.53**
Gram positive isolates		
Staphylococcus aureus	**30**	**6.91**
Staphylococcus saprophyticus	**26**	**5.99**
Staphylococcus epidermidis	**13**	**3.00**
Staphylococcus cohnii subspecies urealyticus	**4**	**0.92**
Staphylococcus chromogenes	**4**	**0.92**
Streptococcus faecalis	**10**	**2.30**
Bacillus subtilis	**8**	**1.84**
Total	**434**	**100**

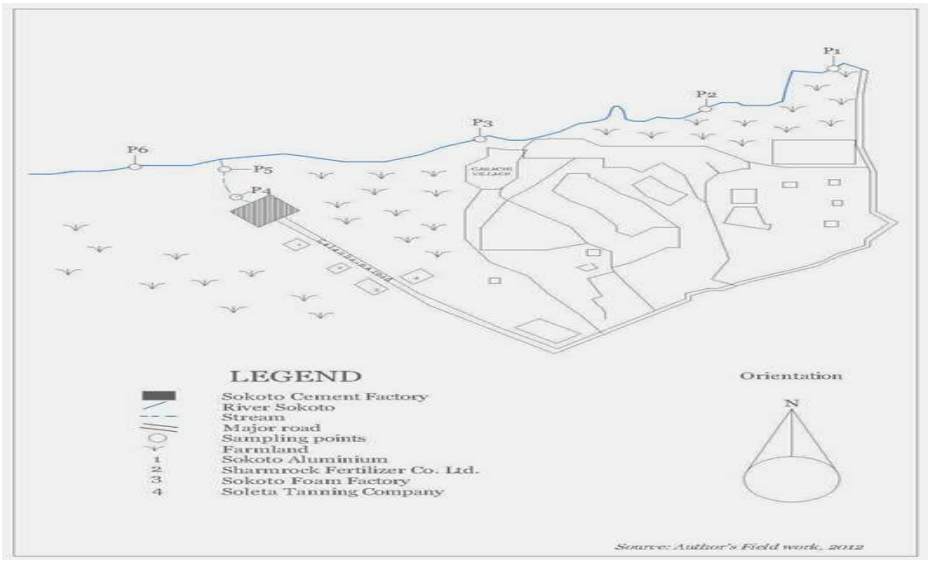

FIG. 1: MAP OF STUDY AREA OF RIVER SOKOTO SHOWING SAMPLING POINTS

Table 2 presents the distribution and types of bacteria isolated from water samples of the river. A total of 434 bacteria organisms were isolated comprising nineteen different species. Among the *Enterobacteriaceae, Escherichia coli* had the highest percentage (11.98%) followed by *Enterobacter aerogenes* and *Klebsiella pneumoniae subspecies pneumonia. Pseudomonas aeruginosa* constituted the majority of non-*Enterobacteriaceae* Gram negative organisms. *Staphylococcus aureus* was the highest

among the Gram positive organisms followed by *Staphylococcus saprophyticus* (5.99%). Other isolates in significant numbers are *Streptococcus faecalis*, *Bacillus subtilis*, *Elizabethkingia meningoseptica* and *Aeromonas sobria*. Some rare bacteria in the study area which are of aquatic habitat like *Elizabethkingia meningoseptica, Klebsiella oxytoca, Providencia rettgeri, Raoultella ornithinolytica, Staphylococcus cohnii subspecies urealyticus* and *Staphylococcus chromogenes* were also isolated.

DISCUSSION

River Sokoto is a major source of water for domestic, agricultural and industrial uses in Sokoto metropolis. It is the source water for the water treatment plant that supplies pipe-borne water need of the people in the metropolis. Residents in the locality use water from the river for washing and bathing. The river water is also used to irrigate adjoining farmland, where crops such as onions, sweet potatoes, carrots, millets, tomatoes and vegetables, some of which are often eaten raw, are cultivated. The factories in the locality use the river as source water for various purposes such as water for cooling and washing. People swim and fish in the river and its sand is being dredged for building construction. All these human activities with other environmental factors would negatively impact the physical, chemical and microbiological quality of River Sokoto. This could also pose serious health and environment hazards to the community, as stated by (24), who inferred that waterborne pathogens present greater health risk to people using river water for drinking, bathing, irrigation of crops eaten raw, fishing, and recreational activities. Because of many activities going on around River Sokoto, it is therefore imperative to study the river to know the impact of these factors on the river with the aim of creating awareness on the quality of the river water for the safety of the people using it. The mean total heterotrophic counts, faecal coliform and faecal streptococci counts of the water samples of River Sokoto, shown in Table 1 were higher than the permissible limit recommended by (25) and (26).

This high bacterial load might be as a result of poor hygiene and sanitation such as bathing, cloth washing and defecating in and around the river, which are common practices in the study area. Lack of proper sanitation in urban cities has been cited as the main cause of high bacterial pathogens in rivers traversing major world cities (27). Unhygienic defecation on ground causing contamination of surface water has also been reported in other studies (28-30). The finding of this study corroborates that of (31) where high level of heterotrophic plate count (HPC), coliform and enterococci counts were also recorded on all stream water samples from sixteen sampling points on Esinmirin Stream in Ile-Ife, Nigeria. High level heterotrophic and coliform counts observed in this study also agree with the findings of (32) where adjoining two drainage streams from industrial and residential areas, impact negatively the water quality of River Kaduna in Nigeria. While sampling point (P1) about 2 kilometers away from farmland had the highest heterotrophic and coliform counts, site P4 on the stream that carried effluents from Sokoto Cement Factory into the river had the least values throughout the year. Cement factories are not normally associated with large volumes of liquid waste.

Large numbers of *Escherichia coli*, among the *Enterobacteriaceae* followed by *Enterobacter aerogenes* and *Klebsiella pneumoniae subspecies pneumonia* isolated from River Sokoto has health implications. *Escherichia coli* are gram-negative bacteria that can survive in an environment with or without air (facultative anaerobes). In fact, human faeces may consist of as much as 50-52% of *E. coli* (21). The fact that large numbers of *E. coli* were isolated from River Sokoto indicated that the river's major source of contamination is human. Presence of other *Enterobacteriaceae* (*Enterobacter aerogenes* and *Klebsiella pneumonia*) also constitute serious threat to the community. The most serious water pollutions in terms of human health worldwide are pathogenic organisms such as *Pseudomonas* and *Salmonella* (21). *Pseudomonas aeruginosa* coincidentally constituted the majority of non-*Enterobacteriaceae* Gram negative organisms isolated from the river. The most important water-related diseases these could cause include typhoid fever, cholera, bacterial and amoebic dysentery, hepatitis, malaria, yellow fever, filariosis and schistosomiasis. Also, large numbers of *Staphylococcus aureus*, *Staphylococcus saprophyticus* and others like *Streptococcus faecalis*, *Bacillus subtilis*, *Elizabethkingia meningoseptica* and *Aeromonas sobria* are of serious concern. Since the largest number of faecal coliform and faecal streptococci is always present in manure (33) then the presence of either of these microbes in a surface water sample is strong evidence of faecal contamination. The presence of coliform bacteria in water does not necessarily indicate water contamination by faecal waste; however the presence of faecal coliform in water may indicate recent contamination by human sewage or animal dropping which could contain other bacteria, viruses, or diseases causing organisms (21, 34) associated water-borne disease occurrences including acute gastrointestinal disease, cholera, dysentery, hepatitis- A, and typhoid with the use of Ganges River in India. Residents around River Sokoto use water from the river for similar purposes. Therefore, isolation of these pathogenic bacteria from River Sokoto could be the cause for rampant cases of water-borne infections in the study area as reported earlier by (35).

CONCLUSION

The fact that water from River Sokoto contained

high level microbial load which were predominantly *E. coli* is an indication of serious faecal contamination and that makes it unsuitable for drinking and agricultural use. The water from this river is therefore not potable, and poses a health risk to residents that rely on it for domestic and agricultural purposes. Government should conduct surveillance and regular monitoring of rivers in order to provide good quality water and people should be encouraged to practice adequate sanitation to ensure human health and protect against a wide range of water-related diseases.

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ORIGINAL ARTICLE

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GENETIC DIVERSITY OF OCHRATOXIGENIC *ASPERGILLUS* SECTION *NIGRI*, USING RAPD AND VCG TECHNIQUES

Thomas, [1]B.T., Ogunkanmi, [2]L.A., Iwalokun, [3]B.A., & Popoola, [1]O.D.

1. Department of Microbiology, Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria, 2. Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos, Nigeria, Division of Molecular Biology and Biotechnology, Nigeria Institute of Medical Research,Yaba, Lagos, Nigeria

Correspondence: B.T.Thomas, benthoa2013@gmail.com

ABSTRACT

This study evaluates the genetic diversity of ochratoxigenic *Aspergillus* section *Nigri* using RAPD and VCG techniques. Results obtained revealed OPX 07 as the most informative of the tested RAPD markers generating 12 polymorphic bands while the least bands were generated by OPR 19. Of the 40 *Aspergillus* section *Nigri* (20 each of *Aspergillus niger* and *Aspergillus carbonarius*), 22 VCGs and 27 RAPD haplotypes were delineated. The two techniques demonstrated similar resolution except in few cases where the RAPD technique further sub divided some VCGs into simpler haplotypes. The average percentage of variable VCG and RAPD reactions were 25 and 50% in that order of sequence while 75 and 50% of the isolates were resolved as same isolates by these techniques respectively. It was also found that the Simpson index of genetic diversity approached one for the isolates from the four geopolitical zones of Ogun State, Nigeria with the mean genetic diversity within isolates (G_L) contributing significantly approximately 89% of the total diversity observed within the isolates ($F=22.23$, $p<0.05$). The remaining 11% of variation could only be allotted to diversity among isolates (G_S). On the whole, the total genetic diversity (H_T) was found to be approximately 48%. In conclusion, RAPD markers provided better resolution than the classical VCG typing technique.

Keywords; Genetic Diversity, Ochratoxigenic *Aspergillus*, RAPD and VCG .

DIVERSITÉ GÉNÉTIQUE DES OCHRATOXIGENIC ASPERGILLUS LA SECTION NIGRI, EN UTILISANT RAPD ET TECHNIQUES VCG

Thomas, [1]B.T., Ogunkanmi, [2]L.A., Iwalokun, [3]B.A., & Popoola, [1]O.D.

1. Département de microbiologie, Université Olabisi Onabanjo, Iwoye, il y a Etat d'Ogun, Nigéria, 2. Département de biologie cellulaire et génétique, Université de Lagos, Lagos, Akoka Nigeria, 3. Division de biologie moléculaire et de la biotechnologie, le Nigeria Institute of Medical Research, Yaba, Lagos, Nigéria

Correspondance à : B.T.Thomas, benthoa2013@gmail.com

Résumé

Cette étude évalue la diversité génétique des ochratoxigenic Aspergillus la section Nigri en utilisant RAPD et techniques VCG. 07 résultats obtenus ont révélé que l'OPX plus informatif de l'essai de RAPD générer 12 bandes polymorphes alors que les bandes ont été générés par le RPT 19. Du 40 Aspergillus la section Nigri (20 chacun d'Aspergillus niger et Aspergillus carbonarius), 22 et 27 haplotypes VCGs RAPD ont été délimités. Les deux techniques ont démontré une résolution similaire, sauf dans quelques cas où la technique RAPD plus de sous-divisé certains haplotypes VCGs en éléments plus simples. Le pourcentage moyen de VCG variable et les réactions ont été 25 RAPD et 50 % dans l'ordre de séquence alors que 75 et 50 % des isolats ont été réglées comme mêmes isolats par ces techniques respectivement. Il a également été constaté que l'indice de diversité génétique Simpson approché une pour les isolats provenant des quatre zones géopolitiques de l'Etat d'Ogun, au Nigeria avec la diversité génétique moyenne dans les isolats (GL), contribuant de manière significative à environ 89 % du total de la diversité observée dans les isolats ($F = 22,23$, $p <0,05$). Les 11 autres % de variation ne peut être attribuée à la diversité parmi les isolats (GS). Dans l'ensemble, la diversité génétique totale (HT) est d'environ 48 %. En conclusion, les marqueurs RAPD a fourni la meilleure résolution que la technique classique VCG.

Mots-clés ; la diversité génétique, l'Aspergillus, RAPD et Ochratoxigenic VCG .

INTRODUCTION

The diversity of filamentous fungi in every spheres of life ranging from agriculture through medicine and biotechnology to the environment has long been documented. In medicine, their role in causing various ailments such as asthma, cystic fibrosis and invasive aspergillosis cannot be overemphasized [1-3]. These organisms has since been indicted in agriculture for their ubiquitous contamination of pre and post harvest food commodities including the ready to eat foods [4-7]. Their significance in different environmental hazards such as flooding, Hurricane Katrina among others has also been recorded both in the United State and Denmark [8]. According to Hawksworth [9 and 10], less is known about the variation of fungal diversity and composition along different gradients such as latitude, altitude, productivity and salinity. On the other hand, Fisher [11] and Rosenzweig [12] pointed out that the distribution of biodiversity along these environmental gradients has been of long-standing interest to ecologists and that most of what is known about how biodiversity varies along environmental gradients stems from research on plants and animals.

As early as 1975, the central goal of using sound techniques and statistics in biodiversity research for delineating the abundance, distribution and processes of species coexistence at different spatial and temporal scales has been pointed out [13] in order not to underestimate the biodiversity scores. Before now, genetic diversity in filamentous fungi is majorly carried out using the vegetative compatibility technique which involves characterization of fungi based on heterokaryon formation between different fungal individuals. Heterokaryon formation is an important component of many fungal life cycles and may serve as the first step in the parasexual cycle and the transmission of hypovirulent factors such as dsRNA [14]

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This technique however is still being used by many researchers to decipher genetic diversity in phytopathogenic moulds even in the presence of growing advances in the field of mycology and science. Our research however was aimed at comparing the ability of this vegetative compatibility technique in typing ochratoxigenic *Aspergillus* section *Nigri* relative to the random amplified polymorphic DNA (RAPD) method.

MATERIALS AND METHODS

Sources of *Aspergillus* section *Nigri*

The *Aspergillus* section *Nigri* used in this study was isolated from processed *Manihot esculenta* (garri) collected from the four geopolitical zones of Ogun State, Nigeria in our previous study. The four geopolitical zones sampled were Yewa, Egba, Remo and Ijebu. The isolates laboratory code, the specie of the *Aspergillus* section *Nigri*, the origin of the isolates, the VCG assignments as well as the RAPD haplotypes were properly delineated in table 1

Vegetative Compatibility Grouping (VCGs)

Recovering of nit mutants
Fungal cultures were grown in solid M3 culture medium as explained earlier [15]. A mycelial fragment was then sub cultured from the grown isolates in the solid M3 culture medium to the center of the petri dishes containing minimal medium with 1.5% potassium chlorate (MMC), using the technique described by Brooker *et al*.[16]. The Petri dishes were incubated at room temperature and examined after 14 to 21 days for sector verification. Fragments from these cultures were transferred to petri dishes containing a minimal medium and sodium nitrate (NaNO3). The isolates that presented poor growth colonies in this medium and little mycelial production were considered to be nit mutants, while those presenting dense aerial mycelium growth, or wild-type, were discarded [17]

Phenotypic classification of the nit mutants

For the phenotypic classification of the nit mutants, mycelial fragments from the same petri dishes containing minimal medium were selected and transferred to the center of dishes containing basal medium (BM) supplemented with sodium nitrite (0,5 g/L), sodium nitrate (2,0 g/L), hypoxanthine (0,5 g/L), ammonium tartrate (1,0 g/L) and uric acid (0,2 g/L). Each nit mutant was transferred to three dishes (100 x 15 mm) with each of the aforementioned media; totaling 15 dishes for each isolate. These dishes were maintained in an incubator at 25^{o}C for a period of 21 days. Two evaluations were carried out: the former on the 14th and the latter on the 21st day. The phenotypic classification was done according to the mycelial growth of the mutants in media supplemented with different sources of nitrogen: BM + sodium nitrate (MM), BM + sodium nitrite (NM), BM + hypoxanthine (HM), BM + ammonium tartrate (AM) and BM + uric acid (UAM). Media supplemented with sodium nitrate and ammonium tartrate were used as negative and positive controls respectively [17]

Heterokaryon formation and VCG classification
The heterokaryons were formed when the colonies of different nit mutants were confronted in petri dishes (100 x 15 mm) at a 1 cm distance in nitrate medium (MM). The dishes were stored in a greenhouse, in the dark at 25^{o}C. After 14 to 21 days, they were analyzed on a weekly basis to verify the existence of heterokaryons. In order to carry out the confrontations, combinations were done whereby each dish contained five different isolates and a mycelial fragment was taken from a determined isolate from the center of the dish and in the other four isolates from the margins, i.e., each mutant selected from a determined isolate was paired with all the other mutants from the other isolates so as to determine the number of complementary groups to which the distinct nit mutants belonged [14].

Random amplified polymorphic DNA analysis (RAPD)

DNA Isolation, Primer Screening and PCR Amplification
DNA was isolated and purified based on the manufacturer's instruction of DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). A total of 26 RAPD primers were screened and optimized for polymorphisms and annealing temperature (Tm) using the isolated ochratoxigenic moulds. Optimal PCR amplification across the isolated organisms was achieved with annealing temperature between 40 and 36^{o}C. Seven primers that shows good and clear polymorphism with the PCR products were therefore used for the study. These primers include OPX 07(GAGCGAGGCT), OPR 16 (CTCTGCGCGT), OPR 19 (CCTCCTCATC), OPR 11(GTAGCCGTCT), OPV 06 (GAACGGACTC), OPA 01(CAGGCCCTTC) and OPA 04(AATCGGGCTG). Each 25 µl PCR reaction contained 12.5 µl master mix (2×) (0.05 45 units/µl *Taq* DNA polymerase in reaction buffer; 4 mM $Mgcl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP), 40 pmol oligonucleotide primer and 1 µg of template DNA. The DNA was first denatured for 2 minutes at 95^{o}C followed by 40 cycles of 15sec denaturation at 95^{o}C, the annealing temperature was progressively decreased by 0.5^{o}C every cycles from 40^{o}C to 35^{o}C for 1 min and 2 min elongation at 72 ^{o}C with a final elongation for 2 min. The amplified products were separated on 3% TBE agarose gels stained with ethidium bromide and viewed under a UV Transilluminator. The analyses of the amplification products were done manually with consideration of the number of fragments and repeatability of the reaction following the procedures described by Roodt *et al*.[18]. Each lane of amplified product was checked manually and scored for presence (+) or absence (-) of fragments.

RESULTS

The table 1 below shows the vegetative compatibility assignment and the random amplified polymorphic DNA haplotypes of the studied *Aspergillus* section *Nigri*. Out of the total 40 *Aspergillus* section *Nigri* analyzed (20 each of *Aspergillus niger and Aspergillus carbonarius*), 22 different VCGs and 27 RAPD haplotypes were found. In our data set, the two methods provided similar resolution except in few cases, where RAPD markers divided some VCG into different haplotypes (Fig.1).

TABLE 1: *ASPERGILLUS* SECTION NIGRI; THEIR ASSIGNMENT AND RAPD HAPLOTYPES

LC	Species	Origin	VA	RH
Y_1	*Aspergillus niger*	Ilaro	Z_1	1
Y_2	*Aspergillus niger*	Owode-yewa	Z_1	1
Y_3	*Aspergillus niger*	Oke Odan	Z_2	2
Y_4	*Aspergillus niger*	Idiroko	Z_2	3
Y_5	*Aspergillus niger*	Aiyetoro	Z_{19}	3
Y_6	*Aspergillus carbonarius*	Imeko	Z_5	4
Y_7	*Aspergillus carbonarius*	Joga Orile	Z_8	5
Y_8	*Aspergillus carbonarius*	Ihubo	Z_8	5
Y_9	*Aspergillus carbonarius*	Igbogita	Z_3	6
Y_{10}	*Aspergillus carbonarius*	Oja Odan	Z_3	7
E_1	*Aspergillus niger*	Owode egba	Z_7	8
E_2	*Aspergillus niger*	Owode egba	Z_7	8
E_3	*Aspergillus niger*	Obantoko	Z_7	8
E_4	*Aspergillus niger*	Itosin	Z_7	9
E_5	*Aspergillus niger*	Itosin	Z_7	10
E_6	*Aspergillus carbonarius*	Orile Imo	Z_{22}	11
E_7	*Aspergillus carbonarius*	Kuto	Z_6	12
E_8	*Aspergillus carbonarius*	Kuto	Z_{10}	13
E_9	*Aspergillus carbonarius*	Owode egba	Z_{10}	14
E_{10}	*Aspergillus carbonarius*	Obantoko	Z_{10}	15
R_1	*Aspergillus niger*	Sagamu/Falawo	Z_4	16
R_2	*Aspergillus niger*	Sagamu/Awolowo	Z_4	16
R_3	*Aspergillus niger*	Sagamu/Sabo	Z_4	16
R_4	*Aspergillus niger*	Ilisan	Z_9	17
R_5	*Aspergillus niger*	Ode-Remo	Z_9	17
R_6	*Aspergillus carbonarius*	Ode-lemo	Z_{11}	18
R_7	*Aspergillus carbonarius*	Ikenne	Z_{11}	18
R_8	*Aspergillus carbonarius*	Ikenne	Z_{11}	18
R_9	*Aspergillus carbonarius*	Irolu	Z_{13}	19
R_{10}	*Aspergillus carbonarius*	Irolu	Z_{21}	20
I_1	*Aspergillus niger*	Ago-iwoye/garage	Z_{16}	21
I_2	*Aspergillus niger*	Ago-iwoye/main mkt	Z_{16}	21
I_3	*Aspergillus niger*	Ijebu –Igbo	Z_{15}	22
I_4	*Aspergillus niger*	Ijebu - Igbo	Z_{15}	22
I_5	*Aspergillus niger*	Oru	Z_{17}	23
I_6	*Aspergillus carbonarius*	Mamu	Z_{20}	24
I_7	*Aspergillus carbonarius*	Oru	Z_{18}	25
I_8	*Aspergillus carbonarius*	Ijebu Ode/Oja oba	Z_{18}	25
I_9	*Aspergillus carbonarius*	Ilese	Z_{14}	26
I_{10}	*Aspergillus carbonarius*	Ilaporu	Z_{12}	27

KEY; LC = laboratory code, VA = VCG Assignment, RH = RAPD haplotypes

There were also cases where both techniques gave equal resolution to certain isolates. However, there were no cases where a RAPD haplotype was further divided by the VCG typing. In the description of the genetic variation and genetic diversity in *Aspergillus* section *Nigri* using VCG typing and RAPD markers. The ratio of VCGs classification to that of the RAPD technique was 22 to 27. The percentage of variable resolution to same resolution was 25 to 75% in VCG typing and 50 to 50 in the RAPD technique. The Simpson's index of genetic diversity approached one in all the four geo political zones of Ogun State for both techniques. However, the sum total of this diversity index for both technique was 0.51 and indifferent (table 2). Table 3 connotes the RAPD band frequencies and genetic diversity of *Aspergillus* section Nigri. The mean genetic diversity within isolates (G_L) contributes approximately 89% of the total diversity ($F=22.23$, $p<0.05$) while the remaining 11% of variation could only be allotted to diversity among isolates (G_S). On the whole, the total genetic diversity (H_T) was found to be approximately 48%.

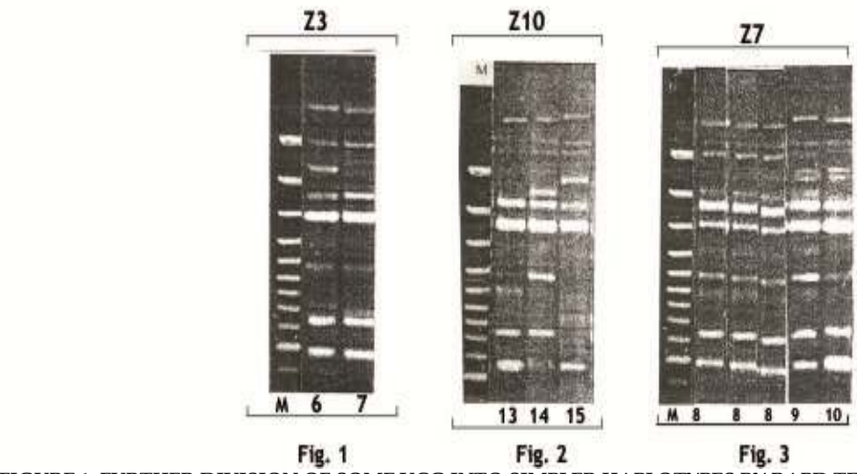

FIGURE 1: FURTHER DIVISION OF SOME VCG INTO SIMPLER HAPLOTYPES BY RAPD TECHNIQUE

TABLE 2: DESCRIPTION OF GENETIC VARIATION AND GENETIC DIVERSITY IN *ASPERGILLUS* SECTION *NIGRI* USING VCG TYPING AND RAPD MARKERS

	VCG TYPING					RAPD TECHNIQUE				
	Y	E	R	I	S_T	Y	E	R	I	S_T
Number of Isolates	10	10	10	10	40	10	10	10	10	40
Number of VCG/RAPD Haplotypes	6	4	5	7	22	7	8	5	7	27
Percentage of Variable VCG/RAPD haplotypes	20	20	20	40	25	40	70	20	40	50
Percentage of Same VCG/RAPD haplotypes	80	80	80	60	75	60	30	80	60	50
Simpson's Index of Diversity	0.87	0.93	0.91	0.81	0.51	0.8	0.7	0.9	0.8	0.51

Table 3: RAPD band frequencies and genetic diversity of *Aspergillus* section Nigri from the four geopolitical zones of Ogun State, Nigeria

RAPD	RAPD band Frequency in the studied organisms						Hier.gene diversity		
	YEWA	EGBA	Remo	Ijebu	Total (n=40)	Probability	HT	GS	GL
OPX07 – 0.1Kbp	0.40	0.20	0.00	0.00	0.15	0.40	0.20	0.02	0.98
OPX07- 0.15kbp	0.20	0.30	0.10	0.10	0.18	0.40	0.15	0.02	0.98
OPX07 – 0.2kbp	0.20	0.30	0.00	0.00	0.13	0.38	0.13	0.04	0.96
OPX 07 – 0.25kbp	0.00	0.00	0.00	0.10	0.03	0.50	0.01	0.03	0.97
OPX07 – 0.3kbp	0.10	0.20	0.20	0.40	0.23	0.40	0.25	0.03	0.97
OPX07- 0.4kbp	0.20	0.00	0.20	0.40	0.20	0.40	0.24	0.10	0.90
OPX07 – 0.5kbp	0.60	0.30	0.60	0.40	0.40	0.40	0.88	0.38	0.62
OPX07- 0.6kbp	0.20	0.20	0.20	0.30	0.25	0.37	0.26	0.22	0.78
OPX04- 0.7kbp	0.20	0.50	0.00	0.40	0.20	0.40	0.24	0.13	0.87
OPX07-0.8kbp	0.70	0.70	0.30	0.60	0.53	0.09	1.19	0.71	0.83
OPX07-0.9kbp	0.70	0.00	0.60	0.40	0.60	0.44	1.50	0.08	0.92
OPX07-1.0kbp	0.70	0.00	0.40	0.20	0.33	0.09	0.69	0.07	0.93

DISCUSSION AND CONCLUSION

The major aim of this research was to use the VCG method to genotype isolates of *Aspergillus* section Nigri relative to the RAPD technique. Our findings however depicts that the VCG typing provided similar resolution as that of the RAPD technique except in few cases where the RAPD method further subdivided some VCGs into RAPD haplotypes. This finding is not unexpected as the vegetative compatibility technique has long been documented as a reliable method for scoring diversity in phytopathogenic fungi [17]. The fact that the RAPD technique subdivided some VCGs into different haplotypes demonstrates the superiority of the latter technique. This observation is not surprising, as the vegetative compatibility techniques scores diversity based on only one marker as against a number of markers that can even be increased to meet specific needs, simply by using more primers [21]. Isolates from most locations were present on different clusters for both VCG and RAPD analyses. This might be the result of gene flow between the geopolitical zones. This is because, garri from which the isolates was obtained are displays in open bowls in markets and various packaging materials use to haul this food from rural to urban areas might exacerbate fungal contamination [22], thereby causing multiple contamination which could be an important prerequisites to evolution of a new fungus as a result of sexual or parasexual recombination. In addition, the values obtained for total gene diversity (H_T), diversity among and within isolates (GS and GL) are similar to those observed in fungi with known sexual life cycles [23, 24]. According to Grypta *et al*.[25], regional population structure of this nature is usually the result of more frequent interbreeding events among isolates within a site than between sites and is more common in diploid or dikaryotic organisim. In summary, the high level of diversity observed in this study may be due to the ability of these isolates to undergo para sexuality under controlled field conditions [26,27] and studies have assessed neither the degree to which parasexuality occurs in natural populations nor the significance of such asexual horizontal gene transfer as an adaptive mechanism relative to migration and genetic drift [28]. Any efforts taken to control fungal contamination should bear in mind the high levels of genetic diversity found from this study before any control measure can be put in place.

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TRANSCRIPTIONAL FACTOR INFLUENCE ON OTA PRODUCTION AND THE QUELLING ATTRIBUTE OF SIRNA ON THE OTA PRODUCING STRAINS OF *ASPERGILLUS* SECTION *NIGRI*

[1]Thomas, B.T., [2]Ogunkanmi, L.A., [3]Iwalokun, B.A., [1]Agu, G.C

1. Department of Microbiology, Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria
2. Department of Cell Biology and Genetics, University of Lagos, Akoka, Lagos, Nigeria
3. Division of Molecular Biology and Biotechnology, Nigeria Institute of Medical Research, Yaba, Lagos, Nigeria

Correspondence to: B.T.Thomas, benthoa2013@gmail.com

Abstract

This study determined the influence of some transcriptional factors on ochratoxin A production as well as investigates the quelling attributes of some designed siRNA on the OTA producing *Aspergillus* section *Nigri* using standard recommended techniques. Results obtained following comparison of the *pks* gene promoter sequences from 15 isolates depicts differences in length and homology with the *pks* gene ranging from 218bp in a strain of the *Aspergillus niger* to 700bp in *Aspergillus carbonarius*. The alignment of the *pks* gene promoter region revealed that six and two of the aligned genes have *Aba A* binding site corresponding to CATTCT and CATTCC respectively while *Brl A* binding site was absent in all the isolates. *Pac C* binding site corresponding to CCTGGC and GCCAAG was also found in two and three of the *pks* gene promoter region respectively. The three designed *siRNA* shows significant impact on OTA inhibitions with no significant statistical differences (80.9, 74.4 and 75.3% for *pks_Ia, pks_Ib* and *pks_Ic* respectively) (F= 3.830, p>0.05). It can be concluded that *Are A* and *Aba A* are potential enhancers for ochratoxin A biosynthesis and none of the investigated transcriptional factors is enough for the activation of ochratoxin A production. However, *pks* gene was seen as a good target gene for inactivation in order to develop efficient means for ochratoxin A control using RNA silencing technology.

Key words: Transcriptional factors, Ochratoxin A, siRNA, Quelling , *Aspergillus* section *Nigri*

FACTEUR TRANSCRIPTIONNEL INFLUENCE SUR LA PRODUCTION D'OTA ET DE LA RÉPRESSION DE L'ATTRIBUT DE SIRNA SUR LES SOUCHES PRODUISANT DE L'OTA DE L'ASPERGILLUS LA SECTION NIGRI

1Thomas, B.T., 2 Ogunkanmi, L.A., 3Iwalokun, B.A., 1AGU, G.C

1. Département de microbiologie, Université Olabisi Onabanjo, Iwoye, il y a Etat d'Ogun, Nigéria, 2. Département de biologie cellulaire et génétique, Université de Lagos, Lagos, Nigeria, Akoka, Division de biologie moléculaire et de la biotechnologie, le Nigeria Institute of Medical Research, Yaba, Lagos, Nigéria

Correspondance à : B.T.Thomas, benthoa2013@gmail.com

Résumé

Cette étude visait à déterminer l'influence de certains facteurs de transcription sur l'ochratoxine A fait enquête sur la production ainsi que la répression de certains attributs conçu sur le siRNA Aspergillus la section Nigri produisant de l'OTA en utilisant des techniques recommandées. Résultats obtenus par la comparaison des séquences promotrices des gènes du pks à partir de 15 isolats illustre les différences dans la longueur et de l'homologie avec des gènes de pks allant de 218bp chez une souche d'Aspergillus niger de 700bp à Aspergillus carbonarius. L'alignement de la région promotrice du gène pks a révélé que six et deux de l'alignement des gènes ont un site de fixation de l'Aba à CATTCT CATTCC et correspondant respectivement alors que Brl un site de fixation était absent de tous les isolats. Cip C binding site CCTGGC GCCAAG correspondant à et a également trouvé dans deux et trois de la région du promoteur du gène pks respectivement. Les trois conçu siRNA montre un impact significatif sur les inhibitions OTA avec aucune différence statistique significative (80,9, 74,4 et 75,3 % pour la pks_Ia, Ib et pks pks__Ic respectivement) (F = 3,830, p >0,05). Il peut être conclu que sont un et de l'Aba A sont pour l'ochratoxine A enhancers potentiels biosynthèse et aucun des facteurs de transcription d'une enquête est suffisant pour l'activation de l'ochratoxine A la production. Cependant, le pks a été considérée comme une bonne cible pour l'inactivation de gènes afin de développer des moyens efficaces pour l'ochratoxine A contrôle en utilisant la technologie de neutralisation de l'ARN.

Mots clés ; facteurs transcriptionnels, ochratoxine A, siRNA, répression , Aspergillus la section Nigri

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INTRODUCTION

Ochratoxins are a group of chemically related fungal contaminants produced by some strains of filamentous fungi. These toxins are classified into three major classes namely Ochratoxin A, B and C with Ochratoxin A being the most popular due to their recent classification as Group 2B human carcinogen following experiments on animals [1,2]. The presence of ochratoxin A in several foods and its accumulated effect such as immunotoxicity, neurotoxicity, genotoxity and possibly carcinogenicity has been well documented [3]. This toxin has also been suspected to be a risk factor for testicular cancer [4].

The organisms that produce ochratoxins are said to be ochratoxigenic and are said to be ubiquitous contaminants of pre and post harvest food commodities including the ready to eat foods [5,6]. The ability of this type of fungi to produce toxin is dependent on their strains, the composition of the food, the conditions of handling as well as storage conditions [7]. These organisms have also attracted attention through the damage it does to plants, animals and humans [1-2,8]. Their secondary metabolites (Ochratoxin A) is a developmental challenge to Africa and the world at large adversely affecting three major sectors namely; public health, trade and economy as well as food and nutrition security.

Some studies suggest that the mycotoxin biosynthesis genes are activated under different environmental conditions and so can easily be induced and not expressed constitutively [9,10]. Their induction can be determined some time before the detection of mycotoxin by analytical methods [11,12]. The signaling processes that switch on ochratoxin biosynthesis during ripening or in poorly stored crops are still not well understood. In order to state whether biosynthesis of OTA may be possible under certain transcriptional factor, *pks* promoter sequences of the isolated organisms were analyzed *in silico* and the effect of RNA interference on OTA silencing was investigated due to the recent evidences suggesting the possibility of some of these ochratoxigenic moulds growing in the presence of both suboptimal and even optimal concentration of both synthetic drugs and plant active ingredients.

MATERIALS AND METHODS

Sources of Processed *Manihot esculenta* Crantz (Cassava Flakes)

Two hundred and fifty (250) samples each of processed *Manihot esculenta* Crantz were purchased between March 2013- December 2014 from the local markets in the four geopolitical zones of Ogun States, Nigeria viz; Yewa, Egba, Remo and Ijebu zones respectively during the dry and wet seasons to represent a total of one thousand samples according to a statistical sampling scheme recommended for microbiological testing of foods [13]. These samples were collected in pre sterilized aluminum pan. The samples in pre sterilized aluminum pan whose lids were opened before getting to the laboratory were rejected. Appearance of the garri samples, the sources of each collection site and the geopolitical zones were noted. autoclaved garri sample was used as control while the remaining one thousand (1000) processed *Manihot esculenta* samples were used as the test samples.

Fungal Isolation and Identification

Microbiological identification

One gram each of the processed *Manihot esculenta* Crantz sample was aseptically seeded in the middle of a sterile potato dextrose agar (PDA) plate in duplicate and incubated for one week at 25^0C.After incubation, fungal isolates of public health significance were identified using rate of growth, colonial and microscopic morphology according to Larone [14].

Molecular Identification of Fungi

DNA Isolation, Amplification and Sequencing

Each specimen (fungal isolate) was stirred directly into 200 ml sterile saline and extracted using a QIAamp DNA mini kit (Qiagen) according to a protocol adapted for extraction of DNA from fungal cells, as described elsewhere [15]. In brief, each sample was pre-incubated at 99^0C for 20 min and then processed as suggested by the manufacturer. After the addition of the cellular lysis buffer, the sample was incubated again at 99^0C for 10 min. The extracted DNA was amplified by PCR using a pair of universal fungal primers (V9D: 59-TTAAGTCCCTGCCCTTTG TA- 39; LS266: 59-GCATTCCCAAACAACTCGACTC-39) encompassing highly conserved regions encoding fungal rRNA [16]. PCRs were performed in 0.2 ml reaction tubes in a final volume of 50 ml containing 2 to 10ng of DNA, 1.5 U Platinum Taq DNA polymerase (In vitrogen), 200 mM each of dATP, dGTP and dCTP, 400 mM dUTP (instead of dTTP), 20 mM Tris/HCl (pH 8.4), 50 mM $MgCl_2$, 0.4 mM each primer and 1 U uracil-N-glycosylase. The amplification reaction included a hold at 50^0C for 5 min to allow uracil-N-glycosylase activity and an additional hold at 95^0C for 5 min for Taq activation, followed by 35 cycles at 95^0C for 30s, 62^0C for 1 min and 72^0C for 2 min, with a final extension step at 72^0C for 5 min. The amplified product was visualized on agarose gels, purified and sequenced using a 310 auto

Genetic Analyzer (PerkinElmer, Applied Biosystems Div., Waltham, USA) with the same primers. For each sample, a pair of primers amplifying the human b-globin gene was included as an extraction/amplification internal control. DNA sequences were analysed using the BLAST database and assigned to the reference isolate sequences with the highest bit score

Determination of level of ochratoxin A in processed *Manihot esculenta* Crantz (cassava flakes)

Five grams each of processed *Manihot esculenta* was weighed into a weighing bottle. Then 25ml of 50% methanol was added. The content was shaken vigorously for 3 minutes on the horizontal shaker . A 5 ml aliquot of the resulting solution was filtered using the Neogen filter (45µm) syringe, into the Neogen collecting tube. The amounts of ochratoxin A (OTA) in the analyzed processed *Manihot esculenta* were determined using a competitive direct enzyme-linked immunosorbent assay (CD-ELISA). Screening method for the analysis was done using Neogen Veratox® testing kits with limits of detection of 1 µg/kg (ppb) for ochratoxin A. Free ochratoxin A in the samples and controls were allowed to compete with enzyme-labelled ochratoxin A (conjugates) for the antibody binding sites. After a wash step, substrate was added, which reacts with the bound conjugate to produce blue colour. More blue colour means less Ochratoxin A while more pink means more Ochratoxin A. The test is read in a microwell reader (Thermo lab system, Thermo, Finland) to yield optical densities. The optical densities of the controls form the standard curve, and the sample optical densities were plotted against the curve to calculate the exact concentration of ochratoxin A.

Ochratoxigenic fungal isolates

Twenty one of the forty isolated *Aspergillus* section *Nigri* are ochratoxin A producing isolates. The *Aspergillus* section *Nigri* used were *Aspergillus niger* and *Aspergillus carbonarius* . The PCR amplification of this isolates yielded a single fragment of an approximately between 600 and 700 bp. BLAST search using Genbank database showed that the isolate percentage similarity of sequence with GenBank range between 98-100% . All the isolates were found with differing levels of OTA

Alignments and phylogenetic analyses

DNA sequences were aligned using Clustal W in DNAMAN (Lynnon Biosoft, Vandreuil, Canada). Data sets included aligned DNA sequences from the *pks* region. Phylogenetic trees were obtained by parsimony analyses using heuristic search methods with stepwise sequence addition and the tree-bisection–reconnection (TBR) branch-swapping algorithm. Node support was assessed with 10000 bootstrap replicates. Gaps were completely deleted. The partition homogeneity test (PHT) in PAUP* was performed on parsimony informative sites only, with 10000 randomized data sets using heuristic search methods with stepwise sequence addition. A two-tailed Kishino–Hasegawa (KH) test using 10000 RELL bootstrap replicates in PAUP* was employed to further assess the likelihood of the different tree topologies.

Protoplast generation from the ochratoxigenic Aspergillus section Nigri

Protoplast was generated as described by Abdel-Hadi *et al.*[17] but with little modification. Briefly, spore suspension of *Aspergillus niger* and *Aspergillus carbonarius* were subcultured in 200 ml of YES broth in 500 ml conical flask. Cultures were incubated for 24 h in the dark at 25°C with shaking at 200 rpm. The mycelium was harvested by filtration through miracloth. One gram of mycelia was transferred into 20 ml of filter sterilized enzyme solution (Per 20 ml: 17 ml of H_2O, 2 ml of 0.2 M $NaPO_4$ [pH 5.8], 0.4 ml of 1.0 M $CaCl_2$, 1.4 g of NaCl, 200 mg of lysing enzyme [Sigma] and 50 mg of driselase [Sigma]. Mycelia were incubated at 30°C before shaking at (80 rpm) for 3 h. Protoplasts were separated from intact mycelia by passage through miracloth into sterile 50 ml tube and 20 ml of sterile STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH7.5]) was added. Protoplasts were pelleted by low-speed centrifugation (1,000 rpm) for 5 mins. The supernatant was carefully removed, and the protoplasts washed again in 20 ml of STC and pelleted by centrifugation as previously described. The protoplasts were then resuspended in 1.0ml of STC buffer and their concentration was adjusted using a spectrophotometer to 10^5 protoplasts ml^{-1}

siRNA design

Prior to siRNA design, the mRNA sequences of *A. niger* (Accession number XM_ 001394521.1) was obtained from NCBI database and used for designing the siRNA sequences. The designed sequences were subjected to i-score bioinformatics analysis [18]. This bioinformatic tool grade the sequences based on their biochemical attributes while the sequences with five higher scores were selected. To avoid off target effects, the selected siRNA sequences were challenged using the nucleotide alignment search tool (Blastn) against all reference sequences available and later against all filamentous fungi sequences [19]. Sequences with high homology to other genes were excluded. The top three siRNA sequences were then selected. These siRNA were named as *pks*_Ia, *pks*_Ib and *pks*_Ic designed to target *pks* genes of *A.niger*

and *Aspergillus carbonarius*. The designed siRNA oligonucleotides were obtained from ambion custom silencer® select (Life technologies). Silencer® Negative Control (Life technologies) with no sequence homology and labelled with pks_I0 was used to assess transfection efficiency and influence on ochratoxin A production. All siRNAs were resuspended in RNase free water at a final concentration of 6.3, 12.5, 25 and 50nM. In a sterile 1.5 mL micro centrifuge tubes, 10 µL of each siRNA was mixed with 1 µL of Lipofectamine™ RNAi MAX (Invitrogen Life Technologies, UK) and allowed to stand for 15 min at 20 °C. 19 µL of protoplasts (1×10^3) were added and mixed gently. The tubes were incubated at 20°C for 24 h to allow transfection to proceed. The efficiency of the transfection protocol was evaluated mathematically relative to control using difference in their fluorescence levels. Fluorescence difference between 0nM siRNA control and the *pks_I0* labelled negative control with and without the presence of transfection reagent permitted the identification of protoplasts by their fluorescence levels. Protoplast regeneration was enhanced by adding 70 µL of YES broth with 1.2 M of sorbitol to the transfection mixture and incubation at 25°C for another 24 h of this suspension. The entire 100 µL of protoplast suspension were spread in wheat agar medium (wheat extract 1L, peptone 20g, yeast extract 10g, glycerine 10g, agar 20g, pH 6.8-7) and incubated at 25 °C in the dark. All experiments were carried out using three biological replicates.

TABLE 1. DETAILS OF SIRNA SEQUENCES USED IN THIS STUDY

siRNA name	siRNA sequence	
pks_Ia	Sense strand	CCUCAUAAAACCAGGUUAA
	Antisense strand	UUAACCUGGUUUUAUGAGG
pks_Ib	Sense strand	UAUUUGAAGUCUCUGGGUA
	Antisense strand	UACCCAGAGACUUCAAAUA
pks_Ic	Sense strand	AUGAGAGACACCGGGUAUU
	Antisense strand	AAUACCCGGUGUCUCUCAU

Effect of siRNA on ochratoxin A production

OTA was extracted as described previously.

RESULTS

Comparison of *pks* promoter region from 15 isolates of ochratoxin A producing *Aspergillus* section *Nigri* showed differences in length and homology. The *pks* gene ranged from 218bp in a strain of the *Aspergillus niger* to 700bp in *Aspergillus carbonarius*. The alignment of the *pks* region of fifteen isolates revealed the presence of putative binding sites for homolog's of some known fungal transcription factors namely *Are A*, *Aba A* and *Pac C* while all the studied isolates lack the binding site for *Brl A*. Consequently, eight of the fifteen studied isolates had at least one *HGATAR/YTATCD* site. Isolate 6 however contained two of these sites separated by 30bp (table 1). Sequences for *Aba A* binding site (*CATTCT*) were found ranging between 79-685bp while the *CATTCC* binding site was found in isolate 4 and 14. Isolates 14 had the CATTCC at 139bp. The other (isolate 4) however, harbored two of these sequence separated by 379bp. The sequence identical to binding site for the transcription factor involved in pH regulation (*Pac C*) was found in five of the isolates, three of the four isolates (isolate 1, 4 and 7) having Pac C binding site corresponding to GCCAAG with isolate 7 also having the binding site CCTGGC together with isolate 6 (table 2). The siRNA designed to inhibit OTA production were successful while no significant variation was observed at concentration range of 6.3 to 50nM on the transfection efficiency. The opposite was the case with OTA inhibition as concentration significantly influence OTA inhibition in *Aspergillus carbonarius* but not on *Aspergillus niger* except for *pks*_Ia that had significant effectiveness at 25nM. Nevertheless, the inhibitory activities of the designed siRNAs on OTA were found to be relative with no significant statistical variation (P>0.05) (Figure 1-3).

TABLE: PRESENCE OF PUTATITIVE BINDING SITES FOR AREA IN THE PARTIALLY SEQUENCED *pks* GENE

Isolates	pks gene length	GATA1	GATA2	GATA3	GATA4	GATA5
		CTATCT	TTATCT	TTATCA	CTATCA	TTATCT
1	700bp	-	-	-	-	141
2	280bp	-	-	-	-	-
3	647bp	419	-	-	-	-
4	697bp	-	-	-	-	-
5	683bp	471	-	-	-	-
6	647bp	455/425	-	-	-	-
7	649bp	-	-	-	275	-
8	220bp	-	-	-	-	-
9	622bp	-	-	-	-	-
10	551bp	433	-	-	-	-
11	650bp	433	-	-	-	-
12	600bp	433	-	-	-	-
13	625bp	557	-	-	-	-
14	218bp	-	-	-	-	-
15	526bp	-	-	-	-	-

TABLE 3: PUTATITIVE BINDING SITES FOR OTHER FUNGAL TRANSCRIPTIONAL FACTORS IN THE PARTIALLY SEQUENCED pks GENE

Isolates	*pks* gene length	Aba site A CATTCT	Aba site 2 CATTCC	Brl A site 1 AGAGGGG	Brl A site2 CAAGGGA	PacC site 1 CCTGGC	PacC site 2 GCCAAG
1	700bp	-	-	-	-	-	141
2	280bp	-	-	-	-	-	-
3	647bp	-	-	-	-	-	-
4	697bp	-	-306/685-	-	-	-	-17-
5	683bp	-79-	-	-	-	-	-
6	647bp	-	-	-	-	-	-594-
7	649bp	-344-	-	-	-175-	-	-491-
8	220bp	-	-	-	-	-	-
9	622bp	328	-	-	-	-	-
10	551bp	109	-	-	-	-	-
11	650bp	109	-	-	-	-	-
12	600bp	109		-	-		-
13	625bp	-	-	-	-	-	-
14	218bp	-	-	-	-	-	-
15	526bp	-	-	-	-	-	-

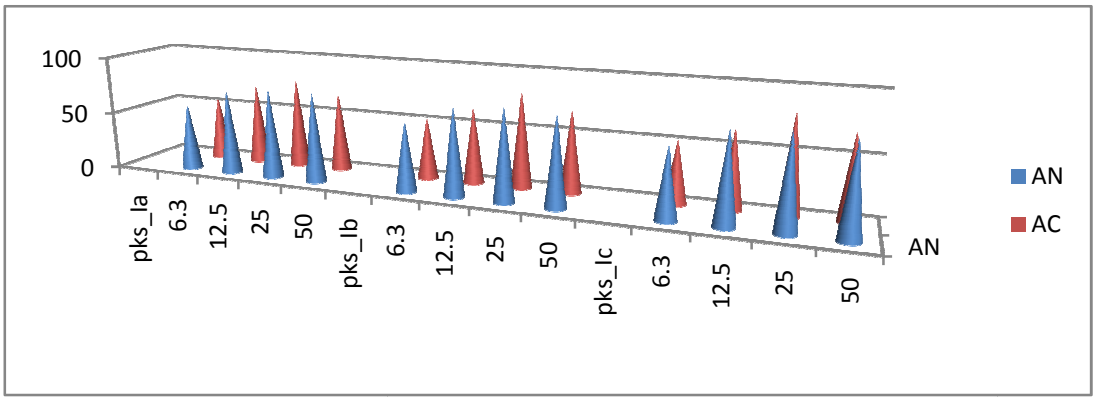

FIGURE 1: INFLUENCE OF DIFFERENT CONCENTRATION OF siRNA ON TRANSFECTION EFFICIENCY

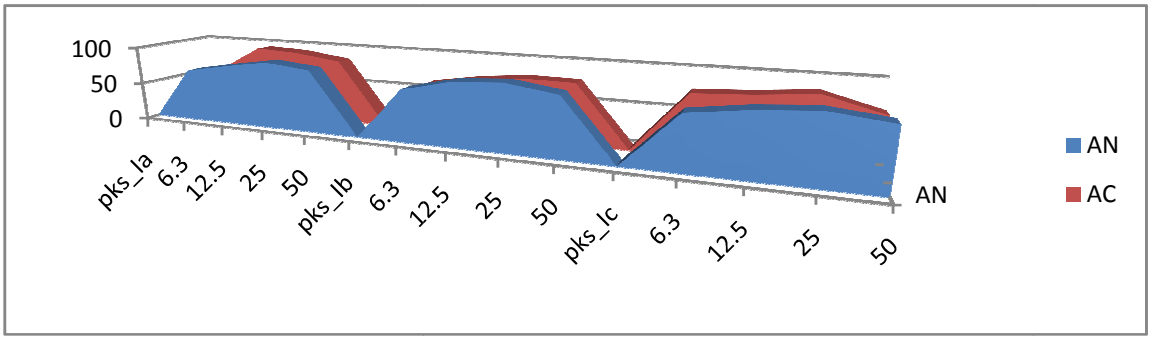

FIGURE 2: INFLUENCE OF DIFFERENT CONCENTRATION OF siRNA ON OTA INHIBITION

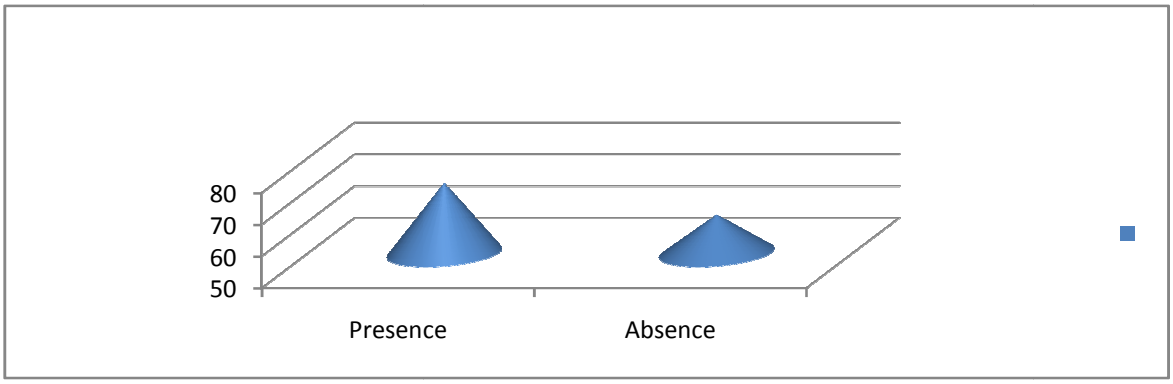

FIGURE 3: INFLUENCE OF THE PRESENCE OF LIPOFECTAMINE ON TRANSFECTION EFFICIENCY

DISCUSSION AND CONCLUSION

The use of bioinformatic tools for sequence comparison in order to deduce important gene structure and functions have been well documented. In this study, *pks* gene promoter sequence as well as several fungal transcriptional factors compared revealed variation in homology and length. This observation is not unexpected as such observation has been reported for *Aspergillus* section *Flavi* [20](Ehrlich *et al.*,1999). However, our findings are a strong pointer that these genetic traits may not be necessary among the factors that triggers ochratoxin A production. Sequence differences in the *pks* region and promoter structure provide a basis for predicting the roles of environmental and developmental cues in

differential regulation of ochratoxin production among ochratoxin A producing fungi. The possibility of inhibition or stimulation of ochratoxin A production by different nitrogen sources was assessed with the presence or absence of *Are A*-binding sites of *pks* gene. Results obtained revealed a considerable 66.7% of the total isolates harboring these gene and associated high level of OTA. The variability of GATA sites in the *pks* region and differences among fungi in ochratoxin A production provides a way to test the role of nitrogen sources on transcriptional regulation of ochratoxin A biosynthesis genes. Transcription factors *Aba A* and *Brl A* may mediate expression of genes involved in development-specific processes in fungi [21], but have not yet been directly implicated in regulation of secondary metabolite biosynthesis. Putative *Brl A*-binding sites was absent in the *pks* region of all isolates. This observation may not be unconnected to the fact that this important gene is not involved in OTA biosynthesis. The presence or absence of *Pac C*-binding sites in the *pks* region could partly account for differential sensitivity of ochratoxin A production to pH in different species of *Aspergillus* [22]. Precedent for interference by *Pac C* in the expression of acid-expressed genes has been reported for the *gab A* gene in *A. nidulans* [23]. In this study, *Pac C* site was found in only five of the total of the fifteen isolates representing only 33.3%. This observation could be suggesting that pH may not be that important in the regulation of ochratoxin A gene. One of such method could be the use of RNA interference which is a post transcriptional silencing technique. In this study, the treatment of fungal protoplasts with synthetic siRNAs directed toward the *pks* gene has been shown to effectively silence ochratoxin A production [17,24]. The siRNA designed to inhibit OTA production were successful while no significant variation was observed at concentration range of 6.3 to 50nM on the transfection efficiency. The opposite was the case with OTA inhibition as concentration significantly influence OTA inhibition in *Aspergillus carbonarius* but not on *Aspergillus niger* except for *pks*_Ia that had significant effectiveness at 25nM . This observation corroborated that of Abdel-Hadi *et al.*[17] that reported 25nM concentration as the most effective for inhibiting biosynthetic gene *AflD* and the regulatory genes *AflR/AflS* in *Aspergillus flavus* and *Aspergillus parasiticus*. Moreover, Goncalo [25] reported concentration of 10nM as the optimum for silencing *Otapks PV* gene while 25nM was reported for *Tri5*. The disparity observed in the various study is an indication that optimum concentration for different siRNA varies according to the targeting gene. Our findings further assert that RNA interference could be the needed technique required to control ochratoxin A production as its effect was immediately noticed after just the fifth day of the experiment. This may not be unconnected to the fact that the fungal protoplast maximally uptake the designed siRNAs [17]. The fact that efficient siRNA transfection was significantly higher in the presence of lipofectamine is not unexpected as this reagent has been reported to enhance uptake of siRNA in a medium [17,25]. The lack of any effect on OTA production observed with the control (*pks_I0*) is an indication that inhibition observed with the designed siRNA in this study are not caused by transfection conditions and/or due to off target effects. Nevertheless, the inhibitory activities of the designed siRNAs on OTA were found to be relative with no significant statistical variation ($P>0.05$). This finding suggest that such inhibitory activities may be due to sequence specific nature of our siRNA. Even though, this technique remain very promising, there is a need to further elucidate the exogenous expression of dsRNA by plant and the uptake of these molecules by their pathogens in future studies .

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REVIEW ARTICLE

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CONTAINMENT OF EBOLA – STEPS TO PREVENT SPREAD OF EMERGING INFECTIOUS DISEASES, THE NIGERIA EXAMPLE

Peter, Y.J.

Department of Medical Microbiology and Parasitology, College of Health Sciences, University of Abuja, Nigeria.

Correspondence: Dr.Y.J. Peter;drjonahpeter@gmail.com

ABSTRACT

Emerging infectious diseases are diseases whose incidence in humans have increased in the past decades or threaten to increase in the near future. The epidemiological and Laboratory techniques needed to detect, investigate and contain a deliberate outbreak are the same as those used for natural outbreaks. The threat to Nigeria posed by the arrival in Lagos of a patient acutely ill with Ebola was potentially enormous. Six response teams were deployed within the Emergency Operations Centre; 1) Epidemiology/ Surveillance, 2) Case Management/ Infection Control, 3) Social mobilization, 4) Laboratory Services, 5) Point of Entry and 6) Management / Coordination. The strategy group reviewed and approved all of the teams work and needed resources. Eleven patients with laboratory confirmed Ebola were admitted and discharged, an additional patient was diagnosed at convalescent stage while eight patients died. Several issues were observed by the response team during Nigeria Ebola outbreak that could in retrospect have been mitigated through additional preparedness planning for public health emergencies:- 1) Financial resources were slow to arrive, 2) Political leadership did not appreciate the enormous consequences that even a small Ebola outbreak could have on civil institutions, 3) Lack of Nigerian health workers willing to care for Ebola because of a lack of information and training on how to care for Ebola patients, 4) Inappropriate coordination of private sector engagement and 5) Partners and parts of government were unfamiliar with EOC/IMS system and its use as a means of streamlining coordination and response elements into one unified approach.

LE CONFINEMENT DE L'EBOLA - MESURES POUR PRÉVENIR LA PROPAGATION DES MALADIES INFECTIEUSES ÉMERGENTES, LE NIGERIA PAR EXEMPLE

Peter, Y.J.

Département de microbiologie médicale et parasitologie, Collège des Sciences de la santé, l'Université d'Abuja, Nigéria.

Correspondance : DrY.J. Peter;drjonahpeter@gmail.com

Résumé

Les maladies infectieuses émergentes sont des maladies dont l'incidence chez les humains ont augmenté au cours des dernières décennies ou menacer d'augmenter dans un avenir proche. Les techniques de laboratoire et épidémiologiques nécessaires pour détecter, enquêter et contenir une épidémie délibérée sont les mêmes que celles utilisées pour les éclosions. La menace pour le Nigéria posés par l'arrivée à Lagos d'un patient gravement malade avec le virus Ebola est potentiellement énorme. Six équipes d'intervention ont été déployés dans le centre des opérations d'urgence ; 1) de l'épidémiologie et de surveillance, 2) La gestion de cas/ Contrôle de l'infection, 3) la mobilisation sociale, 4) Les Services de laboratoire, 5) le point d'entrée et 6) Gestion / Coordination. La stratégie groupe a examiné et approuvé l'ensemble des équipes de travail et les ressources nécessaires. Onze patients atteints d'Ebola confirmé en laboratoire ont été admis et déchargée, un autre patient a été diagnostiqué au stade de convalescence alors que 8 patients sont morts. Plusieurs questions ont été observés par l'équipe d'intervention pendant le Nigeria l'éclosion d'Ebola qui pourrait rétrospectivement ont été atténuées par la planification des mesures supplémentaires pour les urgences en santé publique :- 1) ressources financières ont été lentes à arriver, 2) Le leadership politique n'a pas apprécié les conséquences énormes que même une petite épidémie d'Ebola pourrait avoir sur les institutions civiles,

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3) le manque de travailleurs de santé nigérians prêts à s'occuper de l'Ebola en raison d'un manque d'information et de formation sur la façon de soigner les patients d'Ebola, 4) Pas de la coordination de l'engagement du secteur privé et 5) Partenaires et parties du gouvernement n'étaient pas familiers avec l'EOC/IMS système et son utilisation comme moyen de rationaliser la coordination et des éléments de réponse dans l'une approche unifiée.

INTRODUCTION

Emerging infectious diseases are diseases that: -

1. Ebola cases have not occurred in humans before (this type of emergence is difficult to establish and is probably rare (1), or
2. Have occurred previously but affected only small numbers of people in isolated places (AIDs and Ebola hemorrhagic fever are examples (2);
3. Have occurred throughout human history but have only recently been recognized as distinct diseases due to an infectious agent (3).

Many specialists in infectious diseases include re-emerging diseases as a subcategory of emerging disease (4). An infectious disease emerging in a single country is a threat to all countries (5).

Despite the challenges of emerging and re-emerging infectious diseases, the results of basic research, show that there is a reason for hope (6). Acquired immune deficiency syndrome (AIDs was first described in 1981, and it took two years to identify the retrovirus that causes it, which named Human Immunodeficiency Virus (HIV) (7). The development of polymerase chain reaction (PCR), a powerful new research technique allows rapid identification of causative agents of infections (8). Recommendations for avoiding and/or treating of new infectious diseases become possible when new techniques, developed through basic research are applied to the problem of disease emergence (9).

Ebola Virus Disease

Ebola virus disease (EVD) is a severe, potentially life threatening illness caused by *Ebola virus (10). Ebola virus* is an enveloped RNA virus about 80x800-1400 nm in size whose survival is dependent on an animal reservoir (11). An international team of researchers have sequenced 99 *Ebola virus* genomes and also observed a rapid increase in its genetic variation (9). However, more recent molecular data obtained from the outbreaks in Kikwit and Gabon did not find any molecular evidence for adaptation during human to human transmission (12). Infection results in severe disease with high mortality rate among infected humans and other primates (13). I has no known cure or vaccine; treatment is only supportive (8). Ebola virus is considered a potential biological weapon candidate (11).

On the 23rd of March 2014, the World Health Organization (WHO) issued its first communiqué on a new outbreak of EVD, which began in December 2013 in the republic of Guinea (14). Located on the Atlantic coast of West Africa, Guinea became the first country in this geographical region to report an EVD outbreak with more than one case reported. The 2014 Ebola epidemic is the largest in history, affecting multiple countries in West Africa (15).

The Nigeria Ebola Outbreak

On July 20th 2014, an acutely ill traveler from Liberia arrived at the Murtala Mohammed International airport in Lagos, Nigeria and was admitted to a private hospital (16). Based on the patient's failure to respond to malaria treatment and his travel from an Ebola affected country in the region, treating Physicians suspected EVD. The patient was isolated and tested for *Ebola virus* infection while local public health authorities were alerted about a suspected case of EVD (16). He was later confirmed to have EVD. This index patient potentially exposed 72 persons at the airport and the hospital (14). The Federal Ministry of Health with guidance from the Nigeria Centre for Disease Control (NCDC) thereafter, declared an Ebola emergency. On July 23rd, the Federal Ministry of Health with the Lagos State government and International partners activated an Ebola Incident Management Centre (IMC) as a precursor to the current Emergency Operations Centre (EOC) to rapidly respond to this outbreak. On August 8th, the WHO declared the epidemic to be a public health emergency of International concern (17).

Port Health Services conducted early contact tracing at the airport and worked with airline and partners to ensure notification of the outbreak through the International Health Regulations mechanisms (18).

The EOC case-management team took over the management of each laboratory confirmed or suspected case, triaged potential patients, and decontaminated areas inhabited by them. Patients with suspected infection were isolated in suspected case ward at the Ebola treatment facilities(9), initially in Lagos, Western Nigeria and subsequently in Port Harcourt, South east Nigeria. A contact tracing team staffed and supervised by skilled, dedicated epidemiologists was established to investigate all primary contacts and alert the case management team of symptomatic contacts for assessments and possible reclassification. A suspected case was reclassified as a confirmed case if reverse transcription polymerase chain reaction (RT-PCR) detected *Ebola virus* in a blood specimen, and was ruled out if RT-PCR testing of two blood specimens collected at least 48 hours apart was negative.[16]Additionally, testing for anti-*Ebola virus* immunoglobulin G, indicating an immune response to *Ebola virus*, was added to the testing protocol for PCR-negative suspected cases in persons with some symptoms who were epidemiologically linked to subsequent confirmed cases (19). When a contact became ill with suspected case, the contact tracing team gathered data on persons exposed to that contact from the date of symptom onset in the event the suspected case should become laboratory confirmed (20). Having the capacity to conduct Ebola laboratory diagnosis in-country facilitated rapid identification of confirmed cases and quick discharge of persons with suspected EVD who tested *Ebola virus* negative.

The Nigeria Response

The threat to Nigeria posed by the arrival in Lagos of a patient acutely ill with Ebola virus infection was potentially enormous (21). The implementation of a rapid response that made use of the available public health assets was the highest priority at the onset of the outbreak, as was organizing the response using proven structures for the delivery of public health in Nigeria.[22] Initially, NCDC and the Lagos State Minis try of Health established an IMC which served as the overall implementing arm of the national response. The initial IMC was subsequently recast as the national EOC, in line with Incident Management Systems (IMS) nomenclature and national structures aimed at emergency response. The EOC expanded its operations to Rivers state when cases emerged there, and oversaw the monitoring of contacts in Enugu state with state health officials as part of the early outbreak response.[22]There was a stated expectation that all partner organizations, donors and response teams would work through the EOC structure, reporting to an Incident Manager (IM). In turn, the IM would be responsible to deliver accountable and transparent results to the NCDC and the Federal Ministry of Health. The IM responsible for oversight of the response was selected based on IMS experience and competency rather than rank in government or public service.

The Nigeria response benefited from the rapid use of its national public institution (i.e. NCDC), previous outbreak responses such as a major lead poisoning response in 2010, and its recent experience with polio eradication. In October 2012, responding to the declaration by the World Health Organization of polio eradication as a global public health emergency and to improve its national response, the Government of Nigeria used the IMS to establish a national EOC as part of a new national emergency plan for the global polio eradication initiative. The use of IMS through the EOC changed the operational tempo, accountability measures and success of the polio eradication program. Indicators and dashboard (electronic displays of high level indicators for each response team monitored at the EOC) were developed to increase accountability of the program staff and spending. Through the EOC and the Nigeria Field Epidemiology and Laboratory Training Program (NFELTP) polio activities, State health systems strengthening and preparedness was prioritized.[16]

Six response teams were developed within the EOC specific to an Ebola response including; 1) Epidemiology/Surveillance, 2) Case Management / Infection Control, 3) Social Mobilization, 4) Laboratory Services, 5) Point of Entry, and 6) Management / Coordination (23). Terms of reference and priority activities were developed by the strategy team to guide each operational team's work; operational teams develop their own staffing list, list of materials and financial needs and a goal oriented operational plan (23,24). The strategy group reviewed and approved all of the teams work and needed resources. Technical partners assigned staff throughout the operational teams in technical

advisory roles aimed at building the capacity of the local teams and ensuring quality work (21).

The Impact of the Nigeria Response

The index patient died on July, 25th; as of September 24th, there were 19 Laboratory confirmed Ebola cases and one probable case in two states, with 894 contacts identified and followed during the response (25). No new cases had occurred since August 31, suggesting that the Ebola outbreak in Nigeria might be contained.[25] The EOC established quickly and using an IMS to coordinate the response and consolidate decision making, is largely credited with helping contain the Nigeria outbreak early. National public health emergency preparedness agencies in the region, including those involved in Ebola responses, should consider including the development of an EOC to improve the ability to rapidly respond to urgent public health threats (26).

The Lessons Learnt

Several issues were observed by the response team during the Nigeria EVD outbreak that could in retrospect, have been mitigated through additional preparedness planning for public health emergencies (27).

First, financial resources were slow to arrive at the EOC, a delay that threatened to impede the rapid expansion of containing activities across the response (24). National preparedness efforts should consider how resources can be quickly made accessible to fund the early stage of the response.

Second, there were discrepancies among the levels of political leadership in fully appreciating the enormous consequences that even a small Ebola outbreak could have on civil institutions such as hospitals, airports and public gatherings.[16]Targeted education about the urgent need to fund, staff and supply a response effort was provided to political leadership and should be considered for preparedness efforts elsewhere (27). Similarly, the Nigerian public did not have specific information about *Ebola virus* infection and early information provided by the press, in advance of official information from the health authorities was sometimes inaccurate and created a nationwide scare.[10,26] This scare resulted in some in some persons resorting to extreme and sometimes harmful and ineffective measures to avoid infection such as consuming large quantities of salt water, even in places distant from the outbreak. Both issues could have been addressed through preparedness activities that focused on education and planning, as well as explaining *Ebola virus* infection to the public and describing how to respond should *Ebola virus* arrived in Nigeria (28).

The case management team indicated that early efforts to establish an isolation ward were delayed due to a lack of Nigerian health care workers willing to care for patients with Ebola because of a lack of information and training about how to care for Ebola virus infected patients without getting infected and because care providers had been disproportionately impacted by EVD in other affected countries (29). Preparedness activities should include orientation and training of physicians, nurses and attendants to safely provide services with attention to infection control procedures and quality EVD treatment at an appropriately designed facility.

Another challenge was ensuring appropriate coordination of private sector engagement. The EOC system facilitated improved coordination through the designation of the Management and Coordination Team Lead as the private sector point of contact.

Finally, some partners and parts of government were unfamiliar with the EOC/IMS and its use as a means of streamlining coordination and response elements into on unified approach. The government led EOC process could define opportunities for partners to place staff strategically in the national and local response efforts and could encourage this through the EOC response teams and management system. Further, EOC mechanisms should be tested through strategic exercises and use in otheremerging infection response (17).

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ORIGINAL ARTICLE

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TRICHOMONAS VAGINALIS INFECTION AMONG ADOLESCENT GIRLS IN SOME SECONDARY SCHOOLS IN BENIN CITY, EDO STATE, NIGERIA

Akinbo, F. O. & Oronsaye, I. S.

Department of Medical Laboratory Science, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria.

Correspondence: Frederick O. Akinbo Tel: +2348033796874 E-mail addresses: fgbengang@yahoo.com

RUNNING TITLE: TRICHOMONIASIS AMONG ADOLESCENT GIRLS

ABSTRACT

*Trichomonasvaginalis*is the most common non-viral sexually transmitted disease (STD) and one of the neglected parasitic infections. This study aimed to determine the prevalence of *T. vaginalis*infection among adolescent girls in some secondary schools in Edo State, Nigeria. A total of 272 girls were recruited in this study. The participants' age ranged from 13 to 18 years. Two high vagina swab specimens were collected from each participant. The specimens were analyzed using wet mount examination and culture methods. A prevalence of 9.2% of *T. vaginalis* was observed among adolescent girls in this study. Level of education of parents, occupation of mothers, sexual relationship and clinical manifestation of participants significantly affected the prevalence of *T. vaginalis*infection among adolescent girls attending secondary school. Culture method detected more cases of *T. vaginalis* infection than the wet mount technique. The use of culture method in routine diagnosis of *T. vaginalis*infection is advocated as this will reduce cases of infertility resulting from undetected and untreated infection as well as the risk of HIV transmission and acquisition.

Key words: Adolescent girls,Benin City, *Trichomonasvaginalis*

TRICHOMONAS VAGINALIS INFECTION CHEZ LES ADOLESCENTES DANS CERTAINES ÉCOLES SECONDAIRES À BENIN CITY, EDO STATE, NIGERIA

Akinbo, F O & Oronsaye, I. S.,

Ministère de la Science de Laboratoire Médical, l'École des Sciences Médicales de Base, de l'Université du Bénin, Nigéria, Benin City

Correspondance : Frederick O. Akinbo Tel : 2348033796874 E-mail : fgbengang@yahoo.com

TITRE COURANT: LA TRICHOMONASE PARMI LES ADOLESCENTES

Résumé
Trichomonas est le plus souvent non virale maladie transmise sexuellement (MTS) et l'une des infections parasitaires négligées. Cette étude visait à déterminer la prévalence de l'infection à T. vaginalis chez les adolescentes dans certaines écoles secondaires à Edo State, Nigeria. Un total de 272 jeunes filles ont été recrutés dans cette étude. L'âge des participants variait de 13 à 18 ans. Deux spécimens ont été prélevés du vagin-tige de chaque participant. Les spécimens ont été analysées à l'aide de l'état frais et l'examen des méthodes de culture. Une prévalence de 9,2 % de T. vaginalis a été observée chez les adolescentes dans cette étude. Le niveau de scolarité des parents, la profession des mères, des relations sexuelles et de manifestation clinique des participants touchés de manière significative la prévalence de l'infection à T. vaginalis chez les adolescentes fréquentant l'école secondaire.

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Méthode de culture détecté plus de cas d'infection à T. vaginalis de la technique de montage humide. L'utilisation de la méthode de culture dans la routine diagnostic de l'infection à T. vaginalis est préconisé comme cela va réduire l'infertilité résultant d'une infection non traitée ainsi que le risque de transmission du VIH et d'acquisition.

Mots clés : adolescentes, Benin City, *Trichomonas vaginalis*

INTRODUCTION

Globally, *Trichomonas vaginalis*is the most common non-viral sexually transmitted disease (STD) and one of the neglected parasitic infections (1). According to WHO, an incidence of 276 million new cases each year and a prevalence of 187 million of infected individuals with ages 15 and 49 years old were reported (2). The global prevalence of trichomoniasis is much higher than other curable STDs such as gonorrhea and syphilis, both counting for 36.4 million cases, and *Chlamydia* infection, with 100.4 million of infected adults (3).

Sexually transmitted infections (STIs) are the most common public health problem in Africa and high rates of STI in adolescents and young adults indicate their vulnerability to HIV infection (4). *T. vaginalis*has a cosmopolitan distribution and has been found in all racial group and socioeconomic strata; with no seasonal variability. Trichomoniasis commonly associated with other sexually transmitted diseases (5, 6). The preferred cells infected by *T. vaginalis*are those of squamous epithelium origin involving the vagina, urethra as well as the endocervix (7, 8). This parasitic infection is frequently asymptomatic in adults. However, symptomatic women may complain of vaginal discharge, vulvovaginal soreness, pruritus, odour and irritation (8, 9). The disease is a recognized cause of urethritis associated with scanty, clear to mucopurulent discharge, dysuria, and burning sensation immediately after sexual intercourse (10). Complications of this disease in both men and women have been reported and they include increased risk of HIV, premature rupture of membranes, premature labour, pelvic inflammatory disease, low birth weight, infection of the adnexa, endometrium and bartholin glands (in women), prostatitis, balanoposthitis, epididymo-orchitis (in men) and possibly infertility (9, 11, 12, 13, 14). It has been reported that *T. vaginalis*infection in adolescent girls is likely to remain undetected and untreated, causing increasing morbidity and reducing their fertility as well as increasing their risk of other reproductive tract infection and human immunodeficiency virus (15). This has not been well studied in our locality therefore, this study aimed to determine the prevalence of *T. vaginalis*infection among adolescent girls in some secondary schools in Edo State, Nigeria.

MATERIALS AND METHODS

Study area

This study was conducted at some secondary schools in Benin City, Edo State, Nigeria. Edo State is situated in the Midwestern part of Nigeria. The state is located within the low rain forest zone of Nigeria and has two seasons, dry and wet. The dry season lasts from mid-October to March or April while the raining season lasts from April to September. Edo State has a population of about 3, 218, 332 (National Population Census, 2006). The secondary schools were: Ezomo College, Iyowa; Ebomisi Secondary School, Ugbogiobo; Oba Erediuawa Secondary School,Utekon; Uwelu Secondary School, Uwelu and Egor Secondary School, Egor, Edo State, Nigeria. These secondary schools are situated in Egor and Ovia Northeast Local Government Areas of Edo State, Nigeria.

Study population

A total of 272 girls were recruited in this study. The participants' age ranged from 13 to 17 years. Serial sampling method was used and participants attending secondary schools whose parents or guardians gave consent were recruited for this study. Adolescent girls that did not return their consent form from their parents or guardians were excluded from the study. Information on demographic characteristics was obtained from participants by administering a well-structured questionnaire bothering on biodata, sexual relationship and personal hygiene. The Ethics and Research Committee of the Ministry of Health, Edo State approved the protocol of this study.

Specimen collection

Two high vagina swab specimens were collected from each participant. The specimens were analyzed using wet mount examination and culture methods were employed. Briefly, a drop of normal saline was added to the exudate collected with the swab and mixed. A drop of the emulsified vagina exudate was placed on a grease free slide, covered with a coverslip and examined microscopically for the presence of *T. vaginalis* (16). The Dorset culture medium was the culture medium used where the exudate on the second swab was inoculated on the medium and kept in the incubator anaerobically at 37^{o}C for 3 days. Detection of growth was checked daily for *T.*

vaginalis.The culture plates were examined for growth of *T. vaginalis* on days 1, 2 and 3 (17).

Statistical analysis

The frequency data were compared using the Chi square (X^2) whereas the odd ratio was calculated for the potential risk factors. The INSTAT software (Graph PAD software Inc, La Jolla, CA) was used in the analyses.

RESULTS

Out of the 272 participants, 15 (5.5%) and 25 (9.2%) were positive for *T. vaginalis*infection using wet mount and culture methods respectively. In addition, diagnostic method did not significantly affect the prevalence of trichomoniasis among the secondary school girls (*P*=0.1385) with the culture method recording more cases of *T. vaginalis*(Table 1).

TABLE 1: DIAGNOSIS OF *T. VAGINALIS* BY WET MOUNT AND CULTURE TECHNIQUES

Method of diagnosis	No. sampled	No. positive (%)	OR	95% CI	P value
Wet mount	**272**	**15(5.5)**	**0.5767**	**0.296, 1.120**	**0.1385**
Culture	**272**	**25(9.2)**	**1.734**	**0.893, 3.368**	

***P*<0.05**

TABLE 2: RISK FACTORS ASSOCIATED WITH *T.VAGINALIS* INFECTION AMONG ADOLESCENT GIRLS

Characteristic	No. sampled	No. positive (%)	OR	95% CI	P value
Age					
13-15	129	14(10.8)	1.461	0.637, 3.345	0.4058
16-18	143	11(7.7)	0.684	0.298, 1.568	
Religion					
Muslim	258	23(8.9)	0.587	0.123, 2.787	0.3752
Christian	14	2(14.3)	1.703	0.3588, 8.083	
Educational status of Participants' father					
None	16	0(0)			0.0138
Primary	65	12(18.5)			
Secondary	170	11(6.5)			
Tertiary	21	1(4.8)			
Educational status of Participants' mother					
None	10	0(0)			0.0264
Primary	**51**	10(19.6)			
Secondary	166	13(7.8)			
Tertiary	45	2(4.4)			
Occupation of participants' father					
Farmer	48	5(10.4)			0.1556
Business man	169	18(10.7)			
Civil servant	55	2(3.6)			
Occupation of participants' mother					
Farmer	30	6(20.0)			0.0153
Business man	201	19(9.5)			
Civil servant	48	0(0)			
Location					
Rural	179	21(11.7)			0.0912
Semi-urban	42	3(7.1)			
Civil servant	51	1(1.9)			
Sexual relationship					
Yes	94	21(22.3)	12.514	4.149, 37.740	<0.0001
No	178	4(2.3)	0.079	0.026, 0.241	
Type of toilet					
Bush	26	3(11.5)			0.7328
Pit latrine	71	5(7.0)			
Water cistern	175	17(9.7)			

Age (P=0.4058), religion (*P*=0.3752), occupation of father (*P*=0.2793), location (*P*=0.0913), and type of toilet (*P*=0.7328) did not significantly influence the prevalence of trichomoniaisis among adolescent girls attending secondary schools. Educational level of father and mother of participants significantly

affected the prevalence of *T. vaginalis*infection in this study (*P*=0.0138 and *P*=0.0264 respectively). Occupation of mother affected significantly the prevalence of trichomoniasis among the secondary school girls (*P*=0.0153) with mothers who are farmers having the highest prevalence (20%). Sexual intercourse was a risk factor for acquiring *T. vaginalis*infection among adolescent girls attending secondary school (OR=12.514; 95% CI= 4.149, 37.740; *P*<0.0001). (Table2).

DISCUSSION

*Trichomonas vaginalis*is one of the sexually acquired reproductive tract infections and has been labeled a silent epidemic among females, causing gynaecological morbidity and maternal mortality globally (18). Besides, the main issue concerning trichomoniasis is its relationship with serious health consequences like cancer, adverse pregnancy outcomes, infertility and HIV transmission and acquisition (13, 14, 19, 20, 21, 22). The undetected and untreated *T. vaginalis* infection can result in increasing morbidity and infertility among adolescent girls (15). There is a dearth of information on trichomoniasis among adolescent secondary school girls in Edo State, Nigeria.

T. vaginalis was observed in 25 (9.2%) out of the 272 adolescent girls in this study using the culture methods whereas 15 (5.5%) from the 25 infected participants were positive for *T. vaginalis* using the wet mount preparation. Others reported similar findings (17, 23, 24). Direct microscopic examination of the vaginal specimen remains the most widely used diagnostic test for *T. vaginalis* infection (10). Culture methods have been reported as the current gold standard for the detection of *T. vaginalis* and should be considered for widespread clinical use (17, 25). We suggest the inclusion of culture methods in the screening of *T. vaginalis*.

Generally, an overall prevalence of 9.2% of trichomoniasis was observed among adolescent secondary school girls in this study. The prevalence of *T. vaginalis* observed in this study is similar to the 9.3% reported by Obunge *et al.* (26) in Port-Harcourt, Rivers State. However, the prevalence of 9.2% of *T. vaginalis* infection reported in this study is lower than the 25.9% and 24.6% reported among school girls in Zambia (27, 28). The observed difference in prevalence could be attributed to geographical locations, personal hygiene and diagnostic methods used. There is the need to put in place drastic measures otherwise, the high prevalence of *T. vaginalis*infection observed in adolescent girls is likely to remain undetected and untreated, causing a rising pool of infection in the local population, increasing morbidity and reducing their fertility as well as increasing their risk of other reproductive tract infection and human immunodeficiency virus (15).

It has been observed that the incidence of sexually transmitted diseases including *T. vaginalis* infection is highest among 15 to 30 years age group (29). The 13-15 years age group had the highest prevalence of trichomoniasis (10.8%) when compared with the 16-18 years age group (7.7%). In addition, age was not a risk factor for acquiring *T. vaginalis* infection (P=0.5978) among adolescent girls attending secondary schools.

Adolescent girls that are Muslims presented with the highest prevalence (14.3%) of *T. vaginalis* infection when compared with their Christian counterparts (8.9%). However, religion was not a risk factor for acquiring *T. vaginalis* infection among the adolescent girls attending secondary schools (P=0.3752).

Individuals with poor personal hygiene and low socio-economic status have been reported to have an increased risk of *T. vaginalis* infection (30). Adolescents that are from home with poor educational background are likely to have poor personal hygiene standards. Level of education of parents of the school girls significantly affected the prevalence of *T. vaginalis* infection (P<0.05) with the girls from father and mother that had primary school education having the highest prevalence of 13.3% and 19.6% respectively. Low level of education has been reported to play a major role in epidemiology of parasitic diseases (2).

Occupation of participants' father did not significantly affect the prevalence of *T. vaginalis* infection among the adolescent girls (P=0.2793). However, the occupation of participants' mother greatly affected the prevalence of trichomoniasis among the adolescent girls attending secondary school (P=0.0153) with the girls whose mothers are farmers having the highest prevalence (20.0%). Most farmers are believed to be of poor educational background which is likely to impart on their level of personal hygiene as well as that of their girl child. This may explain the reason for this finding.

Improvement in personal hygiene is more likely to be observed in the urban settings due to access to sexual and reproductive health education from several media as well as the availability of reproductive health services which include treatment and counseling services (15). Girls from the rural settings had the highest prevalence (11.7%) of trichomoniasis when compared with other locations. However, location of adolescent girls did not affect significantly the prevalence of trichomoniasis in this study (P=0.0912). This finding is inconsistent with the report of Nazari*et al.* (17) that had higher prevalence of *T.*

vaginalis infection from the urban dwellers than their rural counterparts. The reason for this difference is unclear.

The relationship between bacterial vaginosis, *Neisseria gonorrhoeae, Chlamydia trachomatis*, genital ulcer disease, and the increase in the risk of HIV transmission make early detection and diagnosis of reproductive tract infection particularly *T. vaginalis*, a major public health imperative (31, 32, 33, 34, 35, 36). *T. vaginalis* primarily infects the squamous epithelium of the genital tract where it replicates by binary fission. It is transmitted among human host mainly by sexual intercourse (37). In this study, the prevalence of trichomoniasis was strongly associated with sexual intercourse. In addition, sexual intercourse appears to have 4-37-fold increased risk of acquiring *T. vaginalis* infection among adolescent girls (P<0.0001). Adolescent girls that have had sexual intercourse presented with the highest prevalence (22.3%) when compared with those that did not have sexual (2.3%). The incidence of *T. vaginalis* infection has been reported to depend mainly on sexual activity (3). This observation may explain our finding.

Participants that used bush as a type of toilet had the highest prevalence (11.5%) of trichomoniasis when compared with other types of toilet (pit latrine 7.0%; water cistern 9.7%). In addition, type of toilet did not significantly affect the prevalence of trichomonias is among adolescent girls attending secondary school (*P*=0.7328). This finding is consistent with the report of Crucitti*et al.* (28). The reason for this finding is unclear.

CONCLUSION

An overall prevalence of 9.2% of *T. vaginalis* was observed among adolescent girls in this study. Level of education of parents, occupation of mothers and sexual relationship of participants significantly affected the prevalence of *T. vaginalis*infection among adolescent girls attending secondary school. Culture method detected more cases of *T. vaginalis* infection than the wet mount technique. The introduction of culture methods to routine diagnosis of *T. vaginalis*infection is advocated as this will reduce cases of infertility resulting from undetected and untreated infection as well as the risk of HIV transmission and acquisition.

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IMPACT OF MALARIA ON INFLAMMATORY PROTEINS, HAEMATOLOGICAL AND BIOCHEMICAL INDICES IN PREGNANCY

Digban, A.K.[a],Digban, T.O.[b],Adelabu, A.O.[b,*], &NdukweaM. V.

[a]Department of Medical Laboratory Science, College of Health Science, Igbinedion University Okada, PMB 001, Okada Edo State, Nigeria; [b] Department of Biochemistry and Microbiology,University of Fort Hare, Private Bag X1314, Alice 5700, Eastern Cape Province, South Africa

•Correspondence: Digban, A. Kester, Department of Medical Laboratory Science, College of Health Science, Igbinedion University Okada, PMB 001, Okada Edo State, Nigeria

Telephone: +2348055276353, E-mail: kdigban@yahoo.com

ABSTRACT

Malaria morbidity and mortality has remained a major health burden in the developing countries especially in tropical Africa. Thus malaria association in pregnancy and its associated complication remains a major health problem to the expectant mothers. In this study a total of five hundred and fifty (550) blood specimens were obtained from both pregnant and non-pregnant mothers with and without malaria *parasitaemia* who consented to the study. Selected biochemical and haematological parameters were assessed using conventional methods. The result showed that the malaria parasite species isolated was *plasmodium falciparium*which accounted for about 80% of the total population; the age group of 26-35years had the highest malaria density for those classified as low (53.7%), average (55.7%) and high (70.4%). This accounted for 60.7% of the total malaria parasite density. Further analysis of the malaria parasite density on pregnancy according to their trimester showed that women on their second trimester of pregnancy had the highest percentage of malaria parasite density of 55.7% and this was statistically significant (P>0.05). The result also show that pregnancy with malaria *parasitaemia* had the highest mean ± standard deviation of 20.37± 15.55 while those grouped as having 'malaria *parasitaemia* without pregnancy' had the lowest (6.09± 3.76) level of C- reactive protein (CRP). This was also statistically significant (P<0.01).

Conclusively, the findings recorded in this study have now shown that malaria parasite infections during pregnancy have a significant impact on both the biochemical and haematological indices and the prevalent species of the parasite is *plasmodium faciparium.*

Keywords: *plasmodium faciparium,* pregnancy, malaria *parasitaemia,* morbidity, mortality.

IMPACT DU PALUDISME SUR PROTÉINES INFLAMMATOIRES, HÉMATOLOGIQUES ET BIOCHIMIQUES DANS AWHARENTOMAH KESTERA DIGBAN GROSSESSE

Digban, A. K.[a],Digban, T. O.[b], Adelabu, A. O.[b,*], &NdukweaM. V.

Un ministère de la science de laboratoiremédical, College of Health Sciences, UniversitéIgbinedion Okada, PMB 001, Edo Okada State, Nigeria; b Département de biochimie et microbiologie de l'Université de Fort Hare, Private Bag X1314, Alice 5700, Province orientale du Cap, Afrique du Sud
•Correspondence: Digban, A. Kester, Department of Medical Laboratory Science, College of Health Science, Igbinedion University Okada, PMB 001, Okada Edo State, Nigeria

RÉSUMÉ

L'auteurdelamorbidité et de la mortalité du paludismeestresté un important fardeau de la santé dans les pays endéveloppement, enparticulierenAfriquetropicale. Ainsi, le paludisme pendant la grossesseetl'associationsa complication associéedemeure un problème de santé majeur pour les futures mères. Danscetteétudeun total de cinq cent cinquante (550) échantillons de sang ontétéobtenus par les femmes enceintes et non enceintes et mèresd'uneparasitémie sans paludisme qui a consenti à l'étude. Certainsparamètreshématologiquesetbiochimiquesontétéévalués à l'aide de méthodesconventionnelles. Le résultat a montré que les espèces de parasites du paludisme a étéisoléfalciparium du plasmodium qui représentaient environ 80 % de la population totale ; le grouped'âge de 26-35ans avait la plus haute densité de paludisme pour ceuxclasséscommefaible (53,7 %), moyenne (55,7 %) et élevé (70,4 %). Celareprésentait 60,7 % de l'ensemble du parasite de la densité. Une analyse de la densité des parasites du paludisme sur la grossesseenfonction de leurtrimestreontmontré que les femmes sur leurdeuxièmetrimestre de grossesseavaient le pourcentage le plus élevé de paludisme la densitéparasitaire de 55,7 % et cen'étaitstatistiquementsignificative (P > 0,05).

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Les résultatsmontrentégalement que la grossesse avec le paludisme la parasitémieavaient la plus haute moyenne ± écart-type de 20,37 ± 15,55 tandis que cellesclasséescommeayant le paludisme la parasitémie sans grossesse' avaient la plus faible (6,09 ± 3,76) niveau de la protéineréactive C (CRP). C'étaitaussistatistiquementsignificative (P < 0,01). D'unemanièreconcluante, les résultatsenregistrésdanscetteétudeontmontré que les infections parasites du paludisme pendant la grossesseontun impact significatif sur les indices biochimiques et hématologiques et les espècesdominantes du parasite Plasmodium estfaciparium.

Mots clés :grossesse, faciparium plasmodium, le paludisme la parasitémie, morbidité, mortalité.

INTRODUCTION

Malaria, a vector-borne disease is an ancient disease probably originating in Africa and is now regarded as a life threatening disease with nearly half of the world population being vulnerable to the infection(1-2). Malaria accounts for an estimated 2-3 million deaths annually and it is also responsible for untold morbidity in approximately 300-500 million people annually (3).

Malaria is caused by *plasmodium*, which is transmitted by mosquitoes and the four species of *plasmodium* cause malaria infection in humans. These are *Plasmodium falciparume*, *P. vivax, P malaria*e and *P. ovale. P. falciparum* (4)andis responsible for most deaths and most of the severe complications (5), including cerebral malaria, anemia and renal failure (6).

Malaria disease generally has severe devastating impact on man and it is commonly associated with poverty, or a cause of poverty (7), which can lead to a major hindrance in economic development. Malaria is one of the most common infectious diseases creating an enormous public health problem. The disease is caused by protozoan parasites of the genus Plasmodium. Most species of the *Plasmodium* parasites can infect humans. The most serious forms of the disease are caused by *Plasmodium falciparum* while those caused by *Plasmodium vivax,Plasmodiumovale* and *Plasmodium malariae* may cause milder disease in humans that is not generally fatal (8).

Plasmodium infection causes an acute febrile illness which is most notable for its periodic fever paroxysms occurring at either 48 or 72 hour intervals. The severity of the attack depends on the Plasmodium species as well as other circumstances such as the state of immunity and the general health and nutritional status of the infected individual. Malaria is a chronic disease which has a tendency to relapse or recrudesce over months or even years (9).

The most common way to obtain malaria is through the natural transmission by mosquitoes. Malaria can also be transmitted via blood transfusions or sharing syringes. Mechanical transmission of infected blood will result in a shorter incubation period since there will be no liver stage. There is also an increased risk of fatality with mechanically-transmitted *P.falciparum*. The lack of the liver stage infection also precludes relapses in *P.vivax*or *P.ovale* infections. Congenital transmission has also been documented, but is believed to be relatively rare despite the heavy infection of the placenta (10).

Symptoms of malaria include fever, shoveringarthralagia (Joint pains), vomiting, anemia caused by haemolysis, hemoglobinuria, retinal damage (11) and convulsion. The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting 4-6hrs, occurring every two days in P.vivax and P.ovale infections, while every three, for P. malariae (12). P. *flaciparum* can have recurrent fever every 36-48 hrs or a less pronounced and almost continuous fever for reasons that are poorly understood, but that may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (13). Malaria has been found to cause cognitive impairments, especially in children as a result of wide spread of anemia during a period of rapid brain development. This direct brain and neurologic damage results from cerebral malaria to which children are more vulnerable (14).

Severe malaria is almost exclusively caused by *P.falciparam* infection and usually arises 6-14 days after infection consequences of severe malaria include coma and death if untreated, young children and pregnant women are especially fumble splenomegaly, severe headache, cerebral ischemia, hepatomegaly hypoglycemia and hemoglobiuria from lysed red blood cells leak into the urine. Severe malaria can progress extremely rapidly and cause death within hours or days (15).

In most severe cases of the disease fatality rates can exceed 20%, even with intensive care and treatment (16) in endemic areas because treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria (17). Chronic malaria is seen in both P.vivax and P.ovale, but not in *P.falciparum*. Here the disease can relapse months or years after exposure, due to the presence of latest parasites in the liver. Describing a case of malaria as cured by observing the disappearance of parasites from the blood stream can therefore be deceptive. The longest incubation period reported for P.vivax infection is 30 years (18).

The etiology of malaria in pregnancy is non-multifactorial of which its causes include poor nutrition and infection with other parasites which together contribute to increases maternal and neonatal morbidity and mortality (19).

In Nigeria, it's believed that in the malaria endemic area, pregnant women could remain asymptomatic despite *falciparum* infection (20). It is also clear that malaria parasites density differs in instances of asymptomatic and clinical malaria while the degree of *parasitaemia* may influence the pathologic and biochemical presentations of individuals presenting with either of these conditions.

Unfortunately not much has been done to establish the degree of renal involvement or other impaired function in malaria association with pregnancy cases in Nigeria and Anambra in particular. Hence, the present study was designed to assess *the influence of P. falciparum parasitaemia among pregnant mothers and its association on certain biochemical and haematological parameters* that could be evidence in apparently healthy pregnant women. Thus, the findings hope to serve as a tool in evidence based health education on the need to intensify efforts at malaria prevention during pregnancy through prompt access to effective treatment, intermittent preventive treatment and the consistent use of insecticide treated nets.

MATERIALS AND METHODS

A total of 550 women comprising of 300 pregnant women with malaria *parasitaemia*, 100 non pregnant women with malaria *parasitaemia* and 150 pregnant women without malaria *parasitaemia* who served as control, were recruited for this study. The control group were selected after data on their health status obtained from questionnaire containing relevant information responded to indicated healthy status before they were screened for the presence of *Plasmodium falciparum parasitaemia* and absence of pregnancy by testing negative to a blood pregnancy testing.

Sample Collection
Using a 10ml sterile syringe, about 8mls of venous blood was collected from each of the subjects and 4mls of the whole blood transferred into a sterile EDTA container while the remaining 4mls was dispense into a sterile plain blood bottle and allowed to clot. A drop of blood from the EDTA bottle was used in making thick and thin blood films. The thick films were stained using Giemsa stains (21) and examined microscopically using 100X objective after applying a small drop of immersion oil. The intensity of infection was also estimated based on the number of parasites counted per high power field of the microscope using the plus sign system. The remaining venous blood was then used in the estimation of some selected haematological and biochemical parameters as well as C - reactive protein.

Plasmodium falciparum malaria parasite screening: The *P. falciparum*malaria parasite was detected by Microscopic examination of giemsa stained thin and thick blood films. The *parasitaemia* was expressed as number of malaria parasites per microliter of blood (22).

Determination of Malaria parasite density
Parasite Density: The parasite density is the number of parasites counted in each microscopic field. The determination of number of parasites per micro-litre of blood is accomplished by counting the number of parasites in relation to a standard number of leukocytes per micro-litre (8000) (23).

Determination of packed cell volume The packed cell volume (PCV) was determined by micro-heamatocrit centrifugation of EDTA-whole blood collected into a capillary tube (24). The volume of the packed red cells was measured in a micro-haematocrit reader and expressed as relative mass of packed red cells present in a sample of whole blood in terms of percentage (%).

Creatinine estimation
The serum creatinine concentration was determined using the modified colourimetric method alkaline picrate-slot (25) in 1965 using the kit supplied Randox (24).

Urea estimation
Serum urea levels were determined using the DiacetyiMonoxine (DAM) (26), using the kit supplied by Randox (24).

Estimation of electrolyte levels (Sodium ion and potassium ion)
Sodium and potassium levels were estimated using flame photometry (27).

Estimation of electrolyte levels (BicarbonateHCO3 – and Chloride Cl-) The levels of bicarbonate and chloride ion were estimated using titrimetric method (27).

Quantitative Estimation of C - reactive protein (CRP)
The C- reactive protein (CRP) levels were assayed by the methodology for Enzyme Linked Immuno-sorbent Assay (ELISA) while the Colour was developed by Sigma-Fast o-phenylenediaminedihydrochloride tablets (supplied by Sigma, St. Louis, Minneapolis, USA). The limit of detection was place at 1µg/ml (CRP).

Determination of White Blood Cell Count (WBC)
0.95ml of WBC diluting fluid was put into bijou bottle and blood sample was mixed by inverting approximately 20 times and 50ml pipette was used

to draw blood up into the tip. The content of the pipette was then expelled into the diluting fluid and the content of the bottle was mixed by inversion. The dilution of blood contained in the bottle was 1:20.

Statistical Analysis

This was performed using Stac-pac Gold package. The mean (±SD) was determined for the variables in both groups while ANOVA was used to evaluate the levels of associations between the variables. The levels of significance was considered at p-value <0.05

RESULTS

The results obtained from this study are displayed in the tables and figures below. Table I showed that the malaria parasite species isolated was *P.falciparium* which accounted for about 80% the total population studied.

TABLE 1: PREVALENCE % OF THE DIFFERENT PLASMODIUM SPECIES ISOLATED AMONG PREGNANT WOMEN IN IHIALA, ANAMBRA STATE

Species	Pregnant women	Non-pregnant women	Total (%)
P. falciparum	300 (60%)	100 (20%)	80
P. ovale	0 (0%)	0 (0%)	0
P. vivax	0 (0%)	0 (0%)	0
P. malariae	0 (0%)	0 (0%)	0

TABLE 2: SHOWED THE AGE GROUP (IN YEARS) OF PERCENTAGE (%) PARITY AND PARASITE DENSITY IN PREGNANCY WITH MALARIA *PARASITAEMIA* ONLY

AGE (YEARS)	Low parasite density N (%)	Average density N (%)	High parasite density N (%)	Total N (%)
15-25 YRS	40 (37.73)	29 (36.70)	25 (21.73)	94 (31.34)
26 – 35 YRS	57 (53.77)	44 (55.69)	81 (70.43)	182 (60.66)
36YRS & ABOVE	9 (08.49)	6 (07.59)	9 (07.82)	**24 (08.00)**
Total	106 (35.3)	79 (26.3)	115 (38.3)	300 (100.0)

X2 = 8.507, DF =4, P- value = P > 0.05

Table 2 showed that the age group of 26-35years had the highest malaria parasite density for those classified as low (53.7%), Average (55.7%) and high (70.4%). This accounted for about 60.7% of the total malaria parasite density. Those above 36 years of age had the lowest malaria parasite density of 8.0%.

TABLE 3: SHOWED THE PERCENTAGE (%) PARITY AND PARASITE DENSITY IN THE DIFFERENT TRIMESTER OF PREGNANCY WITH MALARIA *PARASITAEMIA*

TRIMESTER	Low parasite density N (%)	Average parasite density N (%)	High parasite density N (%)	Total N (%)
First trimester	**20 (26.41)**	**22 (27.84)**	**24 (12.5)**	**74 (24.67)**
Second trimester	**53 (50.0)**	**38 (48.00)**	**76 (3.9)**	**167 (65.67)**
Third trimester	**25 (23.58)**	**19 (24.05)**	**15 (20)**	**59 (19.66)**
Total	**106 (35.3)**	**79 (26.3)**	**115 (38.3)**	**300 (100)**

X2 = 8.959, DF =4, P > 0.05

Further analysis of the malaria parasite density on pregnancy according to their trimester showed that women on their second trimester of pregnancy had the highest percentage of malaria parasite density of 55.7%. whereas those on their third trimester had the lowest (19.7%) as shown in table 3 above. This was statistically significant (P>0.05)

TABLE 4: SHOWED THE MEAN ± S.D OF C REACTIVE PROTEIN (CRP) FOR PREGNANCY WITH AND WITHOUT MALARIA PARASITE AND CONTROL GROUP

Pregnant women			Non Pregnant Women	
Parameter	**Without MP (n=150)**	**With MP (n=300)**	**With MP (n=100)**	**p-value**
CRP (ug/ml)	**14.17±6.09**	**20.37± 15.55**	**6.09± 3.76**	**P<0.01**

Table 4 showed that pregnancy with malaria *parastaemia* had the highest mean± standard deviation of CRP (20.37± 15.55) while those groups as malaria *parasitaemia* without pregnancy had the lowest (6.09± 3.76) level of CRP. This was found to be statistically significant (P<0.01).

TABLE 5: SHOWED THE MEAN ± S.D OF C REACTIVE PROTEIN (CRP) FOR PREGNANCY WITH AND WITHOUT MALARIA PARASITE ACCORDING TO TRIMESTER

	CRP at Different Trimesters of Pregnancy			
Subjects	**First**	**Second**	**Third**	**P value**
Pregnancy withMP(n=300)	21.19±11.81(74)	18.44± 10.46(167)	24.82± 16.94(59)	P<0.05
Pregnancy without MP(n=150)	11.80± 1.40 (19)	15.61± 4.30 (91)	19.93± 6.48 (40)	P<0.05

Further analysis on the CRP levels according to trimester should that the third trimester of the pregnancy with malaria *parasitaemia* group had the highest mean (24.82 ± 16.94) for CRP. While those group under second trimester had the lowest mean (18.44 ± 10.46) for CRP. Those on their third trimester of pregnancy for the pregnancy group without malaria *parasitaemia* had the lowest mean (19.93 ± 6.48). of CRP. This was statistical of pregnancy significant (P<0.01) as shown in table 5 above.

TABLE 6:SHOWED THE MEAN ± SD OF ELECTROLYTE, UREA AND CREATININE IN PREGNANCY WITH AND WITHOUT MALARIA PARASITE AND THEIR CONTROL GROUP (MALARIA PARASITE WITHOUT PREGNANCY).

Parameter	**Pregnant Women**		**Non Pregnant women**	**P value**
	with MP(n=300)	**without MP (n=150)**	**with MP (n=100)**	
Potassium (mmol/l)	**4.55± 4.11**	**5.38± 1.35**	**4.18± 0.64**	**p<0.05**
Soduim ion (mmol/l)	**142.75± 7.26**	**140.50± 1.41**	**141.37± 5.09**	**p>0.05**
Bicarbonate ion (mmol/l)	**24.76±4.30**	**26.13± 1.55**	**22.10± 9.74**	**p<0.05**
Chloride ion (mmol/l)	**101.51± 8.16**	**105.25± 9.28**	**89.66± 8.20**	**p<0.05**
Urea (mg/dl)	**20.66± 6.12**	**30.08±4.62**	**15. 39± 7.70**	**p<0.01**
Creatinine (mg/dl)	**2.75±1.24**	**3.23±1.37**	**0.75±0.24**	**p<0.05**

Table 6 showed that potassium, (5.38±1.35), chloride (105.25±9.28) and bicarbonate (26.13± 1.55) statically increase (P<0.05) for the pregnant women without malaria *parasitaemia* when compared to the non-pregnant women with malaria *parasitaemia* (potassium ion (4.18±0.64) chloride ion (89.66± 8.20) and bicarbonate (22.10±9.74). Similarly, urea (20.66± 6.12) and creatinine (2.75± 1.24), decrease significant (p<0.05) among the pregnant women with malaria *parasitaemia* when compare to those pregnant women without malaria *parasitaemia* (urea=30.08± 4.62 and creatinine =3.23± 1.37)

TABLE 7: SHOWED THE MEAN ± S.D OF ELECTROLYTE, UREA AND CREATININE PARAMETERS IN PREGNANCY WITH OR WITHOUT MALARIA *PARASITAEMIA*

	Pregnant Women		P value
Parameter	**with MP (n=300)**	**without MP (n=150)**	
Potassium (mmol/l)	**4.55± 4.11**	**5.38± 1.35**	**p<0.05**
Soduim ion (mmol/l)	**142.75± 7.26**	**140.50± 1.41**	**p<0.05**
Bicarbonate ion (mmol/l)	**24.76±4.30**	**26.13± 1.55**	**p<0.05**
Chloride ion (mmol/l)	**101.51± 8.16**	**105.25± 9.28**	**p<0.05**
Urea (mg/dl)	**20.66± 6.12**	**30.08±4.62**	**p<0.01**
Creatinine (mg/dl)	**2.75±1.24**	**3.23±1.37**	**p<0.05**

Table 7 showed that in pregnancy, sodium ion increases (142.75 ± 7.26) with malaria *parasitaemia* while potassium ion (4.55 ± 4.11) Bicarbonate ion (24.76 ± 4.30), choride ion (101.51± 8.16), urea (20.66± 6.12) and creatinine (2.75 ± 1.24) decreases respectively with malaria *parasitaemia* This was stastically significant (P<0.05).

TABLE 8: SHOWED THE MEAN ± SD OF ALKALINE PHOSPHATASE, AMINO-TRANSAMINASES & BILIRUBIN LEVELS IN PREGNANCY WITH AND WITHOUT MALARIA PARASITE AND THEIR CONTROL GROUP.

Parameter	**Pregnant Women**		**Non- Pregnant**	**P value**
	with MP (n=300)	**without MP (n=150)**	**women with MP (n=100)**	
Alkaline Phosphate (IU/L)	**42.96±8.12**	**66.88± 6.29**	**15.6± 5.09**	**p<0.01**
Alanine amino-transaminase (IU/L)	**8.27±3.27**	**14.75±2.34**	**7.79±4.32**	**p<0.05**
Asparate amino-transaminas (IU/L)	**10.27±3.70**	**12.72±2.34**	**8.19± 4.02**	**p<0.05**
Total Bilirubin (mg/dl)	**3.77± 1.01**	**2.31± 2.60**	**1.77± 1.01**	**p<0.05**
Conjugate Bilirubin (mg/dl)	**2.33± 1.02**	**1.77± 0.08**	**0.44± 0.20**	**p<0.05**
Unconjugate Bilirubin (mg/dl)	**1.20± 0.11**	**0.48± 0.5**	**0.36± 0.13**	**p<0.05**

Table 8 showed a statistically significant increase (P<0.05) in the pregnancy group without M.P for Alkaline phosphate (66.88± 6.29). Alanine aminotrasarminase (14.75± 2.34), Asparate aminotransminase when campare to their pregnancy with M.P (Alkaline phosphate, 42.96± 8.12 Alamineaminotransminase, 8.27± 3.27 and Asparate aminotransaminase, 10.27± 3.70) and their non-pregnant group with M.P (Alkaline phosphatase, 15.6±6.09, Alamine aminotransaruinase 7.79±4.32 and Asparate aminotransaminase 7.79± 4.32 and Asparate amino transaminase 8.19± 4

TABLE 9: SHOWED THE MEAN ± SD OF ALKALINE PHOSPHATASE, AMINO-TRANSAMINASES & BILIRUBIN LEVELS IN PREGNANCY WITH AND WITHOUT MALARIA PARASITE

Parameter	Pregnant Women		P value
	with MP.(n=300)	without MP.(n=150)	
Alkaline Phosphate(IU/L)	42.96±8.12	66.88± 6.29	p<0.01
Alanine Amino-transaminase(IU/L)	8.27±3.27	14.75±2.34	p<0.05
Asparate Amino-Transaminase(IU/L)	10.27±3.70	12.72±2.34	p<0.05
Total Bilirubin (mg/dl)	3.77± 1.01	2.31± 2.60	p<0.05
Conjugate Bilirubin (mg/dl)	2.33± 1.02	1.77± 0.08	p<0.05
Unconjugate Bilirubin (mg/dl)	1.20± 0.11	0.48± 0.5	p<0.05

Table 9 showed a statistically significant decrease (P<0.05) in akaline phosphate (42.96± 8.12) in pregnant women with malaria parasite when compare to those without malaria *parasitaemia* (66.88± 6.29). While total Bilirubin showed a significant (P<0.05) increase in those with malaria *parasitaemia* (3.77± 1.01) when compared to those without malaria *parasitaemia* (2.31± 2.60

TABLE 10: SHOWED THE MEAN ± S.D. % PCV, HAEMOGLOBIN & TOTAL WHITE BLOOD CELL COUNT LEVELS FOR PREGNANCY WITH AND WITHOUT MALARIA PARASITE AND CONTROL GROUP

Parameter	Pregnant Women	Non Pregnant Women		
	without MP (n=150)	with MP (n=300)	with MP (n=100)	P value
% PCV	4.22±4.22	37.13± 2.17	38.06± 3.76	P<0.01
Hb(g/dl)	11.38± 1.20	14.08± 11.40	12.05± 1.08	P<0.05
WBC-Total	5701.3± 2478.04	6937.50± 1988.49	3958.30± 2049.00	P<0.01

Table 10 showed a significant increase (P<0.01) in the % PCV for non- pregnancy women with malaria parasite (38.06± 3.76), when compare to the pregnant women with malaria parasite (37.13 ±2..17) and those without malaria parasite (34.22± 4.22) The Hb (14.08± 11.40) and total WBC /6937.5± 1988.49 of pregnant women with malaria parasite was increase when compare to both pregnant women without malaria parasite (Hb=11.38± 1.20 WBC-total =5701.3±2478.04) and non-pregnant women with malaria parasite (Hb=12.5±1.08 WBC-total = 3958.30± 2049.00

TABLE 11: SHOWED THE MEAN ± S.D. %PCV, HAEMOGLOBIN & TOTAL WHITE BLOOD CELL COUNT LEVELS FOR PREGNANCY WITH AND WITHOUT MALARIA PARASITE

Parameter	Pregnant Women		P value
	without MP.(n=150)	with MP.(n=300)	
PCV (%)	34.22±4.22	37.13± 2.17	P<0.01
Hb(g/dl)	11.38± 1.20	14.08± 11.40	P<0.05
WBC-Total	5701.3± 2478.04	6937.50± 1988.49	P<0.01

Table 11 showed that % PCV (34.22± 4.22), Hb (11.38±1.20) and total WBC (5701.3±2478.04) of pregnant women without malaria parasite significantly decrease (P<0.05) when compared to their pregnant women with malaria parasite (% PCV (37. 13± 2.17); Hb (14.08± 11.40) and total WBC (6937.50± 1988.49)

TABLE 12: THE MEAN ± S.D. % PCV, HAEMOGLOBIN & TOTAL WHITE BLOOD CELL COUNT LEVELS VERSUS PARITY FOR PREGNANCY WITH MALARIA PARASITE

PARITY	0	1	2	3	4	5	6	
PARAMETER	MEAN±SD	MEAN ± SD	MEAN ±SD	MEAN± SD	(MEAN ±SD)	MEAN± SD	MEAN± SD	P- VALUE
PCV	34.42±3.50	34.1±5.13	34.60±1.93	33.39±5.7	38.1±2.59	31.50±4	37.00±0.00	P<0.05
HB	11.40±1.16	11.33±1.71	11.52±0.63	11.12±1.90	12.70±0.82	10.49±0.82	12.30±0.00	P<0.05
WBC	57.75±38.6	59.63±21	56.19±15.84	52.80±12.81	57.00±10.51	62.00±19.33	68.00±0.00	P> 0.05

TABLE 13: SHOWED THE PERCENTAGE (%) PARITY AND PARASITE DENSITY IN PREGNANCY WITH MALARIA *PARASITAEMIA*

	PARASITE DENSITY							
PARITY	LOW DENSITY		AVERAGE DENSITY		HIGH DENSITY		TOTAL	
	N	(%)	N	(%)	N	(%)	N	(%)
0	31	(29.25)	12	(15.19)	37	(32.17)	80	(26.67)
1	29	(27.35)	37	(46.84)	32	(27.82)	98	(32.67)
2	22	(20.75)	18	(22.78)	23	(20.00)	63	(21.00)
3	17	(16.04)	9	(11.39)	12	(10.43)	38	(12.67)
4	0	0.0)	3	(03.80)	5	(04.35)	8	(2.67)
5	7	(06.60)	0	(0.0)	3	(02.61)	10	(3.33)
6	0	(0.0)	0	(0 .0)	3	(02.61)	3	(1.00)
	106	(35.3)	79	(26.3)	115	(38.3)	300	(100)

X2= 29.217, DF =12, P<0.01

Table 13: Showed that women who were on their first and second pregnancies had the highest percentage of malaria parasite density of 32.7% and 26.7%. While those who had above four pregnancies had the lowest amount of malaria parasite density of 2.7% for four (4), 3.3% for five (5) and 1.0% for six(6) pregnancies outcome.

DISCUSSION

Prevalence of malaria infection

Malaria infection during pregnancy is a major public health problem in tropical and sub-tropical regions around the globe. The result in this study confirms that plasmodium falciparum species of is the prevalent specie of plasmodium that was isolated among pregnant women with malaria *parasitaemia* in Ihiala, Anambra State, This specie accounted for a prevalence rate of 80% a shown on table 1. This finding can be compared to the report ofIsmail(28) who reported that over 90% of the global malaria burden occurs in the Southern parts of Africa. Although, *P.faciparuim* has been reported as the predominate species isolated among pregnant women in the tropics (29)but the presentation of asymptomatic malaria *parasitaemia* is different from that of severe or clinical malaria *parasitaemia* in during pregnancy. This may be due to the differences in malaria parasite density in the blood of the infected individual (30).had a higher percentage of parasite density (26.7% and 32.7%) when compared to those with more episodes of pregnancies (i.e. above four pregnancies outcome) The high parasite density reported may be due to immune suppression among those individuals on their first and second pregnancies which is a consequences of the effect of the *plasmodium faciparuim* infection among these individual and tends to improve as the pregnancies out-come exceed four (4), times. Thus, there is an acquired immunity against malaria (table 2). This can be compared to the report of**Dicko (31)**, who observed that the severity of malaria infection depends largely on the degree of malaria *parasitemia*, and pregnancy may increases the risk of malaria infection (32).

Similar analysis on the severity of high parasite density among the age group of pregnant women revealed that the number of the 2nd trimester pregnant women who visited the antenatal clinic was more than those in their 1st and 3rd trimester possibly because these groups of patient felt there was no clinical need. There was also significantly ($P<0.01$) increase in the parasite density among those with maternal age of 26 to 35 yrs. This finding can be compared to that of Olshake(33) who reported a lower parasite density among maternal age less than 20yrs.

Inflammatory proteins

C-reactive protein (CRP) is a protein found in the blood and whose levels increases in response to inflammation (34). The findings from this study confirms that during the episode of pregnancy there was an elevated CRP (14.17 ± 6.09) as seen among pregnant women without malaria *parasitaemia* and becomes severe when there is malaria *parastaemia* (20.37 ± 15.55) as shown in table 4. This elevated CRP levels were observed among the third trimester of pregnancy for those with malaria *parasitaemia* (table 5). This finding can be compared to the work done by (35) who have reported higher values for CRP during late pregnancy.

Biochemical Indices in Pregnancy with malaria parasitaemia

Alterations in biochemical parameters have been investigated and reported by several authors in malaria infections (1, 36). This has helped to intensify care for this group of patients and thus prevent death that may results from malaria complications. The result obtained from this study shows the impact of malaria and pregnancy on biochemical parameters, in Ihiala, Anambra State. Data presented in this study shows that urea and creatinine was significantly ($P<0.05$) decreased in pregnancy with malaria *partasiteamia* when compared with those without malaria *parasitaemia*. The concurrent decrease in the urea and creatinine reveals that there is a functional glomerular fitteration rate. This finding can be compared to the work done by Etim*et al*. (37) who reported an elevated urea and creatinine level.

In this study, there was a significant ($P<0.05$) increase in sodium ion and a significant ($P<0.05$) increase in sodium ion and a significant ($P<0.05$) decrease in Bicarbonate ion, and chloride ion. These changes may have resulted from the continuous haemolytic effect of the *plasmodium falciparium* parasite on the red cells which in-form will affect the ionic concentration of the whole blood uncosity.

Furthermore, this study also reveals that there is an alteration in the luier enzymes of pregnancy with malaria *parasitaemia* (table 9). There was a significant decrease infection. This can be compared to the work done by Adeosun *et al*.(38) who reported elevated or hyperbilirusinaemia in malaria which is a consequence of hamolysis and concluded that a severe infection can reflect luier damage. Thus, this study reveals a mild elevation in the bilirubin level of pregnant women with malaria *parasitaemia*.

Haematological indices in pregnancy

The impacts of malaria on haematological parameters during pregnancy have been with malaria parastaemia poorly studied in Anambra State. In this study the impact of malaria on haematological parameters, had a significant increase ($p<0.01$) in the %age packed cell volume of the multigravidae with an average mean value of

31.5% with a corresponding mean hemoglobin level of 10.5g/dl These findings can be compared to the work done by *Onyenekwe et al.* (39) who reported a low %age for PCV in the primigraviae. However in areas of stable transmission like Anambra State,

parasites density fifers in instances of asymptomatic and clinical malaria (Onyenekwe*et al* (39).The degree of parasite density and the parasite density threshold could explain the reason for the difference. Similarly there was also no significant difference ($p>0.05$) in the total white cell count (wbc). Thus anaemia as a presenting feature is more common in partially immune multigravida, living in hyper-endemic areas having malaria during pregnancy (40)

The pattern of % PCV among the study group was also observed. In the P.C.V. coded as low, and normal, the highest percentage of the low P.C.V was recorded in the mulliparous 44 (30.8%) and primigravidae 43 (30.1 %). Also the greater percentage of low pcv was recorded among those on 2nd trimester of pregnancy. This may be as the result of haemolysis causes by malaria parasites. This finding can be compared to the work done by (41) that malaria causes neutropemia and anaemia.

CONCLUSION

The findings recorded in this study have now shown that malaria parasite infection during pregnancy has a significant impact on both the most adult women have developed enough immunity that during pregnancy, *P.falciarum* infection does not usually result in fever or other clinical symptoms. It is also clear that malaria

biochemical and haematological indices. The prevalent species of the parasite is *plasmodium faciparium* and the degree of *parasitaemia* may result in inflammatory changes which are observed. Thus, the following contribution to knowledge was made:1. Prevalence rate of *P.faciparium* infection 80%.2. The maternal age of 26-35 years old had the highest degree of parasite density.3. There was significant changes in the liver enzymes especially, Alkaline phosphatase and the amino transaminase of pregnant women with malaria *parasitaemia*.4. There was a significant decrease in the urea and creatinie level of pregnant women with malaria *parasitaemia*. 5. There was a significant increase in the CRP, which serve as a pointer to the underlying inflammatory changes in the pregnant women with malaria *parasitaemia*.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper

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