

ISSN 1595-689X

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY

JANUARY 2019

VOLUME 20

NUMBER 1



Official Publication of the African Society for Clinical Microbiology



AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY (AJCEM) ISSN 1595-689X

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Official publication of the African Society for Clinical Microbiology

January 2019; Volume 20: Number 1

TABLE OF CONTENTS

ARTICLES

Malaria prevention in the Buea health district in Cameroon: factors influencing mosquito bed net use in households

F. H. L. Kamga., P. Nde-Fon., C. Morfaw., N. D. S. Nsagha.....1-8

Profile of septic work up among patients admitted into the intensive care unit in University of Abuja Teaching Hospital Gwagwalada, Abuja

T. Yunusa., A. M. Adeoye., O. A. Akitoye.....9-16

Characterization of methicillin-resistant *staphylococcus aureus* isolates from apparently healthy individuals in Malete, Kwara State, Nigeria

M. I. Bale., S. K. Babatunde., M. R. Adedayo., A. E. Ajiboye., A. T. Ajao.....17-24

The characterization of multidrug resistant type 1 s-fimbriated *Escherichia coli* from women with recurrent urinary tract infections (Rutis) in Bangladesh

M. K. Hasan., F. Momtaz., M. J. Foysal., M. H. Ali., K. Islam., S. H. Prodhon.....25-34

Bacterial contamination of toilet door handles on Baze University campus, Abuja, Nigeria

O. O. Alonge., B. M. Auwal., M. I. Aboh.....35-41

Elimination of mother-to-child transmission of HIV (eMTCT) in western Nigeria: how far have we gone?

S. O. Usmana., G. B. Agboola., A. J. Afeb., G. P. Olubayo., T. Akinmurele., O. T. Oluwaniyi., O. O. Abodunde., O. Adeola., M. Onyema.....42-48

Surveillance of anti-HCV antibody amongst in-school youth in a Nigeria University

M. A. Muhibi., M. O. Ifeanyichukwu., A. O. Olawuyi., A. A. Abulude., M. O. Adeyemo., M. O. Muhibi.....49-53

Screening of selected medicinal plants for their antifungal properties

M. I. Aboh., P. Oladosu., G. O. Adeshina., B. O. Olayinka., S. Olonitola., N. H. Atasie.....54-59

Tube method and congo red agar versus tissue culture plate method for detection of biofilm production by uropathogens isolated from midstream urine: which one could be better?

A. M. Sultan., Y. Nabel.....60-66

A two year old infected with *Dicrocoelium dendriticum*: a case report

J. O. Okwuzu., T. A. Gbaja-Biamila., A. E. Wapmuk., P. C. Austin-Akaigwe., O. O. Odubela., A. N. David., O. C. Ezechi.....67-71

High prevalence of extended-spectrum s-lactamase (blactx-m-15) and new Delhi metallo- β -lactamase-1 (NDM-1) genes among high-level carbapenem resistance *Klebsiella pneumoniae*: an alarm for our health system

S. Yaghoubi., Z. Baseri., A. Rasti., M. Gharani., Y. Erfani.....72-81



AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY (AJCEM) ISSN 1595-689X

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AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/190.1 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.1>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 1-8

MALARIA PREVENTION IN THE BUEA HEALTH DISTRICT IN CAMEROON: FACTORS INFLUENCING MOSQUITO BED NET USE IN HOUSEHOLDS

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ABSTRACT

Background: Insecticide treated bed nets (ITNs), though proven to be effective in preventing malaria will have little impact unless people sleep under them. Several studies have shown that owned ITNs are usually not used and that ITN use is influenced by several factors that vary between communities.

Objective: To investigate the factors influencing the use of mosquito bed nets in households in the Buea Health District (BHD) in Cameroon.

Material and Methods: A cross-sectional study with two-stage cluster sampling included 420 households from 35 sites. Questionnaires adapted from the Malaria Indicator Survey were used. CSPro 4.1 and Epi info 3.5.3 were used to create database and analyze respectively.

Results: ITN ownership in the BHD was high (92.6%; 95% CI: 89.6%-94.9%) but ITN use was less than average (41.2%; 95% CI: 39.2%-43.3%). ITN use was least likely in the age group 5-15 years ($P<0.01$), in educated individuals ($P<0.01$) and in households with less than one ITN for two persons ($P<0.01$). White ITNs were less likely to be used ($P<0.01$). **Conclusion:** There is a gap between ITN ownership and use in the malaria holoendemic BHD and ITN use is associated with age of individual, level of education, colour of ITNs and household net density. Use of the highly owned ITNs could be increased by targeting the least protected 5-15 years age group and schooling individuals; by promoting school-based education on ITN use.

Keywords: Insecticide-treated nets, malaria, Buea Health District

PREVENTION DU PALUDISME DANS LE DISTRICT DE SANTE DE BUEA AU CAMEROUN: FACTEURS INFLUENÇANT L'UTILISATION DE LA MOUSTIQUAIRE DANS LES MENAGES

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Résumé

Contexte: Les moustiquaires, quoique ayant été prouvées efficaces dans la prévention de la transmission du paludisme auront encore peu d'impact sur la population, à moins qu'elles ne soient effectivement utilisées par celle-ci. Plusieurs études ont montré que les personnes possédant les moustiquaires n'en font d'habitude pas bon usage et que leur utilisation est influencée par plusieurs facteurs qui varient selon les communautés.

Objectif: Examiner les facteurs influençant l'utilisation des moustiquaires par les ménages dans le District de Santé de Buea (DSB) au Cameroun.

Matériel et Méthodes: il s'agissait d'une étude transversale avec un échantillonnage groupé en deux étapes comprenant 420 ménages choisis dans 35 sites différents. Les questionnaires adaptés de l'Enquête sur les Indicateurs du Paludisme ont été utilisés. Les logiciels CSPro 4.1 et Epi infos 3.5.3 ont été utilisés pour créer une base de données afin de l'analyser.

Résultats: Le pourcentage de personnes possédant les moustiquaires dans le DSB était élevé (92.6 %; 95 % CI: 89.6 %-94.9 %) celui des personnes en faisant effectivement usage étaient en dessous de la moyenne (41.2 %; 95 % CI: 39.2 %-43.3 %). L'utilisation des moustiquaires était moins probable dans la tranche d'âge 5-15 ans ($P<0.01$), chez les individus ayant un niveau alphabétisation élevé ($P<0.01$) et dans les ménages avec moins d'une moustiquaire pour deux personnes ($P<0.01$). Les moustiquaires de couleur blanche étaient moins susceptibles d'être utilisées que celles d'autres couleurs ($P<0.01$).

Conclusion: Il existe un réel fossé entre la possession des moustiquaires et leur utilisation effective dans le DSB qui est une zone holo-endémique de paludisme. L'utilisation des moustiquaires est associée à l'âge d'individu, le niveau d'éducation, la couleur d'ITN et la densité nette du ménage. L'utilisation des moustiquaires pourrait être augmentée en visant les individus de la tranche d'âge de 5-15 ans et en promouvant la sensibilisation sur l'utilisation des moustiquaires en milieu scolaire.

Mots clés: moustiquaire, paludisme, District de Santé de Buea

INTRODUCTION

Insecticide-treated bed nets (ITNs) have been proven as one of the most effective ways of reducing malaria morbidity and mortality, especially in children and pregnant women [1,2]. They have been shown in several randomized controlled trials in Africa to reduce cases of uncomplicated malaria by 50%, severe malaria by 45%, splenomegaly by 30% and mortality by 17% [3]. ITNs do not only protect those who sleep under, but in communities with a greater than 60 percent ITN use, those who do not sleep under are protected as well by massive killing and decrease in the survival of the anopheles vectors [2]. This implies that what is more important is increased ITN use throughout the entire community and not just by groups most at risk [4]. As suggested by theoretical models, there will be greater benefit in targeting the entire population for ITN use than there will be in targeting only the most vulnerable groups [5]. This is because an estimated 80 percent of human-to-mosquito transmission of *Plasmodium* occurs from human hosts older than 5 years of age [6]. Given that the most promising malaria vaccine to date is just about 50 percent effective [7], ITNs remain one of the most effective methods for malaria prevention. However, ITNs will only be effective in preventing malaria if they are used effectively, and attainment of the desired epidemiological impact is crucially dependent on such effective ITN use [8]. Effective ITN use can reduce malaria transmission by up to 90% [9].

Half of the world's population is at risk of malaria with 89% of the malaria burden on Africa [10]. The disease is the leading cause of illness and death in Cameroon, and accounts for 41% of outpatient consultations, 41% of morbidity in the general population, 49% and 56% of morbidity in pregnant women and children under five years respectively, 43% of deaths in all regions of the country and 14% and 54% of deaths in pregnant women and children under five years respectively [11]. All the ten regions of Cameroon are affected by malaria. The Buea Health District (BHD) is hyperendemic for malaria [12].

As reported in several studies, despite the proven effectiveness of ITNs in preventing malaria, a significant number of ITNs owned are not used [8,13-15]. There is therefore a significant disparity between ITN ownership and ITN use. This means that there are several determinants of or factors associated with the effective use of ITNs even by those who already own them. A particular socio-demographic factor may have different associations with ITN use, depending on the setting. For example, being male was directly associated with net use in Burkina Faso [16] and inversely associated with net use in six other African countries [17].

In several communities, some of these factors associated with ITN use have been identified to be: poor conception and knowledge of malaria [18], knowing that mosquitoes transmit malaria [19], Age [17,20], gender [16,17], level of education [19,21], occupation [21], socioeconomic status [19,22], conical shape of bed net [23], colour of net [19], having to pay for nets [19,23],

sleeping with the mother [24], use of coils for mosquito control [19], and fewer nets in household [8,14,19]. In some communities, studies have shown that ITNs are being misused for other purposes like fishing [25].

There is relatively little data on the factors influencing ITN use in the Buea Health District. Recently, there is was nation-wide campaign to freely distribute ITNs to Cameroonians. It is hypothesized that achievement of the desired epidemiological impact of preventing malaria would depend on effective ITN use. It is therefore necessary to know the local factors associated with ITN use. This study therefore sought to determine the ownership and use of ITNs and the factors associated with their use in the BHD.

MATERIAL AND METHODS

Study Design

This was an observational, cross-sectional descriptive study.

Study Area

This study was carried out in the Buea Health District (BHD). The BHD is located in the Fako Division of the South West Region of Cameroon. It is hyper endemic for malaria [12]. The BHD has a population of 133,092 inhabitants. It is divided into 7 health areas (HA) which are in turn divided into Communities. In the BHD, the percentage of children 0-5 years having received ITNs was 1.2 percent as of March 2010. The national target was 90 percent and the District target was 80 percent. In addition, the percentage of pregnant women having received ITNs was 53 percent as of March 2015. The national target for pregnant women was at least 90 percent and the District target 80 percent [26].

Study Population and Sampling

Sample size

The minimum required sample size was calculated using the formula for proportions as described by Eng [27], thus the study had to include at least 385 households using a prevalence of 50% [28]. It included 420 households in order to account for the cluster sampling technique used. Thus the study included 347 children aged 2 to 9 years.

Sampling technique

A two-stage cluster sampling design was used [28]. In the first stage, Probability Proportional to Size sampling (PPS) as described by Bennett et al. [29], Merg et al. [29], Milligan et al. [30] and Mc Ginn [31] was used to select 35 sites from the 66 communities that make up the 7 health areas in the BHD. The measure of size used was the population of each community [32]. The second stage involved listing and sampling of households within the selected communities [29]. Households in the BHD were listed during the recent nation-wide campaign to distribute ITNs. The household serial numbers were recorded per community. We obtained these numbers from the Health Centres of each Health

Area following authorization from the District Medical Officer. The sampling frame of households was therefore known for each community. In each selected community, 12 households were balloted from the sampling frame. The selected households were visited first to obtain consent, then secondly to obtain data. If no one was available in the household during the first visit, two more visits were carried out. If no one was available after 3 visits, the next randomly selected household was studied until at least 12 households were studied in that community [15,29]. All individuals who spent the previous night in the pre-selected households were studied. However, there was just one respondent of the questionnaire for each household. The respondent of the questionnaire was any adult member of the household who was capable of providing information needed to fill in the questionnaire. A visitor who spent the previous night in the household, though included in the study did not qualify to be a respondent of the questionnaire [29].

Data collection and data quality control

A structured household questionnaire developed from the Malaria Indicator Survey (MIS) was used to conduct interviews in the households [29]. The questionnaire was divided into seven main parts namely Informed Consent Form, Identification, Household Listing, Ownership and Use of ITNs, Perceptions and Attitudes toward Malaria, and Interviewer Summary. To ensure good data quality the questionnaire was pre-tested in a selected sample before being used for the study. Moreover, all questionnaires were checked for errors and completeness in the field and inconsistencies verified with the respondent. Another factor that ensured good data quality was that callbacks were done for households in which no respondent was present during the first visit.

Ethical Considerations

Ethical clearance was obtained from the Institutional Review Board of the Faculty of Health Sciences of the University of Buea and administrative approval from the Regional Delegation of Public Health for the South

West Region, Cameroon. Informed consent was sought from each household before their inclusion in the study. Moreover, for children aged between 2 to 9 years, guardian consent was sought and from those aged 7 to 9 years, assent consent was sought.

Data management and analysis

All questionnaires were checked for filling errors, correct coding and completeness. All inconsistencies were verified with the respondents before leaving the households. Data were entered into CSPro 4.1 every day after returning from the field and counterchecked by double-entry. Logic, skip patterns and consistency checks were used during data entry to prevent entry errors. The data were then exported from CSPro 4.1 to Epi Info 3.5.3 and analyzed. The research was self-weighted by virtue of probability proportional to size sampling (which weights the variables) carried out in the first stage of the cluster sample design [28,30]. All P-values were calculated using the Chi-square test.

RESULTS

A total of 2253 individuals were studied in 420 households. These households had 1110 Insecticide treated bed nets (ITNs). There were more females (54.1%) than males (45.9%). The majority (75.5%) of the participants had attained primary and secondary school and the least proportion had attained higher education. Those who had not had any formal education also made up a good proportion of the study participants (15.6%). More than half of the participants (55.3%) were older than 15 years. A small proportion (1.9%) of the participants was pregnant.

Out of the 1245 adults studied, 511 (41.0%) slept under an ITN ($X^2 = 23.47$, 2 d.f., P-value < 0.01). RR comparing children < 5 years to children 5-15 years = 1.43 (95% CI: 1.24-1.66), $X^2 = 23.36$, P-value < 0.01; RR comparing children < 5 years to adults = 1.24 (95% CI: 1.10-1.40), $X^2 = 11.75$, P-value < 0.01. Thus there is a statistically significant difference in ITN use between children aged less than five years, children aged 5-15 years and adults in the BHD.

TABLE 1 : INSECTICIDE TREATED BED NETS (ITNS) USE ACROSS INDIVIDUALS BY AGE GROUP

Age group in years	ITN use across individuals		Total number of individuals No (%)	RR	P-value
	Used No (%)	Not used No (%)			
< 5 years	199 (50.9)	192 (49.1)	391 (17.4)	1.00	
5-15 years	219 (35.5)	398 (64.5)	617 (27.4)	1.43	< 0.01
> 15 years (adults)	511 (41.0)	734 (59.0)	1245 (55.3)	1.24	< 0.01
total number of individuals	929	1324	2253 (100.0)		

TABLE 2: ITN USE ACROSS INDIVIDUALS BY LEVEL OF EDUCATION

Level of education	ITN use across individuals		Total number of individuals No (%)	RR	P-value
	Used No (%)	Not used No (%)			
None	184 (52.3)	168 (47.7)	352 (15.6)	1.00	
Primary	362 (39.1)	563 (60.9)	925 (41.1)	1.34	< 0.01
Secondary	316 (40.8)	459 (59.2)	775 (34.4)	1.28	< 0.01
Tertiary (Higher)	66 (32.8)	135 (67.2)	201 (8.9)	1.59	< 0.01
Total number of individuals	928	1325	2253 (100.0)		

The use of ITN in individuals who had received no formal education was 52.3% while that in individuals who had attained primary education was 39.1% while that in individuals who had attained secondary education was 40.8% while that in those who had attained higher education was 32.8% ($X^2 = 25.31$, d.f.=3, P-value < 0.01). RR comparing no education to primary

education = 1.34 (95% CI: 1.18-1.52), $X^2 = 17.97$, P-value < 0.01; RR comparing no education to secondary education = 1.28 (95% CI: 1.12-1.46), $X^2 = 12.95$, P-value < 0.01; RR comparing no education to higher education = 1.59 (95% CI: 1.28-1.99), $X^2 = 19.48$, P-value < 0.01. Thus there is a statistically significant difference in ITN use between non-educated, primary-educated, secondary-educated and higher-educated individuals in the BHD.

TABLE 3: ITN USE ACROSS NETS BY ITN COLOUR

ITN colour	ITN use across nets		Total number of nets No (%)	RR	P-value
	Nets used No (%)	Nets not used No (%)			
White	130 (30.9)	291 (69.1)	421 (37.9)	1.00	
Green	37 (52.9)	33 (47.1)	70 (6.3)	0.58	< 0.01
Blue	244 (39.4)	375 (60.6)	619 (55.8)	0.78	< 0.01
Total number of nets	411	699	1110 (100.0)		

TABLE 4: ITN USE ACROSS HOUSEHOLDS BY HOUSEHOLD NET DENSITY

Household net density	ITN use across households		Total number of households No (%)	RR	P-value
	100% ITN Use No (%)	Less than 100% ITN Use No (%)			
≥ 1 ITN per person	19 (43.2)	25 (56.8)	44 (10.5)	1.00	
1 ITN for 2 persons	65 (34.4)	124 (65.6)	189 (45.0)	1.26	0.28
1 ITN for 3 persons	30 (26.3)	84 (73.7)	114 (27.1)	1.64	0.04
1 ITN for ≥ 4 persons	5 (6.8)	68 (93.2)	73 (17.4)	6.30	<0.01
Total number of households	119	301	420 (100.0)		

Of the 421 white nets, 130 (30.9%) had at least one person sleep under; out of the 70 green nets studied, 37 (52.9%) had at least one person sleep under; out of the 619 blue nets studied, 244 (39.4%) had at least one person sleep under ($X^2 = 15.87$, 2 d.f., P-value < 0.01). RR comparing white nets to green nets = 0.58 (95% CI: 0.45-0.76), $X^2 = 12.89$, P-value < 0.01; RR comparing white nets to blue nets = 0.78 (95% CI: 0.66-0.93), $X^2 = 7.93$, P-value < 0.01. Thus there is a statistically significant difference between the use of white, green and blue nets in the BHD.

Out of the 44 households with a household net density of ≥ 1 (one or more ITNs per person), 19 (43.2%) had a 100% ITN use; out of the 189 households with a household net density of 0.50-0.99 (one ITN for two persons), 65 (34.4%) had a 100% ITN use; out of the 114 households with a household net density of 0.33-0.49 (one ITN for three persons), 30 (26.3%) had a 100% ITN use; out of the 73 households with a household net density of 0.00-0.32 (one ITN for four or more persons), 5 (6.8%) had a 100% ITN use ($X^2 = 25.02$, 3 d.f., P-value < 0.01). RR comparing households with net densities of ≥ 1 to those with net densities of 0.00-0.32 = 6.30 (95% CI: 2.53-15.68), $X^2 = 20.05$, P-value < 0.01; RR comparing

households with net densities of ≥ 1 to those with net densities of 0.33-0.49 = 1.64 (95% CI: 1.04-2.59), $\chi^2 = 4.19$, P-value = 0.04; RR comparing households with net densities of ≥ 1 to those with net densities of 0.50-0.99 = 1.26 (95% CI: 0.85-1.86), $\chi^2 = 1.19$, P-value = 0.28. Thus there is a statistically significant difference in ITN use between households having one or more ITNs per person and households having one ITN for three or more persons in the BHD.

DISCUSSION

Data analysis revealed that 92.6 % of the households in the BHD have at least one ITN. This finding indicates that the ownership of ITNs by households in the BHD is high. This could owe to the recent nation-wide campaign to freely distribute ITNs. The ownership of ITNs by children less than five years and pregnant women in the BHD were respectively 1.2% and 53% [33]. These proportions are expected to have greatly increased following the nation-wide free distribution of ITNs to Cameroonian households.

The second objective was to determine the proportion of individuals in the BHD who slept under an ITN the previous night. Data analysis and interpretation revealed that 41.2% of the population in the BHD slept under an ITN. This finding indicates that less than half of the population of the BHD uses ITNs and ITN use in the BHD is less than the 60% target above which a meaningful impact on malaria transmission is expected [34]. It also shows that most people who own ITNs in the BHD do not use them. This finding of a gap between ITN ownership and use is consistent with findings in other studies elsewhere [8,13-15].

The main reported reason for not sleeping under an ITN in the BHD was rumour that ITNs killed people. It may be useful therefore to educate the population and reassure them that ITNs do not kill. They should also be reminded to expose the ITNs outside in a shaded place for at least 24 hours before starting to use [34].

In a study carried out in Cameroon before the nation-wide campaign to freely distribute ITNs [35], the reported problems experienced by families using ITNs were: difficulty in finding chemicals for retreatment, insufficient financial means to buy new bed nets to replace old ones, insufficient bed nets for all in the household and excessive heat when sleeping under net. In a study in Ethiopia [27], the main reported reasons for not using owned ITNs was the perception that ITN had lost its effectiveness or needed retreatment. In another study in the Democratic Republic of Congo [36], the main reason for not using ITNs was heat/discomfort within a bed net. These varied reasons for non-use of ITNs in different communities further confirm the need for studying local environmental and socio-cultural factors influencing ITN use, so that proper cost effective measures can be taken to improve use.

Children under five years were significantly more likely to sleep under an ITN than children aged 5-15 years and adults. Children aged 5-15 years were significantly least likely to sleep under an ITN. These findings indicate that there is an association between ITN use and age of individual in the BHD and children less than 5 years are more likely to sleep under an ITN while children aged 5-15 years are least likely to sleep under an ITN. The association of bed net use with age has also been found in other African studies [17,19,20,37]. The greater ITN use in children less than five years could owe to two reasons: Firstly, they form one of the groups at greater risk of malaria and have been targeted for a long time now (before campaigns targeting the whole community) for ITN distribution at Infant Welfare Clinics. They are therefore more used to ITNs and probably more likely to sleep under [19]. Secondly, as previously reported by Mugisha and Arinaitwe [24], children who shared a bed with their mothers are 21 times more likely to sleep under an ITN; they are more likely to share a bed with their parents whereas older children are more likely to sleep on separate beds or on the floor [20].

In a study carried out in six African countries, Baume and Marin [33] showed the children 5-14 years were least likely to use ITNs. In another study by Noor et al. [20], children 5-19 were least likely to use ITNs. In this BHD study, the age group least likely to use ITNs was 5-15. The relatively lower protection against malaria in children above 5 years and in adolescents has great implications. These children above five years constitute the most important reservoir for malaria with more than 80% of human to mosquito transmission of *Plasmodium* occurring from humans above 5 years [21]. The protection of these children above 5 years by ITNs therefore needs to be scaled up, probably by school based education as suggested by Noor et al. [20]. This finding is contrary to Ndjinga and Minakawa [37] who reported that children aged 5-15 years were more likely to use bed nets than other age groups in the Democratic Republic of Congo. They attributed the increase ITN use in this age group to education on disease prevention and sanitation carried out in primary and secondary schools and further emphasized the importance of education in schools on ITN use especially in the age group 5-15. Although children less than five years constituted the age group with the highest ITN use in the BHD, they had only 50.9% ITN use. This is less than the 60% target set for this age group at the African Summit on Roll Back Malaria held in Abuja, Nigeria, on April 25, 2000 [36]. Therefore, increasing ITN use by the least protected 5-15 year age group should be coupled to improving use in the more protected but more at risk less than five years age group.

Another finding in the present study is that individuals in the BHD who had received no formal education were significantly more likely to use ITNs than individuals who had gone to school while individuals who had attained higher education were least likely to use ITNs. These findings indicate that there is an association between ITN

use and level of education in the BHD. This does not support the findings from previous reports carried out in Africa [18,19,21,36]. These studies found that households whose heads or caregivers were more educated were more likely to use ITNs. Our finding may be explained by the fact that individuals who have attained higher education are exposed to health ideals which enhance promotion of their social health, they are more likely to get good and clean houses in areas with fewer mosquitoes whereas those with no formal education are more likely to get mosquito prone houses. The pain and nuisance caused by these mosquitoes to the people with no formal education may then push them more to seek mosquito preventive measures than their more educated counterparts.

The present study also revealed that white ITNs were significantly least likely to be used compared to green and blue ITNs. Green ITNs, though fewest in number, were significantly more likely to be used, showing the association between ITN use and ITN colour. These findings are consistent with findings in Ghana by Baume and Franca-Koh [19]. A possible reason for these findings is that coloured ITNs do not easily show dirt when dirty as compared to white ITNs and that coloured nets may be considered decorative and attractive [19]. People are therefore more likely to use coloured ITNs. For effective and consistent use of ITNs to be achieved, the choices of the local population must be considered. It may therefore be useful to distribute coloured (especially green) ITNs in subsequent ITN distribution campaigns so that maximum use is achieved.

Households with more ITNs per person were significantly more likely to have a 100% ITN use. However, the difference in ITN use between households with one or more ITNs per person and those with one ITN for two persons was not significant. These findings indicate that there is an association between ITN use and household net density in the BHD and although household ITN use is likely to increase with more nets in the household, there is likely to be no further increase in use above a net density of 0.5 (more than one ITN for two persons). In previous studies of households in many Sub-Saharan African countries [8], bed net use by children under five years and pregnant women was found to increase with increase household net ownership. The reason for this was that in households with few bed nets, the few nets were more likely to be used by the household heads and adults but not children under five and pregnant women. So as the number of household bed nets increased, access to nets for children and pregnant women also increased. In another study in Ghana, using bed nets as the unit of analysis, Baume and Franca-Koh [19] found that a bed net was more likely to be used if there were fewer bed nets in the house. The former studies differed from Baume and Franca-Koh's study in that they used children under five years and pregnant women as their unit of analysis.

The present study used all the household members as well as the ITN as the units of analysis. The findings of this BHD study could be explained by both findings from the two sets of studies above [19,38]. As household net ownership increases, dependent groups like children and pregnant women tend to also have access to ITNs, increasing household ITN use to 100%. This explains the increase in households with a 100% ITN use with an increase in household net density. However, above a net density of 0.5 (more than one ITN for two persons), the extra ITNs are more likely to be kept unused because there is no room to hang them or saved as a reserve net for those already hanging [35]. This explains why there is likely to be no further increase in household ITN use when household net density is above 0.5. The implications of these findings are that it may be useful to distribute more ITNs to households with less than one ITN for two people, with the goal of achieving at least one ITN for two persons. On the other hand, it may not be useful adding more nets to the households already having one or more ITNs for two people.

This study did not find any significant association between ITN use and gender, pregnancy, use of other methods than ITNs for malaria prevention, respondent's perception of malaria as a serious problem, knowledge by respondent that mosquitoes transmit malaria and the respondent's perception on the effectiveness of ITNs in preventing malaria in the BHD.

There are some limitations to this study. A few households in the BHD were not numbered during the household listing that was done to distribute vouchers for ITNs in the BHD. However, this household listing was the best available sampling frame given that there is no better numbering of households in the BHD. Potential inflation in the reported ownership and use of bed nets was suspected. To prevent this, ITNs were directly observed in as many households as possible and noted if they were hanging. It was assumed in this study that the respondent's knowledge, perception of ITN efficacy and perception of malaria as a serious problem applied equally to all household members.

Conclusion: This study investigated the factors influencing the use of mosquito bed nets in the BHD. It was intended to determine any gap between ITN ownership and use as well as determine any factors associated with ITN use, so that ITN use could be improved. This was in relation to the relatively little data in the BHD and in Cameroon on the factors associated with ITN use. The study established that the ownership of ITNs in the BHD is high (92.6%; 95% CI: 89.6%-94.9%) but the use is less than the 60% target at which a meaningful impact on malaria transmission is expected (41.2%; 95% CI: 39.2%-43.3%). The main reported reason for non-use is fear that ITNs kill. It also established that ITN use is significantly least likely in the age group 5-15 years, in educated individuals and in households with less than one ITN for two persons. Moreover green and blue ITNs

are significantly more likely to be used compared to white ones. Furthermore the study established that malaria has gone from being hyperendemic to being holoendemic (splenic index= 81.6%, AES= 4.12) in the BHD. In view of these findings we conclude that there is a gap between ITN ownership and use in the malaria holoendemic BHD and ITN use is associated with age of individual, level of education, colour of ITNs and household net density.

The highly owned ITNs in the malaria holoendemic BHD would be a major tool in reducing malaria transmission provided the less than average use is improved. Improving use would imply targeting the least protected age group of 5-15 years and the school going individuals of the health district using school-based education on ITN

use. It would also imply ensuring that every household in the BHD has at least one ITN for two persons while considering the persons' colour preference.

Competing interests: The authors declare that they have no competing interests

Authors' contributions: KFHL and PNF conceived the study and participated in its design and implementation and drafted of the final manuscript. NPF participated in the design and implementation of the study. CM implemented the study. All authors read and approved the final manuscript

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1902 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.2>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 9-16

PROFILE OF SEPTIC WORK UP AMONG PATIENTS ADMITTED INTO THE INTENSIVE CARE UNIT IN UNIVERSITY OF ABUJA TEACHING HOSPITAL GWAGWALADA, ABUJA

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ABSTRACT

Background: Several infectious agents are responsible for sepsis in all age groups presenting with fever which can have devastating consequences if not adequately treated. Sepsis may arise from bacteria, fungi and viral origin but are localized in particular organ or system with systemic affection. Febrile illness is a leading reason for admission to the intensive care unit of hospitals in the tropics and these patients comes mostly from inpatients rather than from outpatients. Diagnostic apparatus needed for sepsis work up are usually not available in most cases and the data regarding septic work up are very scanty. Therefore, this research set out to determine the pattern of isolates from septic work-up among patients admitted to the intensive care unit in Abuja.

Methodology: This was a descriptive cross-sectional study. Sixty-four consecutive patients admitted to the intensive care unit with symptoms such as fever were involved in the study in a view to determine the septic state of the patients. Samples were collected. Blood culturing was performed using the BACTEC 9050® system and biochemical analytical profile index were used for identification and confirmation of bacterial isolates.

Results: The mean age of the patients admitted to ICU was 40.9±3.2 with the highest proportion within the age range of 31-40 years accounting for 31.7% of the patients enrolled and the lowest proportion being 10-20 years group accounting for 5.0%. Out of the 64 patients investigated in the ICU 60 patients had clinical and positive cultures with an overall positive and negative infection rate of 93.8% and 6.3% respectively. From the positive cultures yields 86.7% were bacteremia and 13.3% were fungaemia. Multiple infections were observed among the male patients, Multi-drug resistance bacteria were observed among *Klebsiella pneumoniae*, *E. coli* and *P. aeruginosa* isolates.

Conclusion: Bacterial and fungal isolates were found in this study but increased rate of polymicrobial isolation and nosocomial infections calls for concern.

Key words: Profile of infectious agents, sepsis, fever, septic work up, Abuja

PROFIL DU TRAVAIL SEPTIQUE CHEZ LES PATIENTS ADMIS À L'UNITÉ DE SOINS INTENSIFS DE L'UNIVERSITÉ D'ABOUJA, HÔPITAL D'ENSEIGNEMENT DE GWAGWALADA, ABUJA

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ABSTRACT

Contexte: Plusieurs agents infectieux sont responsables de la sepsie dans tous les groupes d'âge présentant une fièvre pouvant avoir des conséquences dévastatrices s'ils ne sont pas traités de manière adéquate. La sepsie peut être d'origine bactérienne, fongique ou virale, mais elle est localisée dans un organe ou un système en particulier avec une atteinte systémique. La maladie fébrile est l'une des principales causes d'admission dans les unités de soins intensifs des hôpitaux tropicaux et ces patients proviennent principalement de patients hospitalisés plutôt que de patients externes. Les appareils de diagnostic nécessaires au traitement de la septicémie ne sont généralement pas disponibles dans la plupart des cas et les données concernant le traitement septique sont très rares. Par conséquent, cette recherche visait à déterminer le type d'isolats provenant d'un traitement septique chez des patients admis dans l'unité de soins intensifs d'Abuja

Méthodologie: Il s'agissait d'une étude descriptive transversale. Soixante-quatre patients consécutifs, admis dans l'unité de soins intensifs avec des symptômes tels que fièvre, ont été impliqués dans l'étude afin de déterminer leur état septique. Les échantillons ont été collectés. La culture de sang a été réalisée à l'aide du système BACTEC 9050® et un indice de profil analytique biochimique a été utilisé pour l'identification et la confirmation des isolats bactériens.

Résultats: L'âge moyen des patients admis aux soins intensifs était de $40,9 \pm 3,2$ ans, la proportion la plus élevée des 31 à 40 ans représentant 31,7% des patients inscrits et la plus faible proportion des 10 à 20 ans représentant 5,0%. Sur les 64 patients étudiés en USI, 60 patients avaient des cultures cliniques et positives avec un taux d'infection global positif et négatif de 93,8% et 6,3% respectivement. Parmi les cultures positives, 86,7% étaient des bactériémies et 13,3% des cas de fongémie. Des infections multiples ont été observées chez les patients masculins. Des bactéries multirésistantes ont été observées parmi les isolats de *Klebsiella pneumoniae*, *E. coli* et *P. aeruginosa*.

Conclusion: Des isolats bactériens et fongiques ont été trouvés dans cette étude, mais l'augmentation du taux d'isolement polymicrobien et d'infections nosocomiales est préoccupante.

Mots-clés: profil des agents infectieux, sepsis, fièvre, traitement septique, Abuja

INTRODUCTION

Sepsis which indicates changes in the patient status is a systemic response both to infection and hemodynamic, metabolic and inflammatory derangement, which can present in mild cases as systemic inflammatory response syndrome (SIRS) or progress to severe sepsis and septic shock[1]. Sepsis is a leading cause of morbidity and mortality worldwide and particularly in Africa where awareness is low and resources are limited[2], cause of mortality in about 30% to 65% in the tropics[1,2] but this figure is much more reduced in well developed countries. Therefore, the presence of SIRS with a high laboratory suspicion of positive microbial cultures is considered sepsis. In the Intensive Care Unit (ICU) severe sepsis is defined as a state of sepsis with concomitant organ dysfunction and hypoperfusion which are often than not reversed with appropriate use of antimicrobial agents in the ICU [1-4]

The Intensive Care Unit (ICU) in any developing countries is an epicenter of infectious agents and that of infection control[3]. Therefore any cases of infection in the ICU are capable of triggering sepsis. Patients admitted to the ICU comes with some level of infection and reduced immune systems which means that there must be aggressive means of eradicating the infection patients coming into the ICU and prevention of new nosocomial infection which is important in critical care management of patients

[4,5]. In a study conducted in the tropics, 66.2% had sepsis and blood culture positivity guided therapy in 12.5% [5].

Point prevalence studies in the ICU are the simplest approach to describing the epidemiology of sepsis. For example, 32.8% of 895 patients in 254 Mexican ICUs had sepsis on a single day in 1995 [6-8]. The threshold of eligibility for treatment almost certainly differs by country and levels of sophistication such as availability of ICU beds, varying levels of universal health insurance, and other cultural and economic factors. For instance, only 27.3% and 27.1% of sepsis patients were admitted to the ICU in Brazil and the UK respectively compared to 32% and 51.1% in Spain and the USA respectively [8]. There are several reasons for increase in sepsis in patients admitted to the ICU; workload in the critical care unit and the increase number of tubes such as nasogastric tubes, the oxygen tubes, central line and the intravenous line which are meant to alleviate fluid accumulation that may lead to infections may contribute to multiple site infections [9-11].

The most common infection in the ICU is septicemia but urinary tract infection and gastrointestinal tract infections do exist which may be nosocomial in nature. The hazard of patients acquiring nosocomial infection in the ICU is high [12-13]. There should be a define time for admission to the ICU not transferring patients when every management fails with

deteriorating health of the patients. Physiological host immune state can also determine the progress of sepsis in patients admitted to the ICU, viral and fungi infections flourishes in immune-compromised patients and the elderly [5, 9, 13-14].

Intensive care unit-acquired bloodstream infection was associated with an increased intensive care unit mortality rate [15]. Several studies of bloodstream infections in those admitted to the ICU in African hospitals suggest that the prevalence of bacterial bloodstream infections among patients in the ICU with fever or clinical sepsis exceeds that described in developed countries and bacterial isolates exceed that of fungal infections [2,5,11,12,15-18]. Bacteraemia is a common cause of illness in the ICU in areas of high and low malaria prevalence [19-20]. Gram-negative organisms, especially *Eschericia coli*, exceed Gram-positive organisms such as *Stapylococcus aureus* in importance in several published reports on bloodstream infections in both adults and children from African countries. The use of blood culture to assess septicaemia seriously ill patients has hasten the diagnostic process with fast recognition of presence sepsis, quick isolation of the infective agents and resolution of antimicrobial agents [9,11-12,19-21].

Ceftriaxone and levofloxacin are antibiotics with good susceptibility profile against most of the bacteria isolates. Meronem have been used with success in the ICU [12, 21-22]. The uses of antiviral and antifungal have been used with caution but have been associated with good result [22]. Bacterial are well known cause of antibacterial resistance in our environment, resistance such as β -lactamases and extended spectrum β -lactamases were well documented among enterobacteriaceae [21, 23-24]. The aim was to determine the profile of etiology agents resulting from septic work up among patients admitted into the intensive care unit in Abuja.

MATERIALS AND METHODS

Study background

This study was carried out at the Microbiology research laboratory unit, Microbiology and Parasitology department and the Intensive Care Unit (ICU) of University of Abuja Teaching Hospital (UATH) Gwagwalada, Federal Capital Territory (F.C.T). The Hospital is located in Gwagwalada whose geographical coordinates are 8° 56' 29" North and 7° 5' 31" East. It has an area of 1,043 km². The ICU is a 10 bedded care unit. The Federal Capital Territory had a projected population of 1,406,239 inhabitants in the year 2006, of which 157770 (11.22% approximately) inhabitants reside in Gwagwalada [25]. Projected population of Gwagwalada city in 2012 was over 1 million people. The hospital provides

health care services to the inhabitants of Abuja and neighboring states including Niger, Kaduna, Kogi and Nassarawa states.

Study population

Sixty-four consecutive patients admitted to the intensive care unit with symptoms such as fever were involved in the study in a view to determine the septic state of the patients.

Study design

This was a descriptive cross-sectional study conducted from December 2015 to October 2017.

METHODS

Diagnosis was achieved in collaboration with the Intensivist. Patients admitted to the ICU were recruited. The purpose of this study was explained to the subjects and/or with their relatives and consent to participate was sought. Interviewer-administered, structured questionnaires were used as the study tool. The questions outlined in the data forms were explained to the subjects and completed forms which contain information that included the bio-demographic data. Also, provisional diagnosis and laboratory processes, such that the eventual result was noted in the data forms and communicated to the Intensivist and the patients or the relatives.

SPECIMEN COLLECTION, TRANSPORTATION AND PROCESSING

Septic areas were identified especially along the central line and urinary catheter. Specimens were collected from septic areas and blood for blood culture. Each specimen received was examined for quality, in terms of amount, sterility and presence or absence of debris.

The BACTEC culture bottles were inspected and the top of the culture bottle was cleansed with a sterile swab containing ethanol alcohol. With vacutainer needle, about 5mls of blood was moved into the culture bottle and scanned into the BACTEC chamber to await positive alert. Those with positive bacterial and fungal alert were subcultured on Chocolate, Blood, MacConkey and Sabouraud agar plates. After overnight incubation on these agars, the growth characteristics were noted and pure growth was Gram stained. Colonies that were Gram positive were further characterized using the catalase, coagulase and novobiocin disc tests. Those with Gram negative were further characterized using the API[®] 20 (Oxoid, 211667 Hampshire, UK). Gram negative identification and iMMVPC test (indole, motility, methyl red, voges-proskeuer and citrate) [23]. Culture plates with

positive results were tested for antimicrobial sensitivity using the Kirby-Bauer Diffusion Susceptibility test protocol. Three well-isolated colonies of similar appearance from the isolates and the controls were emulsified in separate 4ml of sterile physiological saline each, labelled, test and controls. The turbidity of the suspensions (both test and controls) was compared to 0.5 Macfalan standards. Muller-Hinton media for both the test and the control were streaked with test suspension and control suspension respectively using sterile swab. After 5mins, sterile forceps were used to place the antibiotic disc, evenly distributed on both plates with similar antibiotics tested in both the test and the controls. Within 20 minutes of applying the discs, the plates were incubated at 35°C for 18 to 24 hours. After overnight incubation, the test and the control plates were examined. Using a transparent ruler on the underside of the plates the diameter of each zone were measured in mm. Interpretation; The zone diameter of each antibiotic of control were compared with the CLSI standards, if within the CLSI acceptable limits for Quality control strains, then the zone diameter of each antibiotic of the test were compared with CLSI zone diameter breakpoints and was recorded sensitive, intermediate or resistance. Antibiotic susceptibility pattern was determined using the Muller-Hinton media by the disk diffusion method. Materials used were; Muller-Hinton media, Petri dish, Antibiotic disks (Oxoid, Hampshire, UK), Macfalan standard, sterile swab stick, control strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212).

DATA

The data obtained was coded on entering and analyzed using Epi Info version 3.5.1 package. Confidence interval was 95% and the p value was 0.05.

RESULTS

This study was carried out among 64 patients who had ICU stay for treatment between the ages of sixteen years and eighty-one years and the outcome of the laboratory results were discussed with the patients/or their relatives before submitting to the Consultant anesthiologist/intensivist. The mean age of the patients admitted to ICU was 40.9±3.2 with the highest proportion within the age range of 31-40 years accounting for 31.7% of the patients enrolled and the lowest proportion being 10-20 years group accounting for 5.0%. Eleven ICU patients representing 15.0% were within the 31-40 years age group, 10 and 8 patients were from the 61-70 and >70 years age group representing 20.0% and 15.6% respectively. However, this distribution was statistically significant ($P < 0.05$, $df=6$ Table 1). There were predominantly

female patients 43 (67.2%) admitted to the ICU with male to female ratio of 1:2. The infection rate among the female was 58.2% while the overall infection rate in male was (25) 41.7% of the total patients enrolled with 21 patients had one infection while four male patients had multiple infections. This distribution was statistically significant ($P < 0.05$, Table 11)

Out of the 64 patients investigated in the ICU 60 patients had clinical and positive cultures with an overall positive and negative infection rate of 93.8% and 6.3% respectively. From the positive cultures yields 86.7% were bacteremia and 13.3% were fungaemia. Multiple infections were observed among the male patients, Multi-drug resistance bacteria were observed among *Klebsiella pneumoniae*, *E. coli* and *P. aeruginosa* isolates and polymicrobial isolates was observed in 35.0% of the total patients admitted to the ICU. C-reacting protein and Procalcitonin assay were 100.0% sensitive and positive predictive value for bacteria sepsis using blood culture as standard. Of the 60 isolates obtained from 64 culture positive, 13 were isolated from the 41- 50 years age group giving a prevalence of 51.9%, 10 (61.0%) each from the 31-40 and 61-70 years age group respectively. This was statistically significant ($p=0.001$, Table 1). Out of the total eight fungi isolates from this study, 6 (75.0%) were candidaemia while 2 (25.0%) were *Cryptococcus neoformans* isolate.

There were colonization of 21(32.8%) urinary catheter, 15(23.4%) intravenous lines, 6 central lines and 9 NG tubes during this study but the infection rate of these materials were 100% for urinary catheter and the central line. This was not statistically significant ($p=0.08$, Table 11).

Gram negative bacteria were the most isolated bacteria among the patients investigated following blood culture with 28 isolates representing 53.8% while the total Gram positive were 15 bacteria representing 28.8%. There were nine (17.3%) anaerobes and were all bacillus species (Figure 1). Patients with gram negative sepsis had longer ICU stay and were predominantly associated with polymicrobial infection than gram positive sepsis. This relationship was statistically significant ($p=0.002$; positive spearman correlation= 0.251).

Sixty-four blood culture were analyzed from the ICU which yielded single and multiple isolates characterized using the API system. The following organism were isolated; *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E.coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) as Gram negative bacteria. *Staphylococcus aureus* (*S.aureus*) as Gram positive bacteria (Figure 2). Among the organism isolated, *Escherichia coli* (26.9%) was the most predominant

bacteria recovered, *S. aureus*, *P. aeruginosa* and *Coagulase Negative Staphylococcus* (CONS) isolation rate were 15.4%, 17.3% and 9.6% respectively (Figure 2). The rate of susceptibility of the bacteria isolates were represented in table 4, levofloxacin, augumentin

and meronem had a good susceptibility profile to ICU isolates. Three (33.3%) *P. aeruginosa* isolates were multi-drug resistance while two (40%) *K. pneumoniae* and 4 (28.6%) *E. coli* isolates were Extended spectrum beta Lactamases (ESBL) producers.

TABLE I: DISTRIBUTION OF ISOLATION AMONG AGE GROUP IN ABUJA

Age group Percent (years) Culture(%) (%)	Total	Percent	Positive
10 – 20 51.9	5	15.0	5
21 – 30 61.1	9	20.0	7
31 – 40 35.0	11	31.7	10
41 – 50 47.8	14	12.8	13
51 – 60 21.4	7	15.6	7
61 – 70 55.5	10	5.0	10
>70 55.5	8	5.0	8
Total 100.0	64	100.0	60

df = 6

P = 0.001

TABLE II: DISTRIBUTION OF PATIENTS ADMITTED TO THE ICU IN ABUJA

Gender	Infection Rate (%)	Frequency	Percent (%)
Males	21	32.8	25 (41.7)
Females	43	67.2	35 (58.3)

TABLE III: DISTRIBUTION OF PATIENTS ADMITTED TO THE ICU AND THE SITE OF COLONIZATION

Sites	Colonization	Percent	Infection	P-value
	Colonization (%)	Rate(%)		
Urinary Catheter	21	32.8	100	
Intravenous line	15	23.4	93.3	
Central Line	6	9.4	100	
Feeding Tube	13	20.3	84.6	
NG Tube	9	14.1	88.9	0.08
Total	64	100.0		

TABLE IV: ANTIBIOTICS SUSCEPTIBILITY PATTERN OF THE ISOLATES FROM ICU PATIENTS IN GWAGWALADA

Bacteria	CLIN	CEFTA	AUG	LEV	CEFTR	MER	GEN	CEFP
<i>K. pneumoniae</i> n=5	100.0	70.0	90.0	100.0	91.8	100.0	60.8	98.0
<i>E. coli</i> n=14	90.0	80.0	90.0	100.0	96.5	100.0	69.8	100.0
<i>P. aeruginosa</i> n=9	44.0	95.0	96.5	50.0	70.5	50.0	33.0	50.0
<i>Bacillus spp</i> n=6	96.0	60.0	82.0	43.0	44.0	100.0	76.3	80.0
<i>S. aureus</i> n=8	50.0	90.0	70.0	100.0	90.0	100.0	30.0	100.0
<i>Acinetobacter</i> n= 5	70.0	80.0	100.0	90.0	90.0	100.0	70.0	80.0
CONS n=5	60.0	80.0	60.0	60.0	60.0	90.0	30.0	30.8

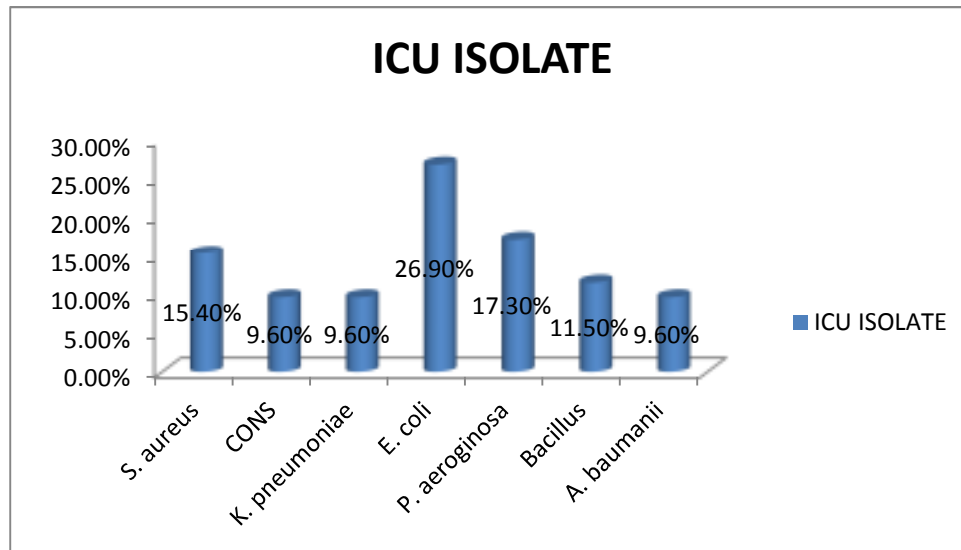


FIGURE 1: DISTRIBUTION OF ISOLATES OBTAINED FROM PATIENTS IN THE ICU

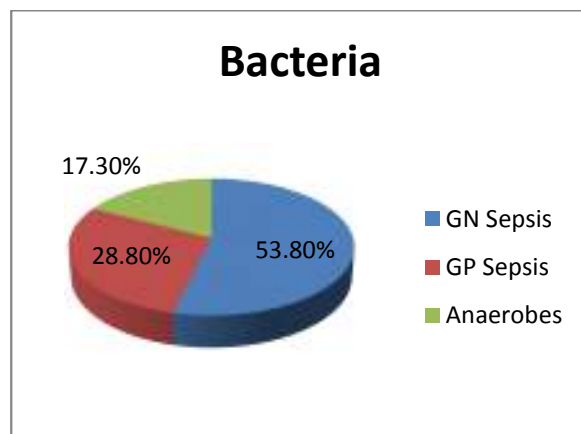


FIGURE 2: DISTRIBUTION OF ISOLATE AMONG PATIENTS ADMITTED TO THE ICU.

DISCUSSION

The infection rate among patients admitted to the ICU in this study was 93.8% and negative culture was 6.3%. This figure varied with findings in Africa [5] and other parts of the world [8,9,13]. The finding of 93.8% in this study was higher than rates of 66.2% in Jos, north-central Nigeria [5], and 27.3% reported in Spain, 51.1% reported in the US, 27.3% in Brazil [8], 20.0% among ICU patients reported by Adria and colleagues in France [9] and 25.4% reported by Stephan [13]. These differences may be due to the methodology employed in our study, invariably the study area and literacy level of the population. The study in Jos [5] expanded the scope of sepsis in relation to those patients with HIV/AIDs in other wards of the hospital. The study in the US, Spain and

France [8, 9] took place in the developed world and are not short of sophisticated equipment's which are lacking in our environment. Therefore, ICU infection rate is low in those studies. Idoko [11] and Alausa [12] had demonstrated that bloodstream sepsis was higher in malaria endemic area of the tropic.

In addition, the high infection rate might be due to the low level of literacy among the populace. Often than not, most patients present late to the hospital with worsen clinical condition. Early presentation may decrease hospital stay and reduce the number of patients for ICU admission. Adria in France [9] established a correlation between workload in the ICU and increase ICU acquired infections. In our setting more patients requires ICU admissions because of late presentation in the hospital.

Age of admission into the ICU have a relationship with rate of ICU acquired infection, in this study infection rate among the older age group was 100.0% (51years to > 70 years) whereas infection rate among the younger age group was lower than 100%. This relationship was statistically significant. The finding was contrary to the knowledge that the older age groups were more predisposed to nosocomial infection because of reduced immunity. Although, Stephan concluded that age >75 years by itself does not appear to be a significant predictor of ICU-acquired nosocomial infection or mortality rate [13]. The finding in this study was contrary to a cohort study by Blot who found that the incidence of bloodstream infection (per 1000 patient days) decreased with age: 8.4 per thousand in middle-aged, 5.5 per thousand in old, and 4.6 per thousand in very old patients [14]. This might be due to differences in

sample size. Female gender predominates in our study with 67.2% and infection rate of 58.3%. This was contrary to study by Alqarni where male predominate with 54% [16] and Sanusi with 62.7% males [27].

In this study, bacteria were more isolated than fungi isolates (81.2% Vs 12.5%) and isolation rate of polymicrobial was 35.0%, this finding was similar to findings by Crowe [17]. The high polymicrobial isolation might be due to the methodology employed in specimen collection and meticulous processing mechanism that minimizes low microbial yield. Urinary catheter was the most colonized in the ICU with highest infection rate while the central line was the least infected in this study. This finding was not supported by Crowe in Nottingham where respiratory tract was the most predominant site of origin of infection with 39.7% of total ICU infections [17].

All the fungal isolates were unicellular yeast cell (*C. albicans* and *C. neoformans*). This might be due to the small samples size. From our study, there were predominant gram negative sepsis than gram positive sepsis (53.8% Vs 28.8%), anaerobes isolated in this study were 17.3%. This findings was similar to 62.2% Vs 46.8% by Florian [8], 47.0% Vs 34.0% isolated by Alqarni [16] and 73.3% Vs 26.7% by Sanusi [27] but contrary to findings by Crowe who got more gram positive [17]. Moreover, Gram negative organisms were frequently isolated from ICU patients. Gram negative bacteria were completely responsible for fever. The distribution was statically significant ($p=0.001$) and correlate positively with high pyrexia and positive Bactec blood culture (spearman correlation=0.408). Gram negative bacteria have potent lipid which has the capacity to induce endotoxic sepsis and consequently increased interleukin 11 and tumor necrotic factor. Increased gram negative isolations might be due to nosocomial infections. The diagnostic mechanism employed was Bactec blood culture which has abundant nutrient for virtually all fast going bacteria to thrive. Notification of culture positivity will lead to prompt diagnosis and reduce patient's hospital stay.

Gram positive sepsis is increasingly becoming common in our environment due to increased rate of antibiotics resistance. Anaerobes isolated in this study

contribute to the rate of polymicrobial isolation in this study.

E. coli was the most frequently isolated among patient in the ICU with 26.9% of the total isolates. This was consistent with other finding [16]. However the findings of 26.9% for *E. coli* was contrary to studies by Florian where *Staphylococcus* predominate with 20.5%, Crowe revealed *Staphylococcus aureus* as most isolated with 22.5% of the total isolates and findings by Sanusi where *Klebsiella pneumonia* Predominates with 26.7%. In our study, there was high isolation rate of *Pseudomonas aeruginosa* and Coagulase negative *Staphylococcus* (CONS) with 17.3% and 9.6% of the total bacteria isolated. The unique nature of *Pseudomonas aeruginosa* will promote its colonization of the various tubes used in the ICU such as IV line and urinary catheter. The difference in the isolation rate of the bacteria might be due its pathogenesis most especially virulence factor rather than differences in their cell wall.

Critical care antibiotics are mainly bactericidal in action and taken into consideration the golden hour Gram negative and Gram positive isolates were sensitive to levofloxacin, augumentin, meronem, ceftriaxone. This is consistent with other studies [21-23] where most of the isolates were sensitive to cell wall acting antibiotics. From this study *E. coli* isolates exhibit significant extended bête lactam resistance while two of the *Pseudomonas aeruginosa* exhibited multidrug resistance and therefore increase the patient's hours of hospital stay. Indiscriminate use of antibiotics, irrational administration of antibiotics and poor administration of drugs may be responsible for this increasing antibiotic resistance.

Conclusion: The nature of Infection in the Intensive Care Unit (ICU) can be used as a predictor of infection in the hospital. Bacterial and fungal isolates were found in this study but increased rate of polymicrobial isolates and nosocomial infections calls for concern. Therefore, increased awareness on hand hygiene among ICU caregivers is necessary to make a big difference.

Acknowledgment: No financial support received in any form

Conflict of interest: There is no conflict of interest

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1903 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.3>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 17-24

CHARACTERIZATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM APPARENTLY HEALTHY INDIVIDUALS IN MALETE, KWARA STATE, NIGERIA

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common and continuously growing cause of nosocomial and community-acquired staphylococcal infections around the world. Screening for colonization with MRSA is a major aspect of control and limiting the spread of infections cause by this organism. We investigated the carriage of MRSA among apparently healthy individuals in four rural villages: Eleburu, Tapa, Atere and Apo all around semi-urban town-Malete, in Moro Local Government of Kwara State, Nigeria.

Methods: Nasal swabs were collected from volunteered individuals and were cultured on mannitol salt agar and blood agar for isolation and identification of *Staph aureus* using standard microbiological techniques. Susceptibility to cefoxitin disc (30 µg) was used to determine MRSA status of the isolates. Molecular method was used to detect the gene responsible for resistance among MRSA isolates. Antimicrobial susceptibility test to commonly prescribed antibiotics was carried out using discs diffusion method.

Results: Total number of individuals carrying *Staph aureus* in their nostrils was 42 (37.2 %). Antibiotics susceptibility profile of *Staph aureus* isolates showed 100 % resistance to cefuroxime, cefotaxime, cloxacillin and augmentin, and were 87 %, 81 %, 69 % and 23.8 % and 19 % resistant to tetracycline, ceftriaxone, erythromycin, ofloxacin and gentamicin respectively. A total of 6 (14%) Community -Acquired MRSA (CA-MRSA) isolates were recovered from individuals living in these villages. Molecular method detected *muc* and *mecA* genes in all the 6 (100%) CA-MRSA isolates and *lukS-lukF* was detected in 3 (50%) of the isolates.

Conclusion: Detection of CA-MRSA strains among these rural dweller indicates that they are harbouring enhance virulence organism that may manifest a more severe disease condition. The danger associated with high prevalence of multidrug resistant *Staph aureus* and CA-MRSA; and its consequential effects of poor drug administration in Nigeria was discussed. There is need to establish a more strict legislation and enforcement on drug control; and a body that would monitor production and appropriate use of antibiotics in the Nigeria.

KEYWORDS: CA-MRSA, *Staph aureus*, Antibiotics, Rural Villages and Molecular Characterization

CARACTÉRISATION DES ISOLATS DE *STAPHYLOCOCCUS AUREUS* RÉISTANTS À LA MÉTHICILLINE PROVENANT DE PERSONNES APPAREMMENT SAINS À MALETE, ÉTAT DE KWARA (NIGÉRIA)

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ABSTRAIT

Contexte: Le *Staphylococcus aureus* résistant à la méthicilline (SARM) est une cause commune et en augmentation constante des infections staphylococciques nosocomiales et d'origine communautaire dans le monde. Le dépistage de la colonisation par le SARM est un aspect majeur du contrôle et de la limitation de la propagation des infections causées par cet organisme. Nous avons enquêté sur le transport de MRSA chez des individus apparemment en bonne santé dans quatre villages ruraux: Eleburu, Tapa, Atere et Apo tout autour d'une ville semi-urbaine-Malete, dans le gouvernement local de Moro dans l'État de Kwara, au Nigéria.

Méthodes: Des écouvillons nasaux ont été prélevés sur des individus volontaires et ont été cultivés sur gélose au sel de mannitol et sur gélose au sang en vue de l'isolement et de l'identification de *Staph aureus* à l'aide de techniques microbiologiques standard. La sensibilité à la céfoxitine (30 µg) a été utilisée pour déterminer l'état de SARM des isolats. La méthode moléculaire a été utilisée pour détecter le gène responsable de la résistance parmi les isolats de SARM. Un test de sensibilité aux antimicrobiens aux antibiotiques couramment prescrits a été réalisé à l'aide de la méthode de diffusion sur disques. Résultats: Le nombre total d'individus porteurs de *Staph aureus* dans leurs narines était de 42 (37,2%). Le profil de sensibilité aux antibiotiques des isolats de *Staph aureus* présentait une résistance à 100% au céfuroxime, au céfotaxime, à la cloxacilline et à l'augmentation, et à 87%, 81%, 69%, 23,8% et 19% respectivement.

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Au total, 6 isolats (14%) de SARM acquis dans la communauté (CA-SARM) ont été retrouvés chez des individus vivant dans ces villages. La méthode moléculaire a détecté les gènes *muc* et *mecA* dans les 6 isolats (100%) de CA-MRSA et a détecté le *lukS-lukF* dans 3 (50%) des isolats. Conclusion: La détection de souches de SARM-CA chez ces habitants des zones rurales indique qu'elles hébergent un organisme de virulence qui pourrait se manifester par une maladie plus grave. Le danger associé à la prévalence élevée de *Staph aureus* multirésistant et de CA-MRSA; et ses effets consécutifs à une mauvaise administration du médicament au Nigéria ont été discutés. Il est nécessaire de mettre en place une législation et une application plus strictes en matière de contrôle des drogues; et un organisme chargé de surveiller la production et l'utilisation appropriée des antibiotiques au Nigéria

MOTS-CLÉS: SARMAC, *Staph aureus*, antibiotiques, villages ruraux et caractérisation moléculaire

INTRODUCTION

Methicillin-resistance *Staph aureus* (MRSA) refers to isolates of *Staph aureus* that are resistant to antibiotics with B-lactam ring such as penicillins, cephalosporins and their derivatives from healthy individuals or patients. *Staph aureus* is an opportunistic pathogen that asymptotically colonizing the nostrils, skin, glands and mucous membrane of both human and animal. It possesses a wide range of virulent factors and are responsible for a wide range of infections such as rashes, endovascular infections, inflammations of bones and the meninges, pneumonia, skin soft tissue infection (SSTIs), septic arthritis, endocarditis, sepsis, bloodstream infection and osteomyelitis [1], [2].

The first recorded emergence of methicillin resistance was in England in 1961 two years after introduction of methicillin [3]. MRSA organisms are also found to be resistant to other methicillin related antibiotics such as oxacillin, amoxicillin and penicillin. These organisms also acquire resistance to other groups of antibiotics that include clindamycin, cotrimoxazole, erythromycin and gentamicin [4]. The resistance is as a result of acquisition of resistant genes known as *mec A* gene which is a membrane-bound enzymes that catalyzes the transpeptidation reaction and encodes the production of PBP-2a a penicillin-binding protein-2a which is essential for cross-linkage of peptidoglycan layer [5]. Lately, a new methicillin resistance gene known as *mecC* gene has been reported and MRSA carrying this gene has been isolated from both human and animal [6], [7], [8].

Hospital acquired MRSA (HA-MRSA) has always been associated with prior exposure to hospitals and healthcare centres, however, recently MRSA infection in healthy individuals in community is on increase and it has been confirmed to be responsible for morbidity and mortality in various parts of the world [9], [10], and is referred to as community acquired methicillin-resistant *Staph aureus* (CA-MRSA) [11], [12].

HA-MRSA strains are genetically distinct from CA-MRSA, CA-MRSA strains are always resistant to the B-lactam and sometimes to erythromycin but largely remain susceptible to several other antibiotics. Many outbreaks of CA-MRSA have been reported in healthy populations and institutions such as Daycare centers, Military quarters and prisons [13].

Malete, a semi-urban town is surrounded by four rural villages; all are in Moro Local Government and Kwara

North Senatorial District of Kwara State of Nigeria. These villages: Apo, Eleburu, Tapa and Atere have population of less than 500 each [14]. The residents are mainly farmers and herders. There are no schools or healthcare facilities in the villages. The pupils attend primary and secondary schools at Malete and the sick ones are cared for at Cottage Hospital, Malete.

In Nigeria, there is paucity of information on MRSA, particularly CA-MRSA in rural and semi-urban populations with its antecedent danger of ignorance. In other to meet with this need, we investigated the carriage of CA-MRSA stains in the nostril of some apparently healthy individuals in rural communities of Malete, Kwara State. We also screened to detect genes responsible for MRSA in individuals that were positive to MRSA.

MATERIALS AND METHODS

Sample Collection

Visits were made to each village heads (Baale) and his elders in council and obtained consent to collect nasal swab from only volunteered members of the villages. Further approval to carry out this research was obtained from Kwara State University research and ethical committee under the Community Development Centre (CDC). All subjects that have been admitted into the Hospital or Healthcare centre and those that were on any antimicrobial treatment in the past three months prior to sample collection were excluded from study. Samples were collected with sterile cotton swab pre-wetted with sterile saline; the swab was inserted into the two anterior nares and gently rotated. Samples were collected randomly from 113 apparently healthy individuals in the four rural villages of Malete that included Apo, Eleburu, Atere and Tapa between first and third weeks of August, September and October, 2017.

Culturing method

Samples collected were transported in ice pack box within one hour of collection to the Microbiology laboratory, Department of Biosciences and Biotechnology, Kwara State University, Malete. The samples were cultured on mannitol salt agar and blood agar plates. They were incubated aerobically at 35-37 °C for 18-24 hours. On blood agar, colonies that were light yellow to whitish cream and were 1-2 mm in diameter with some producing β -haemolysis or colonies on mannitol salt agar that were pinkish-yellow and were 0.5-1 mm in diameter were picked for further

tests. Further tests carried out to confirm that the isolates were *Staph aureus* are Gram staining, catalase, coagulase and DNase tests as previously described [15], [16].

Antimicrobial Susceptibility Test

Antimicrobial susceptibility profile of each isolated *Staph aureus* was determined using standard disc diffusion method on Mueller-Hinton agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK) incubated aerobically at 37 °C for 18-24 hours using the following antibiotics discs; Cefuroxime (CXM₃₀), Cefotaxime (CF₃₀), Cloxacillin (CX₅), Augmentin (AU₃₀), Ceftriaxone (CRO₃₀), Tetracycline (TE₃₀), Erythromycin (E₁₅), Gentamicin (GEN₁₀) and Ofloxacin (OF₅). Zones of inhibition were measured in millimeters and interpreted following criteria of the Clinical and Laboratory Standard Institute [17].

Detection of Methicillin Resistance

Resistance to methicillin was determined using disc diffusion susceptibility method as described by guideline of the [18]. This was performed with Cefoxitin_{30µg} antibiotics disc (Oxoid, UK) on Mueller-Hinton agar plate (Mast Diagnostics, Mast Group Ltd, Merseyside, UK); where there are no zone of inhibition or zone radius measures 2 mm or less, isolates were regarded as resistant [18]

Molecular Screening and Characterization

The genomes of the isolates were extracted using QIAamp DNA Mini Kit according to manufacturer's instructions. Methicillin resistant *Staph aureus* isolated that were phenotypically identified were subjected to molecular screening for the presence of *muc* gene [19] *mecA* gene, Panton valentine leucocidin (PVL) gene and *lukS-lukF* genes [19] using the appropriate primers as listed in Table 1. The PCR condition were initiated for denaturation at 95 °C for 2 minutes followed by 30 cycles of amplification with 94 °C for 30 minutes, annealing at 50 °C for 30 seconds, extension at 72 °C for 30 seconds and final extension at 72 °C for 4 minutes. The PCR products (10 µg each for *muc*, *mec* and PVL genes) were analyzed in 1.5% agarose gel. Electrophoresis was performed in TBE buffer at 180 volts for 1 hour, and gel was subsequently stained with ethidium bromide (10 mg/ml) (Sigma, UK). DNA bands were visualized using UV-illuminator and photographed.

RESULTS

Distribution of *Staph aureus* among villages

Staph aureus was recovered from the volunteered individuals from four rural villages studied. The total numbers of apparently healthy individuals carrying *Staph aureus* in their nostrils were 42 (37.2%) n=113; however the numbers recovered varied from one village (Table 2) to the other.

TABLE 1: SEQUENCE OF PCR PRIMER SET FOR IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS*, METHICILLIN RESISTANT GENES AND PVL GENE

Target genes	primer set	Primer sequence (5'→3')	Amplicon Size (bp)
<i>Staphylococcus aureus</i>	<i>Nuc</i>	F-TCAGCAAAATGCATCACAACAG R-CGTAAATGCACCTTGCTTCAAG	200bp
Methicillin resistance	<i>Mec A</i>	F-AAAATCGATGGTAAAGGTTGGC R-AGTTCTGCAGTACCGGATTGC	500
Panton valentine leucocidin	(<i>lukS-lukF</i>)	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R-GCATCAAGTGTATTGGATAGCAAAAGC	433

TABLE 2: *STAPH AUREUS* DISTRIBUTION IN VILLAGES

Villages	Total Sample Collected		Staph aureus isolated	
	Number	Percentage (%)	Number	Percentage (%)
Apo	38	34.0	13	34.2
Eleburu	27	24.0	9	33.3
Tapa	23	20.0	12	52.2
Atere	25	22.0	8	32.0
Total	113	100	42	37.2

Resistance Profile of CA-MRSA isolates to antibiotics

The percentage distribution of CA-MRSA showed that 42 (100 %) were resistance to cefuroxime, cefotaxime, cloxacillin and augmentin, while 34 (80.9 %) were resistant to ceftriaxone, 35 (87.5 %) are resistant to tetracycline, 29 (69.1 %) were resistant to erythromycin, 8 (19.1 %) were resistant to gentamicin and 10 (23.8 %) were resistant to ofloxacin.

Molecular analysis of the isolated MRSA

Molecular analysis on the 6 isolates that were methicillin resistant *Staph aureus* showed that *muc* and *mec A* genes were detected in all the 6 isolates (Plates 1; 2). While only 3 of the six Methicillin resistant isolates had *lukS-lukF* (Plate 3) and only 3 of the six methicillin resistant *Staph aureus* isolates had PVL gene detected (Plates 3).

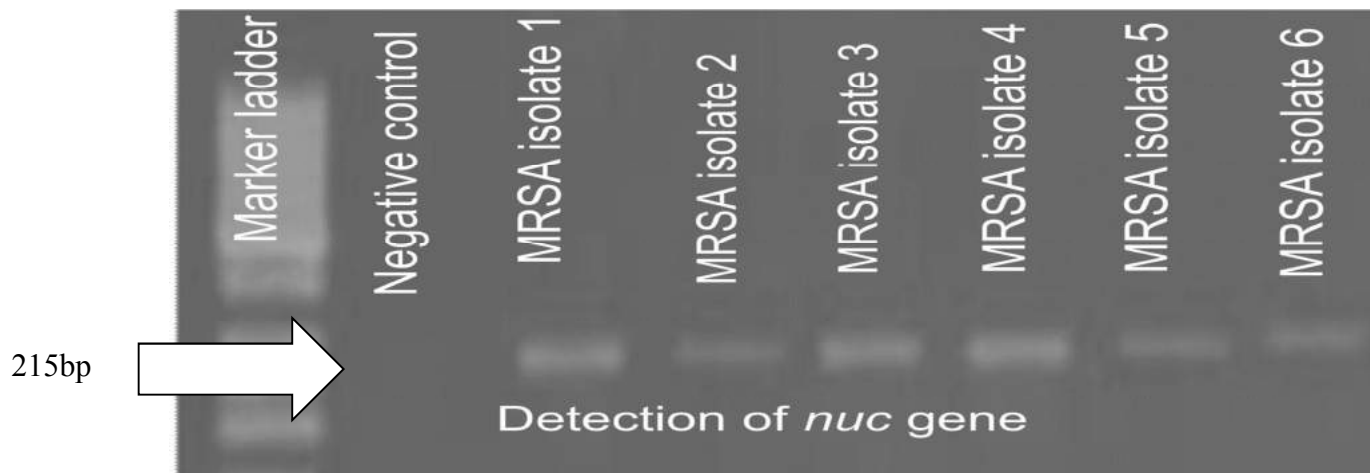


PLATE 1: PCR PRODUCTS AFTER GEL ELECTROPHORESIS SHOWING AMPLICONS DETECTION OF THE *MUC* GENES OF *S. AUREUS* ISOLATES.

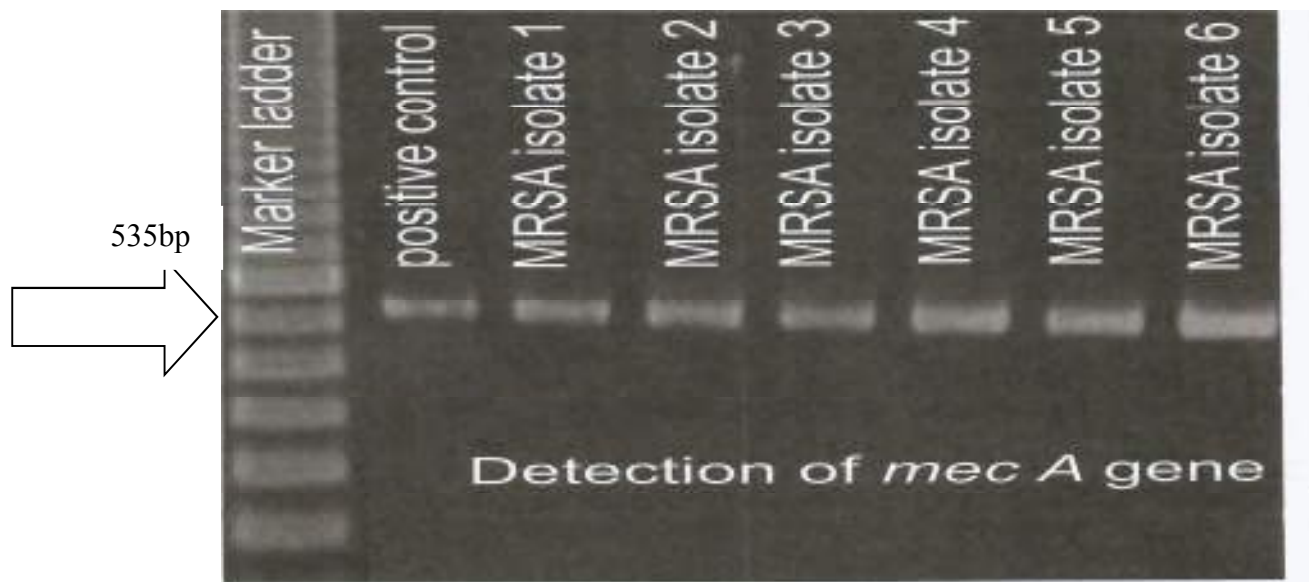


PLATE 2: PCR PRODUCTS AFTER GEL ELECTROPHORESIS SHOWING AMPLICONS FOR *MEC A* (535BP).
DNA MOLECULAR WEIGHT MARKER (M) (535BP)



PLATE 3: PCR PRODUCTS AFTER GEL ELECTROPHORESIS SHOWING AMPLICON FOR PVL GENE DETECTED IN ONLY THREE OUT OF SIX CA-MRSA ISOLATED FROM MALETE COMMUNITIES. DNA MOLECULAR WEIGHT MARKER (M) (435BP)

DISCUSSION

Community acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has emerged as an important human pathogen in various parts of the world. This pathogen has varied distribution in both urban and rural community settings. In this study, we reported a prevalence of 37.2 % of *Staph aureus* in nostrils of apparently healthy individuals in rural communities of Malete. This prevalence is lower than a prevalence of 56.3 % reported by Chibuike and colleagues [20] in rural communities of Ururu among healthy individuals. Also a lower prevalence of 36.5 and 13.9% were reported by Egwuata *et al.* [21] in health workers in Lagos and Okwu *et al.* [22] among healthy individuals in Okada, Edo State Nigeria respectively. Various authors have recommended eradication of nasal carriage of *Staph aureus* to reduce transmission to others with the use of systemic antimicrobials, normal bacterial flora augmentation, antiseptic washes and topical antimicrobials [23], [24].

Antimicrobial resistance is one of the major threat posed by microorganisms to infection management in this twenty first century and *Staph aureus* has always been one of the major pathogen that possess ability to develop resistance to newly developed antimicrobial agents [25]. In this study, *Staph aureus* isolated were resistant to commonly available and recommended antibiotics such as cefuroxime, cefotaxime, cloxacillin and augmentin, but susceptible to ceftriaxone, tetracycline, erythromycin, gentamicin and ofloxacin in varying degrees. Susceptibility of erythromycin ranges from 5 to 64% in different geographic areas [26], while the susceptibility in this study is 30.9 %. There are many factors that may be adduced to the high resistance of this organism to antibiotics in these rural communities. Such factors included self-medication,

availability and use of antibiotics without Doctor's prescription, irrational consumption rate of antibiotics, noncompliance to prescription and sales of fake or substandard drugs and transmission of resistant strains between individuals within the community.

Methicillin resistance is mediated among *Staph aureus* by the penicillin-binding protein (PBP)- 2a encoded by *mecA* gene. It was reported that all β -lactam antibiotics have poor affinity when PBP is altered, and such microbe would not be killed when exposed to therapeutic concentration. The *mecA* gene is found to be located on a mobile genetic element of staphylococcal chromosome cassette (SCCmec) [27]. In this study, the prevalence of methicillin resistance *Staph aureus* was 14.3 %. This prevalence is lower when compared with what other researchers reported among apparently healthy individuals. Prevalence of 22.0, 47.6 and 60.6 % were reported by Akerele *et al.* [28] in Ekosundun, Benin City; Adelowo *et al.* [29] in Maiduguri and Chibuike *et al.* [21] in Uturu rural community respectively.

The major objective of screening for *mecA* gene is to compare results of antibiotic susceptibility by disc diffusion method with gene analysis results in *Staph aureus* isolates. The phenotypic expression of antibiotic resistant genes has been reported to be affected by different conditions such as the incubation temperature and time, medium inoculated, inoculum size, test agents, pH, and ionic strength of NaCl [30]. The *mecA* gene may be heterogeneously expressed and, therefore, all MRSA strains may not be detectable with phenotypical methods and the test require at least 24 h for evaluation of the results. However, the detection of *mecA* gene by PCR techniques is considered the gold standard method. All the six isolates that were cefoxitin resistant were also positive for this gene detection method;

confirming that they are all methicillin-resistant *Staph aureus*

The present study showed strong correlation between genotypic and phenotypic analysis, the results were consistent with previous studies which showed a perfect and strong correlation between the results obtained by the phenotypic antibiotic resistance determination and PCR-based assays [31], [32]. The critical parameters for success of a PCR-based assay for the detection of multidrug resistant bacteria like MRSA are the reliability, accuracy, fast and sensitivity and results were obtained within hours [33]. However the cefoxitin disc has been shown to have specificity 97-100 % to detected *mec A* gene [34].

Previous reports on MRSA in North-central, Nigeria were based phenotypic method, to our best knowledge this is the first report based on the detection of *mecA* gene in CA-MRSA among healthy individuals in rural communities of North-central, Nigeria. However, *mecA* gene has been discovered from other different part of Nigeria such reports included, a study in Benin city, Nigeria, 4 isolates representing 11 % were confirmed to carry *mecA* gene using molecular technique [35], Esan *et al.* [36] confirmed only one MRSA isolate from health care institutions from Ekiti and Ondo states and Shittu *et al.* [37], detected two MRSA isolates with *mecA* gene were detected in Ile-Ife, one from Lagos and two from Ibadan (all in South-western Nigeria) In the North-eastern Nigeria, five MRSA isolates with *mec A* gene were detected in Maiduguri by Okon *et al.* [38] reported the detection of 12.5 % MRSA from clinical specimens from six tertiary hospitals.

PCR result for the detection PVL (*lukS-lukF*) gene in this research showed that out of 6 MRSA isolates screened only 3 of them were PVL positive, representing a percentage of 50 % (n=6). This finding was higher compared to the report of Sani *et al.* [39] who detected PVL gene prevalence of 3.25 and 8.13 % amongst MRSA from hospitalized and non-

hospitalized patients in Benin. However, this difference may be due to the size of our samples. Another report by Breurec *et al.* [40] detected a 57 % *lukS-lukF* genes encoding Pantone-Valentine Leukocidin (PVL) prevalent in MRSA isolates from Cameroon, Niger, and Senegal (West and Central Africa).

It appears that misuse and overuse of these antibiotics could have contributed to increase in antibiotics resistance in these rural communities as it has been observed in other part of the world. Therefore, to prevent resistance and treatment failures, we advocate proper monitoring of antibiotics usage and collection of data on antibiotic susceptibility testing pattern, public enlightenment on the effectiveness of various antibiotics and the enactment of drug policies in Nigeria; are urgently needed to move in line with the global trends.

Multidrug resistant organisms are defined as organisms that have acquired non-susceptibility to at least one agent in three or more classes of antimicrobial [41], [42], [43], [44]. By this definition, all the six CA-MRSA isolated in this community are multidrug resistant *Staph aureus*.

Conclusion: The detection of methicillin-resistant *Staph aureus* carriage among individuals in these remote rural communities of Maleté indicated the extensive distribution of this organism and also indicated that the abuse of drug is on high side in Nigeria. Unlicensed patent medicine dealer and drug hawkers may be the main source of drugs distribution to these villages.

Acknowledgment: We acknowledged the cooperation of members of Apo, Eleburu, Atere and Tapa communities, Director of Community Development Centre (CDC), Kwara State University, Maleté and technical staff of Microbiology laboratory for their support.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1904 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.4>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 25-34

THE CHARACTERIZATION OF MULTIDRUG RESISTANT TYPE 1 S-FIMBRIATED *ESCHERICHIA COLI* FROM WOMEN WITH RECURRENT URINARY TRACT INFECTIONS (RUTIS) IN BANGLADESH

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The characterization of multidrug resistant type 1 S-fimbriated *Escherichia coli* from women with recurrent urinary tract infections (RUTIs) in Bangladesh

ABSTRACT

Background: *Escherichia coli* is a bacterial species that is most frequently associated with urinary tract infections (UTIs) worldwide. Recurrent UTIs (RUTIs) pose a major threat to health, especially in women. However, studies on the pathogenic potentials of *E. coli* isolates associated with RUTIs has yet to be done. The present study was designed to characterize the isolates of *E. coli* from women, suffering from repeated UTIs in Bangladesh.

Methods: A total of 15 isolates of *E. coli*, collected from women with RUTIs in the Sylhet city of Bangladesh, were analyzed by a series of biochemical and molecular tests.

Results: The PCR amplification of the mannose-resistant type 1 S-fimbriae gene (*sfa1*) confirmed all *sfa1*⁺ isolates of *E. coli*. In an antibiogram assay, all the isolates of *E. coli* were found to be completely resistant to at least five of the ten commercial antibiotics and drug resistance were found to be correlated with the weight of the RUTIs patient. Analysis of the 16S rRNA data of the two most drug-resistant and clinically significant isolates were found to be clustered with the drug-resistant UTI-causing *E. coli* isolates, circulated in India.

Conclusions: This study could pave the way of RUTIs diagnosis and treatment strategy for women in Bangladesh.

Keywords: RUTIs; *E. coli*; *sfa1*; multidrug-resistant isolates; 16S rRNA gene sequencing; correlation analysis.

LA CARACTERISATION DES *ESCHERICHIA COLI* MULTIRESISTANTES DE TYPE 1 S-FIMBRIEES CHEZ DES FEMMES AYANT DES INFECTIONS URINAIRES RECIDIVANTES (IUTR) AU BANGLADESH

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ABSTRAIT

Contexte: *Escherichia coli* est une espèce bactérienne associée le plus fréquemment aux infections urinaires dans le monde entier. Les infections urinaires récurrentes (IVU) constituent une menace majeure pour la santé, en particulier chez les femmes. Cependant, les études sur le potentiel pathogène des isolats d'*E. coli* associés aux RUTI n'ont pas encore été réalisées. La présente étude visait à caractériser les isolats d'*E. coli* provenant de femmes souffrant d'infections urinaires répétées au Bangladesh.

Méthodes: Au total, 15 isolats d'*E. coli*, prélevés chez des femmes atteintes de RUTI dans la ville de Sylhet au Bangladesh, ont été analysés par une série de tests biochimiques et moléculaires.

Résultats: L'amplification par PCR du gène S-fimbriae résistant au mannose (*sfa1*) a confirmé tous les isolats de *sfa1* + d'*E. coli*. Dans un essai d'antibiogramme, tous les isolats d'*E. coli* se sont révélés complètement résistants à au moins cinq des dix antibiotiques commerciaux et la résistance aux médicaments s'est avérée être corrélée au poids du patient RUTI. L'analyse des données de l'ARNr 16S des deux isolats les plus résistants aux médicaments et cliniquement significatifs s'est avérée être regroupée avec les isolats d'*E. coli* causant des infections urinaires résistantes aux médicaments, diffusés en Inde. **Conclusions:** Cette étude pourrait ouvrir la voie au diagnostic et à la stratégie de traitement des RUTI chez les femmes au Bangladesh.

Mots-clés: RUTIs; *E. coli*; *sfa1*; des isolats multirésistants; Séquençage du gène de l'ARNr 16S; analyse de corrélation.

INTRODUCTION

Urinary tract infections (UTIs) are the systemic bacterial infections that are known to affect the urethra, urinary bladder, and kidneys. Females are mostly infected due to their anatomical arrangement- a shorter urethra, resulting in an easier travel by the bacteria (1). Previous studies have revealed that around 50–60% of women are likely to develop UTIs in their lifetime (2). The numerous causative agents, responsible for this disease; however, *E. coli* alone accounts for 80–85% of the global UTIs (3, 4). Recurrent urinary tract infections (RUTIs) are the reinfections that are generally caused by the original bacterial isolates at a young age. Frequent sexual intercourse increases the chance of getting RUTIs (5). Approximately one third of women are found to be positive for RUTIs by the same bacteria (6, 7). In some cases, RUTIs can be lethal when the bacteria persist for a long time in a position (5).

Drug resistance in *E. coli* is one of the most common barriers for treating UTI patients worldwide. However, the problem is more severe in countries like Bangladesh due to an improper tendency of frequently prescribing antibiotics for the treatment of UTIs (8). The multidrug-resistant strains of *E. coli* have been reported to further add to the complications in the UTI patients and decrease the effectiveness of the treatment (9, 10). In addition, patients with RUTIs have been reported to have a higher prevalence of antimicrobial resistance due to the evolution and spread of more virulent strains by various genetic

mechanisms (11). The frequency of RUTIs caused by the multidrug resistant strains of *E. coli* has increased recently and has sparked strong attention from the government, medical practitioners, and health agencies (12). Therefore, regional studies on the patterns of antibiotic sensitivity are much needed for selecting proper treatment strategies to overcome the massive problem of drug resistance.

Virulence factors play a major role in the pathogenicity of *E. coli* associated RUTI infections. The S- fimbriae adhesin (*sfa*) genes, encoded by *sfa* operon are common in all the types of UTIs found to be strongly associated with *E. coli* pathogenicity (13). The *sfa* gene is associated with UTIs, gestational pyelonephritis, recurring cystitis, pregnancy complications, and diarrhea (13, 14). The isolates of *E. coli*, especially from the uropathogenic (UPEC) and the diffusely adhering (DAEC) groups are known to produce this virulence factor that mediates the host-pathogen interactions (13, 15).

Recent development in molecular techniques, especially the 16S rRNA gene sequencing tool has been extensively used for the analysis of bacterial species in clinical samples (16, 17). In addition, the introduction of computer-aided bioinformatics tools in sequence analysis has simplified the understanding of the strains that are poorly characterized and rarely isolated. Therefore, the aim of the present study was the characterization and phylogenetic positioning of the isolates of *E. coli* from women with RUTIs, by using the

computer-aided bioinformatic analysis of their 16S rRNA gene sequences.

MATERIALS AND METHODS

Ethics statement

This work has been conducted in accordance with “The Code of Ethics of the World Medical Association”. The Graduate Research Ethics Committee (GREC) of the School of Life Sciences, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh, approved and monitored the study. The patients consent data were handled according to human privacy rights.

The collection and culture of the bacterial isolates

A total of 15 isolates of *E. coli* (E1-E15) were collected from three different hospitals: the Popular Medical Diagnostic Centre (PMDC), Sylhet, Bangladesh, the Jalalabad Ragib-Rabeya Medical College and Hospital (JRRMCH), Sylhet, Bangladesh, and the MAG Osmani Medical College and Hospital (MOMCH), Sylhet, Bangladesh from January, 2017 to December, 2017. After screening the patient data, samples were collected from only those women, who were having a history of RUTIs. In addition, other information about the patients, including patient ID (PID), age, and the types of infection were also recorded from the patient consent forms (Table 1). Primarily, all the patient samples were inoculated onto a chromogenic medium, containing Eosin Methylene Blue (EMB) and incubated at 37 °C for 24 h at the Biochemistry and Microbiology laboratory of PMDC, Sylhet, Bangladesh. The samples were then transported, at a low temperature, to the laboratory for further studies.

The biochemical characterizations

All the isolates of *E. coli* were subjected to several biochemical and microbiological tests, following the Bergey's manual for the presumptive identification of *E. coli* (18). The isolates were assayed for Gram staining, catalase test, oxidase test, oxidative-fermentative (OF) test, H₂S production test, Methyl Red (MR) test, Voges-Proskauer (VP) test, citrate test, urease test, gelatin test, gas production test, etc. Following these biochemical tests, the positive isolates of *E. coli* were preserved and cultured for further identifications through the PCR technique.

The extraction and quantification of genomic DNA

Using a commercial bacterial genomic DNA extraction kit (Bio Basic Inc., Markham, Ontario, Canada), genomic DNA from the isolates of *E. coli*

was extracted. Following the manufacturer's instructions, Proteinase K and RNase A were added to remove impurities from the DNA samples. Using a lambda (λ) DNA molecular weight marker, the quantification of the extracted genomic DNA samples was done on an agarose gel and considering protein-DNA absorbance recorded using NanoDrop UV-Visible measurements for nucleic acid (ThermoFisher Scientific, 2000c). The DNA was then diluted accordingly to make the final concentration 30 ng/μl. The extracted genomic DNA samples were then preserved in an ultrafreezer at -20° C for further use.

The amplification of the *sfa* gene

PCR amplification was performed in 50 μL reaction mixtures, containing 25 μL of the prepared 2X master mixtures (Fermentas, Gene-Ruler™, USA), 2.5 μL of each of the forward and reverse primers (*sfaF*, 5'-CTCCGGAGAACTGGGTGCATCTTAC-3' and *sfaR* 5'-CGGAGGAGTAATTACAAACCTGGCA-3'), 5 μL of the template DNA (100 ng), and 15 μL of nuclease-free water. The PCR conditions were optimized as follows: an initial denaturation step at 94 °C for 4 min, a denaturation step at 94 °C for 1 min, an annealing step at 65 °C for 1 min, an extension period at 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min, followed by storage at 4 °C. A total of 35 serial cycles of the amplification reaction were performed in a MultiGene Gradient Thermal Cycler (Labnet International Inc., USA). The PCR products were separated by electrophoresis on an agarose gel, stained with ethidium bromide solution, and visualized in a gel documentation system(8).

The antibiogram profiles of the isolates of *E. coli*

The antibiotic profiles of the *E. coli* isolates against ten commercial antibiotic discs were determined by the Kirby-Bauer Disc Diffusion Method (19). An aliquot of 30 μL of overnight bacterial cultures (10⁶ CFU/mL) was spread on Tryptic Soy Agar plates (Micromaster Laboratories Pvt. Ltd., Maharashtra, India) and the antibiotic discs were placed aseptically onto the culture media, in order to observe the antibiogram profiles of all the isolates of *E. coli*. The antibiotic discs, used in this study were ampicillin (10 μg/disk), cotrimoxazole (10 μg/disk), tetracycline (30 μg/disk), erythromycin (15 μg/disk), ciprofloxacin (30 μg/disk), cephradine (25 μg/disk), gentamicin (10 μg/disk), streptomycin (10 μg/disk),

chloramphenicol (10 µg/disk), and sulfamethoxazole (25 µg/disk). After 24 h of incubation at 37 °C, the zones of inhibition were measured (8).

The amplification and sequencing of the 16S rRNA gene

The amplification of the target sequences of the 16S rRNA gene specific for the isolates of *E. coli* was carried out following the standard method (20). The PCR reaction mixture was prepared in the same concentration as that used for the amplification of the *sfa* gene with 2.5 µL of each of the universal primers, 27F and 1492R (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGATACCTTGTACGACTT-3'). For this reaction, a total of 30 serial cycles were programmed and the PCR parameters were adjusted like the previous amplification reaction. The amplified full-length PCR products, having a size of 1465 bp were visualized in an agarose gel and purified with the PureLink PCR Purification Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The purified 16S rRNA PCR products were then sequenced from the "1st BASE" sequencing center, Malaysia. The raw sequences were extracted, edited, assembled, and run for further analysis, using the various sequence analysis tools (16).

Analysis of the 16S rRNA gene sequence data and the status of phylogenetic relationship

According to the antibiogram assay, the two most multidrug-resistant isolates of *E. coli*, E8 and E10 – also associated with other complications in the patients such as cystitis, hypertension, and pregnancy – were selected for the universal 16S rRNA gene sequencing to analyze their phylogenetic positions. After sequencing, the raw sequences were extracted and assembled using the BioEdit (v7.0.4), Chromas (v2.01), and SeqMan Pro (v15.0) sequence editing tools. The web-based database, DECIPHER (v9.20), was used to remove the chimeras from the raw sequences. Using BLASTn in the NCBI database, the edited DNA sequences were then run for the homology matching of the highly similar sequences. Using the ClustalW program in MEGA (v7.0), the assembled sequences were aligned and using the nearest neighbor method with 1,000 bootstrap

replicates, a phylogenetic tree was constructed. The GenBank accession numbers of the similar sequences were indicated in parenthesis to most of the *E. coli* species. The evolutionary divergence among the sequences was calculated as the Kimura J-Model in MEGA (v7.0).

Statistical analysis

Data collected from RUTI patients were plotted in corrplot (v0.84) for finding of graphical correlations among variables in RStudio of R package. A total of 15 observations from four variables were tested for Pearson(s) correlation ($p < 0.5$), structured in matrix (mat) form, then mixed (corrplot.mixed) and finally visualized using color method where blue signified positive correlations and red demonstrated negative correlations.

RESULTS

The biochemical characterization of the isolates of *E. coli*

Although all the isolates were initially supplied as *E. coli* in the EMB agar medium, the specific details of the morphological and biochemical characterizations confirmed that all of them belonged to the same species. The results showed isolates were positive for catalase, indole, methyl-red, and motility tests while the alpha-hemolysin producing assay exhibited partial lysis of the sheep blood agar and appeared greenish in color following overnight incubation. The isolates were negative for Gram's test, oxidase, voges-proskauer tests; couldn't utilize citrate, urease, gelatin and H₂S for production of gas.

The amplification of the *sfa* gene in the isolates of *E. coli* by PCR

In the PCR amplification, all the 15 *E. coli* isolates were positive for the *sfa* gene and gave an expected product size of 410 bp upon visualization on the agarose gel. The results of the PCR amplification of the *sfa* gene in the isolates of *E. coli*, collected from RUTIs women are shown in **Figure 1**.

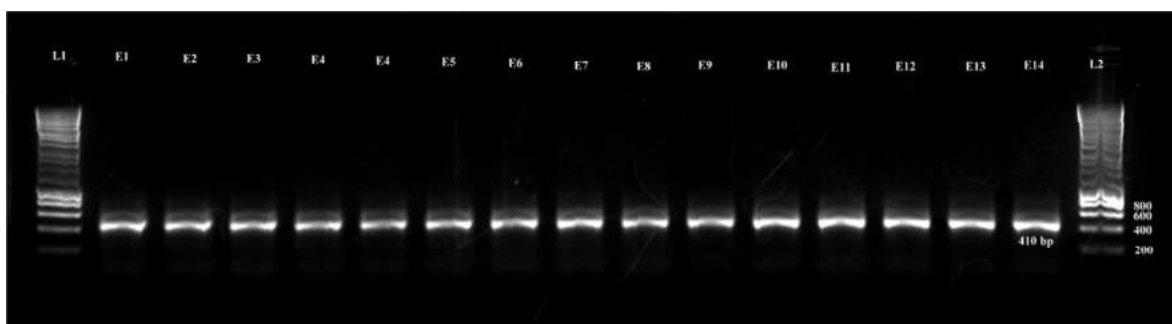


FIGURE 1: PCR AMPLIFICATION OF AFA1 GENES IN *E. COLI* ISOLATES. ALL OF THE ISOLATES WERE POSITIVE FOR SFA1 AND SHOWED 410 BP PCR PRODUCTS ON AGAROSE GEL.

The antibiotic sensitivities of the isolates of *E. coli*

Among the ten antibiotics tested by the Kirby-Bauer Disc Diffusion Assay, all the isolates of *E. coli* were completely resistant to ampicillin (AMP), erythromycin (ERY), streptomycin (STP), sulfamethoxazole (SXT), and tetracycline (TET).

Out of 15 isolates, five (E2, E5, E8, E9, E10) were resistant to gentamicin (GEN) and cephadrine (CEP) and two (E8, E10) displayed resistance to cotrimoxazole (COT). The isolate E8 showed sensitivity to only ciprofloxacin (CIP) and E10 was inhibited by chloramphenicol (CPL) and ciprofloxacin (CIP).

TABLE 1: LIST OF SAMPLES AND INFORMATION

<i>E. coli</i> isolates	Age	No. of antibiotic resistance (out of 15)	Weight	Sources
E1	37	6	56	1*
E2	40	7	48	2†
E3	34	5	52	3□
E4	40	6	58	1*
E5	35	7	50	1*
E6	33	6	54	2†
E7	38	6	49	2†
E8	35	9	62	1*
E9	43	7	58	3□
E10	33	8	60	2†
E11	36	6	52	1*
E12	42	6	54	1*
E13	45	6	53	2†
E14	48	5	50	2†
E15	40	6	56	3□

Footnotes: *Popular Medical and Diagnostic Centre, Sylhet, Bangladesh; †Jalalabad Ragib-Rabeya Medical College and Hospital, Sylhet, Bangladesh; □MAG Osmani Medical College and Hospital, Sylhet, Bangladesh.

The amplification of the universal 16S rRNA gene sequences

The genomic DNA of the isolates of *E. coli* was used to amplify the universal 16S rRNA gene sequences by PCR. The multidrug resistant and complications-associated isolates, E8 and E10, were amplified by the universal amplification primers, 27F and 1492R. Both the isolates gave positive amplifications and displayed an expected product size of 1465 bp on the agarose gel.

Analysis of the 16S rRNA gene sequences of the multidrug-resistant isolates of *E. coli*

In the NCBI database, BLASTn of the assembled sequences of the *E. coli* isolates, E8 and E10, showed 99.0% homology to the sequences of the *E. coli* isolates, extracted from clinical samples. The evolutionary divergence score revealed no

significant distances (0.000) among the isolates in the present study and the two most recent UTI-causing strains of *E. coli* (IBB1 and U744) are circulating in India since August 2017. However, evolutionary distances were found to exist among the study isolates and the other strains, previously circulating in countries such as Pakistan and the USA (0.001–0.002). The phylogenetic tree also supported the close relationship of the four strains—E8, E10, IBB1, and U744, circulating in 2017, according to the cluster they formed (Figure 2). The outgroup bacteria, *Klebsiella pneumoniae*, were positioned in a completely separate cluster in the phylogenetic tree and had significant evolutionary distances with the study isolates (0.048). The sequences of the isolates of the present study are now available at the NCBI database under the GenBank accession numbers, MG857840.1 (*E. coli* strain E8) and MG857757.1 (*E. coli* strain E10).

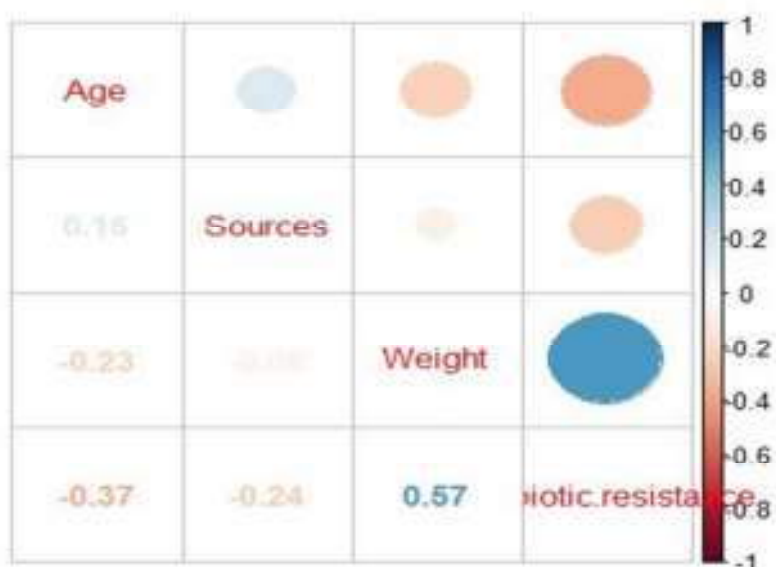


FIGURE 2. CORRELATION MATRIX PLOT SHOWED ASSOCIATION AMONG OBSERVATIONS. DRUG RESISTANCE WERE FOUND TO HAVE AN ASSOCIATION WITH WEIGHT BUT INDEPENDENT OF AGE AND SOURCES (HOSPITALS).

Correlations among variables

Among four variables, antibiotic resistance was found to be positively correlated (+0.57) with the weight of the RUTIs patients. The variables for

antibiotic resistance pattern were independent of patient's age and sources of sample collection while the correlation between age and sources was insignificant to perspectives of the present study (Figure 3).

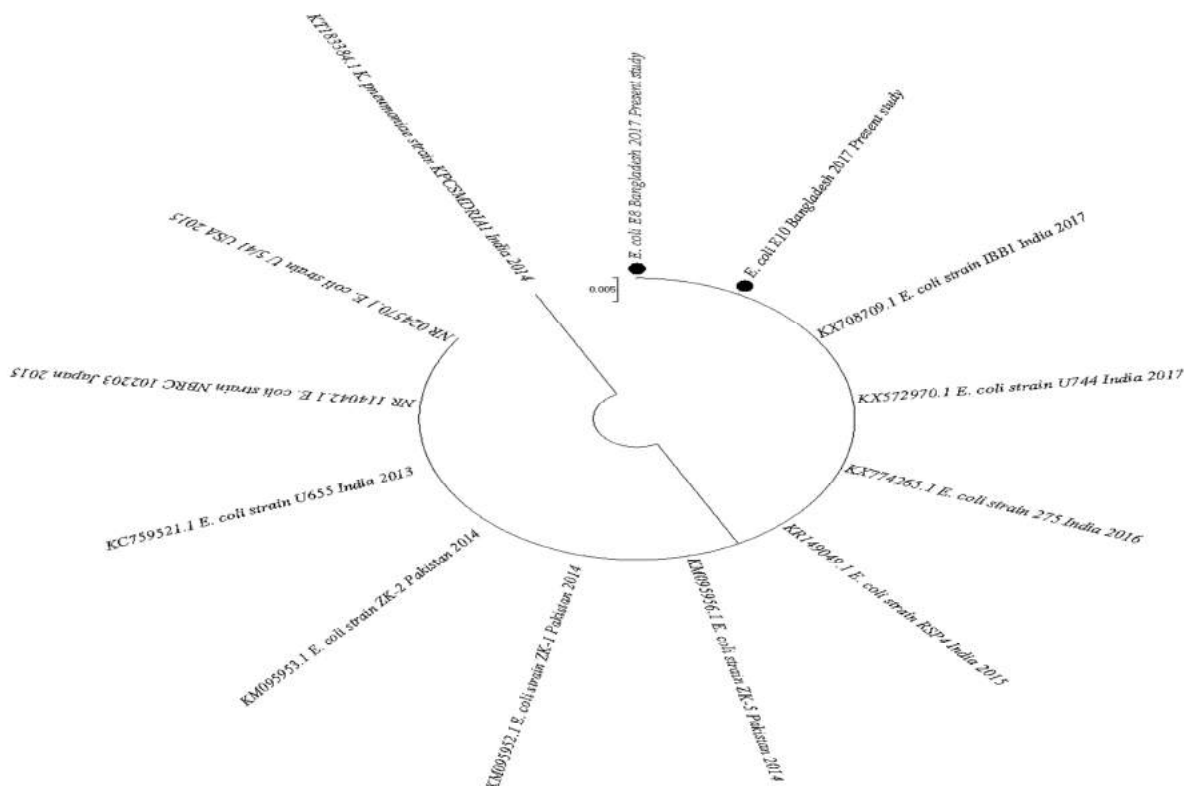


FIGURE 2: THE PHYLOGENETIC RELATIONSHIP OF *E. COLI* STRAINS E8 AND E10, ON THE BASIS OF 16S RRNA SEQUENCES, WITH THE *E. COLI* ISOLATES CAUSING UTI IN OTHER PARTS OF THE WORLD.

The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. all positions containing gaps and missing data were eliminated. *Klebsiella pneumoniae* signifies an outgroup bacteria in the tree. evolutionary analyses were conducted in mega7.

DISCUSSION

The accumulation of the multidrug-resistant clinical isolates results in significant health challenges worldwide, especially in women. Particularly in low-income countries, the prevalence and occurrence of the multidrug-resistant isolates of *E. coli* are much higher and more common (4). Previous studies have revealed that 69% of the UTIs are caused by *E. coli* in Bangladesh, where 83–85% of the infected patients are females (21, 22). Although many of these women have faced RUTIs in their lifetime, most of them were literally unaware of their reinfections (5). Currently, no clinical data about the percentages of women, suffering from RUTIs in Bangladesh are available because of the improper diagnostic and treatment strategies. Hence, we found only seven isolates of *E. coli* after 12 months

of screening across three different hospitals. Due to the frequent use of antibiotics and rupture in the antibiotic dose regimens, the clinical isolates of *E. coli* have recently emerged as multidrug-resistant variants all over the country, thereby making the treatment of UTIs and RUTIs challenging (21, 23). Previous studies have discovered the failure of the commercial antibiotics against the clinical isolates of *E. coli* in the major cities of Bangladesh, including Dhaka, Rajshahi, and Sylhet, where 50–70% of drug resistance was noticed from 2010–2015 (24, 25). The present study found no effective agent that can potentially inhibit all the isolates of *E. coli*. Our results revealed that the pattern of drug resistance was increasing very rapidly in Bangladesh and varied even within a city, where drug resistance to the UTI-causing isolates of *E. coli* increased by

approximately 5–10% in every five years (23). Therefore, a careful use of the commercial antibiotics needs to be executed through appropriate monitoring systems only after a proper diagnosis of bacteria, in order to minimize the spread of the drug-resistant isolates.

In Bangladesh, the diagnosis of the isolates of *E. coli* from the UTI patients depends solely on the EMB Agar Base plate culture method, which often gives false positive results and requires significant technical skills, in order to confirm the bacteria (26). Although the 16S rRNA gene sequencing method is routinely used alongside plate-based screening for the analysis of bacterial sequences and phylogenetic relationship, the details of the virulence properties, molecular characterizations, and phylogenetic analysis, using PCR and the 16S rRNA gene sequencing tools are yet to be undertaken in Bangladesh. The detection of virulence factors is always a key aspect in analyzing the pathogenicity of any bacterial isolate because these factors act multifunctional (27). In the present study, we found very high frequency of the *sfa* gene in the hospitalized RUTIs patients, where all the isolates were positive in PCR amplification. Previous studies have concluded that more than 50% of the *sfa1+* isolates of *E. coli* in the UTIs have a high prevalence of virulence factors and pose a serious threat to the health of the patients (28). Adhesion and fimbrial virulence factors in *E. coli* such as *sfa*, *afa*, *fim*, etc., cause further risks in the development of RUTIs, especially in women (29). In 13 years (2002–2015), studies found approximately 13% increase of *sfa* prevalence in *E. coli* causing UTI (13). In contrast to the previous studies, our study revealed that the *sfa* gene frequencies are much higher (100%) in the Eastern part of Bangladesh and is probably

responsible for other complications in women. In phylogenetic studies, the *E. coli* isolates in the present study clustered very closely to the *E. coli* strains found in India in 2017, suggesting the same evolutionary origin for these bacteria. The changing phylogenetic position of the isolates of *E. coli* each year indicates the accumulation of the more multidrug-resistant isolates in South Asia. Similar to the *E. coli* isolates, E8 and E10, the multidrug-resistant strains, IBB1 and U774 were also characterized from the chronic UTI patients and had a number of virulence factors (30, 31). In addition to drug resistance, obesity also increases the risk of RUTIs infection in women. One study revealed significant correlation of obesity with RUTIs ($p < 0.001$) where fatness increases the risk of recurrent infections in women by 27% and drug resistance by 20% (32). In present study, the association between weight and drug resistance also significant compared to other variables. Thus, the overall trend and pathogenicity of the drug-resistant isolates of *E. coli* are truly alarming in Southeast Asia for the upcoming days.

CONCLUSION: In this comprehensive study, we effectively used the various traditional and molecular biology tools for the precise identification and analysis of the isolates of *E. coli* from women with RUTIs in the eastern part of Bangladesh that can be used for the further molecular analysis of these isolates, associated with UTIs and RUTIs in Bangladesh.

ACKNOWLEDGMENT: The authors are thankful to the Ministry of Science and Technology (MoST), Bangladesh for supporting this research project (R&D-005) for the fiscal year of 2015–2016.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1905 <http://www.ajol.info/journals/ajcem>
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AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 35-41

BACTERIAL CONTAMINATION OF TOILET DOOR HANDLES ON BAZE UNIVERSITY CAMPUS ABUJA NIGERIA

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ABSTRACT

Aim: Contracting infectious diseases from microbial contaminated toilet door handles is a potential threat to public health and safety. Therefore we performed microbiological screening of toilet door handles in twelve public toilets on Baze University campus for bacterial contamination.

Methodology and Results: Biochemical analysis of bacterial isolates from entrance toilet door handles in six building blocks on Baze University campus, revealed a general contamination by mainly seven bacterial species associated with human gut and skin flora in order of decreasing frequency as follows: *Staphylococcus aureus* 42.9%; *Salmonella typhimurium* 21.4%; *Escherichia coli* 14.3%; *Pseudomonas aeruginosa* 9.5%; *Proteus mirabilis* 4.8%; *Klebsiella oxytoca* 4.8%; and *Klebsiella pneumoniae* with 2.3% prevalence.

Results of the total aerobic mesophilic count showed A-Block with the highest amount of contamination - 768*10⁵CFU/ml, while B-Block had the least amount of bacterial contamination - 473*10⁵CFU/ml. The levels of bacterial contamination in the other blocks were as follows: C-Block - 710*10⁵CFU/ml, D-Block - 695*10⁵CFU/ml, E-Block - 567*10⁵CFU/ml, and F-Block - 743*10⁵ CFU/ml.

Conclusion: Overall of the seven isolated bacterial species five were mainly gut-associated bacteria, suggesting fecal contamination. The others were skin-associated bacteria (*Staphylococcus aureus*), suggesting routine touch by hands, and soil-associated bacteria (*Pseudomonas aeruginosa*) suggesting contamination from settling dust particles. This study is relevant for public health and safety, as its findings reveal the presence of bacterial pathogens on toilet door handles, which is vital in preventing the spread of infectious disease.

Keywords: Toilet door handles, bacterial pathogens, fecal contamination, antibiotic resistance.

CONTAMINATION BACTÉRIENNE DE POIGNÉES DE PORTE DE TOILETTES SUR LE CAMPUS UNIVERSITAIRE DE BAZE AU NIGERIA D'ABUJA

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ABSTRAIT

Objectifs: Le fait de contracter une maladie infectieuse à partir de poignées de porte de toilettes contaminées par des microbes est une menace potentielle pour la santé et la sécurité publiques. Cette étude inédite à l'université Baze d'Abuja, au Nigéria, a pour but d'analyser des poignées de portes de toilettes sélectionnées provenant de six bâtiments du campus de l'Université pour détecter une contamination bactérienne.

Méthodologie et résultats: L'analyse biochimique des isolats bactériens provenant des échantillons de portes de toilettes sélectionnés a révélé une contamination générale des toilettes échantillonnées, principalement par sept espèces bactériennes associées à des maladies d'origine alimentaire et hydrique: *Staphylococcus aureus* 42,9%; *Salmonella typhimurium* 21,4%; *Escherichia coli* 14,3%; *Pseudomonas aeruginosa* 9,5%; *Proteus mirabilis* 4,8%; *Klebsiella oxytoca* 4,8%; et *Klebsiella pneumoniae* avec une prévalence de 2,3%. Les résultats de la numération mésophile aérobie totale sur des plaques de gélose nutritive ont montré la plus grande quantité de contamination dans le bloc A - 768 * 10⁵CFU / ml, alors que le B-Block présentait le moins de contamination bactérienne - 473 * 10⁵CFU / ml. Les niveaux de contamination bactérienne dans les autres blocs étaient les suivants: C-Block - 710 * 10⁵CFU / ml, D-Block - 695 * 10⁵CFU / ml, E-Block - 567 * 10⁵CFU / ml et F-Block - 743 * 10⁵ CFU / ml. Conclusion, importance et impact de l'étude: Les agents pathogènes bactériens isolés des poignées des portes de toilettes échantillonnées sont une source de préoccupation, car ils présentent un risque grave pour la santé et la sécurité publiques.

Mots-clés: poignées de portes de toilettes, agents pathogènes bactériens, microorganismes indicateurs, résistance aux antibiotiques

INTRODUCTION

The transmission of infectious diseases from fomites in the surrounding environment is a potential threat to the public health and safety [1, 2, 3]. Remarkably, one of the sources of transmission of these infectious diseases is the previously unknown and seemingly harmless toilet door handles, which are often teeming with microorganisms due to the frequent and inevitable use [4, 1, 5]. Recent studies have shown the presence of bacterial pathogens on hard, non-porous surfaces such as kitchen surfaces, floor surfaces, toilet surfaces, door handles, etc., [6, 7, 8, 1, 9, 10, 5], from which pathogens are easily transmitted to unsuspecting members of the public posing a potential risk to vulnerable, immune-compromised individuals [11, 2, 5]. Currently, some of these bacterial pathogens have become antibiotic resistant, which is a major public health crisis facing the world today [12, 13, 14, 15]. Therefore there is a need to improve standards of toilet hygiene and toilet door handles in order to reduce the spread of infectious diseases [16, 11]. Hence the aim of this research was to examine twelve (six male and six female) public toilet door handles on Baze University campus for bacterial contamination. Our objectives for this research were to (i) ascertain the diversity and distribution of bacterial species on the toilet door handles on Baze University campus; (ii) determine the sources of bacterial contamination on the toilet door handles and, (iii) examine the susceptibility of the isolated bacterial species to antibiotics therapy. This study is expected to increase awareness of the university campus community on the potential threat posed by toilet door handles as a source of infectious disease transmission. The results of which will serve as a baseline data for future studies and reference, as well as improving the ways the public toilets are cleaned. Furthermore, identifying the sources of bacterial contamination on the door handles could be used to track the transmission of pathogens and help prevent the spread of infectious disease.

MATERIALS AND METHODS

Sample Collection and Study Area: A total of 24 microbial samples were collected aseptically from both the internal and external parts of the main toilet door entrances leading to the male and female toilets of each selected block using swab rinse method (Reynolds et al., 2005). The geographical coordinates of the six selected building blocks on Baze University, Abuja campus, are as follows: Block A (240° SW, 9°23'N, 7°24'17"E); Block B (145° SW, 9° 23'N, 7°24'17"E); Block C (328° NW, 9° 0'20"N, 7° 24'16"E); Block D (102° SE, 9° 0'20"N, 7° 24'16"E); Block E (242° SW, 9° 0'20"N, 7° 24'16"E); Block F (17° N 9° 0'20"N, 7° 24'16"E).

Microbiological Analyses: The cultural, morphological, biochemical and physiological characterization of the bacterial isolates were

performed using standard biochemical analysis [17]. Further antibiotic susceptibility test was performed by disc diffusion method [18]; and total bacterial count by aerobic plate counting [19]

The analyses were carried out in the microbiology laboratory of Baze University, Abuja from August to November, 2017. Statistical analysis of the experimental results was performed using OriginPro 8.5© 2010.

Methods of Biochemical Characterization of Isolates:

Door handles were swabbed, after which the swab stick were soaked in 0.9% of saline water for 10 minutes. A 1ml of 10^3 serial dilution of the microbial solution was pipetted aseptically into the surface of sterile solid nutrient agar, Salmonella Shigella agar, MacConkey agar, Centrimide Agar, Eosin Methylene Blue agar and Mueller Hinton agar, plates and incubated in an inverted position at 37° C for 48 hours.

IDENTIFICATION OF BACTERIAL PATHOGENS IN THE SAMPLES

Detection of *Staphylococcus aureus*: A 1ml of 10^3 dilutions of microbial solution was pipetted aseptically into the surface of a sterile mannitol salt agar plate, it was then spread and the plate incubated at 37°C for 24 hours. Yellow colonies were taken as *Staphylococcus aureus*.

Detection of *Salmonella* and *Shigella*: A 1ml of 10^3 dilutions of microbial solution was pipetted aseptically into the surface of a sterile plate of salmonella shigella agar and incubated at 37°C for 24 hours. Colorless colonies with black spots at the center were taken as *Salmonella typhimurium*.

Detection of Coliforms: A 1ml of 10^3 dilutions of microbial solution was pipetted aseptically into sterile eosine methylene blue agar plate and spread with a sterile spatula, after which the plates were incubated at 37°C for 24 hours. Colonies with small green-metallic sheen were presumptively taken as *Escherichia coli*.

Detection of *Pseudomonas aeruginosa*: A 1ml of 10^3 dilutions of microbial solution was pipetted aseptically into the surface of sterile centrimide agar plates, it was then spread and plates were incubated at 37°C for 24 hours. Colonies that were blue-green in color were taken as *Pseudomonas aeruginosa*.

Antibiotic Sensitivity Test by Disc Diffusion Method: A 100 µl of standardized suspension of the total number of isolates of each identified bacteria isolate was dispensed into separate sterile petri dishes, and then 25ml of warm molten Mueller hinton agar (MHA) agar was poured into each, swirled, and allowed to solidify. Antibiotic disc (M&B Multi disc, Abtek biological Limited) containing Cepharox 30µg-Ceftriaxone; Oxavid 5µg-Ofloxacin; CIP-M & B CIPRO 5µg-

Ciprofloxacin; Aluclox 30µg- Ampicillin/Cloxacillin; Levotil 5µg-Levofloxacin; Loxaprim 30µg-Cotrimoxazole; Loxaclav 30µg-Amoxicillin/Clavulanate; Ceftin 30µg-Cefuroxime axetil; IPM - 5µg Imipenem and FOR - 5µg Cefoxitin; was placed in the plates and incubated at 37°C for 24hrs. Furthermore, the results were expressed in percentage of susceptibility/resistance according to the absence or presence / diameter of inhibition: - = No zone of inhibition (Resistant); + = 5mm zone of inhibition (low resistance), ++ =10-14mm (intermediate), +++ =15-17mm zone of inhibition (sensitive) [20, 21].

Aerobic Plate Count: A 1ml of 10⁻⁵ serial dilution of the microbial solution was pipetted aseptically into

the surface of a sterile solid blood agar plate and spread using a sterile spatula. The plates were incubated in an inverted position at 37°C for 48 hours after which colonies were counted using J-2 colony counter and results expressed using the formula: $CFU \times 10^5/1ml$ [19].

RESULTS

Biochemical analysis of bacterial isolates from the selected toilet door samples revealed a general contamination of the sampled toilet door handles by mainly seven bacterial species (Table 1) associated with human gut and skin flora as follows: *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*.

TABLE 1: CHARACTERISTICS OF BACTERIAL ISOLATES FROM SELECTED TOILET DOOR HANDLES

Cell morphology	Rod	Cocci	Rod	Rod	Rod	Rod	Rod
Gram reaction	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Catalase test	+	+	-	-	-	-	-
Coagulase test	-	+	-	-	-	-	-
Indole production	-	-	+	-	-	+	-
Citrate utilization	+	-	-	+	+	+	+
Urease test	-	-	-	-	+	-	+
Oxidase test	+	-	-	-	-	-	-
Motility test	+	+	+	+	-	-	+
Lactose test	+	+	+	-	+	+	-
Glucose test	+	+	+	+	+	+	+
Manitol	-	+	-	-	-	-	-
Kliger Iron Agar	Slope	R	-	Y	R	Y	R
	Butt	R	-	Y	Y	Y	Y
	H ₂ S	-	-	-	+	-	+
	Gas	-	-	+	+	+	+
Probable Identity	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Klebsiella pneumoniae</i>	<i>Klebsiella oxytoca</i>	<i>Proteus mirabilis</i>

Key: + = positive; - = negative; KIA-Kliger Iron Agar: slope: Butt, H₂S, Gas R- Red-pink (alkaline reaction), Y - Yellow (acid reaction)

Unsurprisingly *Staphylococcus aureus* with the highest frequency of prevalence - 42.9%, was the sole bacterial specie isolated from all examined toilet door handles. While, *Klebsiella pneumoniae* had the lowest frequency of prevalence - 2.3%. The

prevalence rates of the other bacterial isolates were as follows: *Salmonella typhimurium* 21.4%; *Escherichia coli* 14.3%; *Pseudomonas aeruginosa* 9.5%; *Proteus mirabilis* 4.8%; and *Klebsiella oxytoca* 4.8% (Table 2).

TABLE 2: PREVALENCE OF BACTERIAL ISOLATES ON TOILET DOOR HANDLES IN THE SELECTED BLOCKS

Bacterial Isolate	Frequency of Isolates on Toilet Door Handle Samples by Blocks						Total	% Frequency
	Block A	Block B	Block C	Block D	Block E	Block F		
<i>Pseudomonas aeruginosa</i>	2	-	-	2	-	-	4	9.5
<i>Staphylococcus aureus</i>	3	4	2	3	3	3	18	42.9
<i>Escherichia coli</i>	-	-	-	3	1	2	6	9.5
<i>Salmonella typhimurium</i>	2	-	1	3	3	-	9	21.4
<i>Klebsiella pneumoniae</i>	-	-	1	-	-	-	1	2.3
<i>Klebsiella oxytoca</i>	-	-	-	-	-	2	2	4.8
<i>Proteus mirabilis</i>	-	-	-	-	-	2	2	4.8
Total Number of Isolates per Block	7	4	4	11	7	9	42	100

Further antibiotic sensitivity testing on these bacterial isolates by disc diffusion method (Table 3) showed a general susceptibility to Ciprofloxacin, Ofloxacin, Levofloxacin, Imipenem, and Cefoxitin by all isolates. *Salmonella typhimurium* showed total resistance to Ampicillin/ Cloxacillin, Co-

trimoxazole, Amoxicillin/ Clavulanate, and Cefuroxime axetil. Ciprofloxacin was the most effective against all isolates: *Salmonella typhimurium* (77.8%), *Staphylococcus aureus* (100%), *Escherichia coli* (100%), *Klebsiella pneumoniae* (100%), *Klebsiella oxytoca* (100%), and *Proteus mirabilis* (100%).

TABLE 3: ANTIBIOTICS SENSITIVITY TEST ON BACTERIAL ISOLATES BY DISC DIFFUSION METHOD

Antibiotic	S. aureus (n=18)		S. typhimurium (n=9)		E. coli (n=6)		P. aeruginosa (n=4)		P. mirabilis (n=2)		K. pneumoniae (n=1)		K. oxytoca (n=2)	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Ceftriaxone	13	72.2	8	88.9	2	33.3	1	25.0	1	50.0	1	100.0	0	0.0
Ofloxacin	15	83.3	2	22.2	5	83.3	2	50.0	2	100.0	1	100.0	1	50.0
Ciprofloxacin	18	100.0	7	77.8	6	100.0	4	100.0	2	100.0	1	100.0	2	100.0
Ampicillin/ Cloxacillin	14	77.8	0	0.0	2	33.3	2	50.0	0	0.0	1	100.0	1	50.0
Levofloxacin	12	66.7	5	55.6	2	33.3	2	50.0	2	100.0	1	100.0	1	50.0
Co-trimoxazole	14	77.8	0	0.0	2	33.3	3	75.0	1	50.0	1	100.0	0	0.0
Amoxicillin/ Clavulanate	13	72.2	0	0.0	2	33.3	1	25.0	1	50.0	1	100.0	1	50.0
Cefuroxime axetil	10	55.6	0	0.0	2	33.3	0	0.0	0	0.0	0	0.0	0	0.0
Imipenem	18	100.0	4	44.4	5	83.3	3	75.0	2	100.0	1	100.0	2	100.0
Cefoxitin	18	100.0	7	77.8	5	83.3	2	50.0	1	50.0	1	100.0	2	100.0

Results of the total aerobic mesophilic plate count, showed a general trend of a considerably higher amount of bacterial contamination on the internal part of the entrance toilet door handles than the external parts of both male and female toilets in all the selected blocks as follows: Block A: male toilet - 10×10^5 CFU/ml, female - 6×10^5 CFU/ml; Block B:

male - 10×10^5 CFU/ml, female - 5×10^5 CFU/ml; Block C: male - 2×10^5 CFU/ml, female - 10×10^5 CFU/ml; Block D: male - 18×10^5 CFU/ml, female - 50×10^5 CFU/ml; Block E: male - 18×10^5 CFU/ml, female - 39×10^5 CFU/ml; Block F: male - 17×10^5 CFU/ml, female - 10×10^5 CFU/ml (Figure 1: A & B).

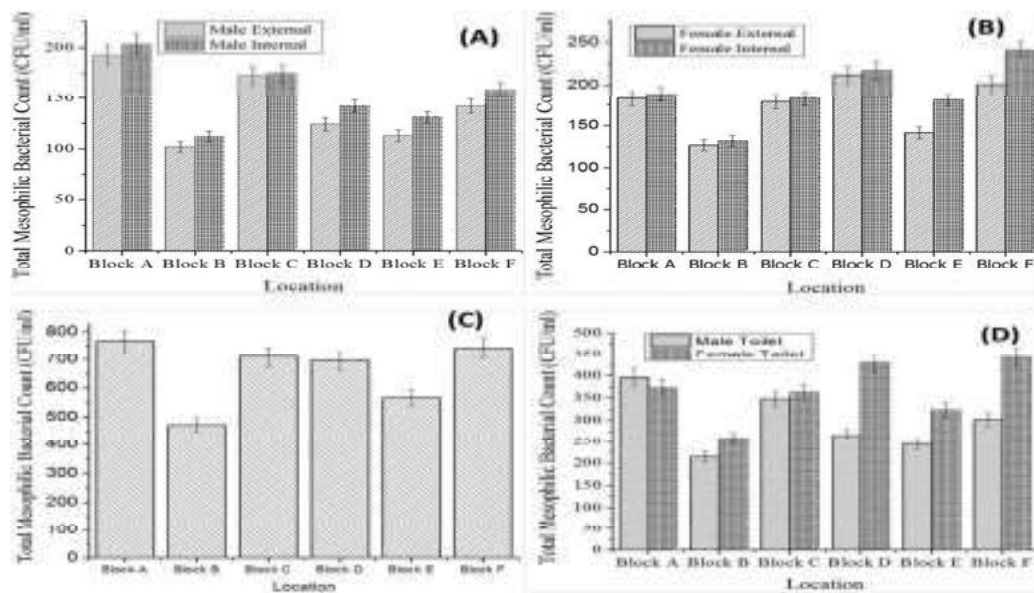


Figure 1: Comparative analysis on the level of bacterial contamination on toilet door handles: A- between the external and internal parts of male door handles; B - between the external and internal parts of female door handles; C - total amount of contamination by Blocks; D - between male and female toilets in all the Blocks

Comparative analysis of the combined total of bacterial contamination on door handles (internal and external) of both male and female toilets by blocks, showed Block A with the highest amount of contamination - $768 \times 10^5 \pm 38.4$ CFU/ml, while Block B had the least amount of bacterial contamination - $473 \times 10^5 \pm 23.7$ CFU/ml. The levels of bacterial contamination in other blocks were as follows: Block C - $710 \times 10^5 \pm 35.5$ CFU/ml, Block D - $695 \times 10^5 \pm 34.8$ CFU/ml, Block E - $567 \times 10^5 \pm 28.4$ CFU/ml, and Block F - $743 \times 10^5 \pm 37.2$ CFU/ml (figure 1C).

Furthermore, with the exception of A-Block, the frequency of bacterial contamination on female toilet door handles was about 1.04 - 1.61 times higher than the frequency of prevalence on door handles of the male toilets (figure 1D).

DISCUSSION

The presence of *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*, infer contamination by fecal matter, suggesting poor hygienic practices and the settling of suspended microorganisms in the air after flushing of water closet systems without covering the lid [4, 1]. The presence of *Pseudomonas aeruginosa* a predominant soil bacterium suggest transmission from toilet floor surfaces brought in from foot wears, and settling dust suspensions [1, 3, 22].

The isolated bacterial pathogens from the toilet door handles in this study are consistent with the findings of other researchers [4, 9, 10, 5], with *Staphylococcus aureus* being the most prevalent [9, 10, 23]. Contamination by *Staphylococcus aureus*, a bacterium of the skin flora, suggests direct contact

of the toilet door by individual handlers [24, 1, 3]. Bacterial pathogens such as *Salmonella typhimurium* isolated from virtually all the blocks is a major cause for concern, causing typhoid fever which is a leading cause of disease and death in Nigeria today [25]. Opportunistic pathogens of the coliform group, *Staphylococcus* and *Pseudomonas* have been linked to urinary tract infections, bacterial diarrhea, bacterial meningitis and bacterial pneumonia (*K. pneumoniae*) [26].

Indications from the antibiotic sensitivity test, showed that the Quinolones (Ciprofloxacin, Ofloxacin, Levofloxacin), whose mode of action involves the inhibition of nucleic acid synthesis of Bacteria, were most effective against both the gram positive (*Staphylococcus aureus*) and gram negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella oxytoca*, and *Klebsiella pneumoniae*) bacterial isolates. In contrast the Cephalosporins (Ampicillin/ Cloxacillin, Amoxicillin/ Clavulanate, and Cefuroxime axetil) with the exception of Ceftriaxone, Imipenem and Cefoxitin, were not as effective, especially against the gram negative isolates, these results were consistent with the findings of other researchers [14, 15]. This could be as a result of a growing resistance to the β -lactams component of these antibiotics, by β -lactamase producing bacteria such as *Staphylococcus aureus* [27], suggesting the development of Methicillin Resistant *Staphylococcus aureus* (MRSA); and especially extended-spectrum β -lactamases (ESBLs) producers such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli* and *Proteus mirabilis*, which possess grave consequences to public health [28, 12, 29].

The high prevalence of bacterial contamination on the internal parts of the entrance toilet door handles than the external parts of both male and female toilets in all the selected blocks can be attributed due to the close proximity of the internal parts of the toilet handles to the water closet system and basin sink. Hence contamination is more likely as a result of being exposed to settling air borne microbes from coughing, sneezing, flushing, vector borne spread (flies) and contact with unwashed human hands [1, 2].

The high frequency of bacterial contamination on toilet door handles in A-Block can be attributed to the high traffic of staff, students, and visitors using these toilets. A-Block houses both the administrative department and the largest Faculty in Baze University, the faculty of Management & Social Sciences. B-Block which houses the smallest faculty by departments had the least amount of contamination.

Finally, the higher abundance of bacterial contamination on female toilet door handles was also reported by other researchers in their work [9, 1]. This occurrence suggests additional source of contamination from the high concentration of vaginal-associated bacterial species contained in female discharges as reported by Flores et al., (2011).

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Conclusion: There is a high level of bacterial contamination on the sampled toilet door handles. The isolated bacterial species are mainly associated with human gut flora and skin flora, suggesting fecal contamination, and routine contact by hands. Most of which showed resistance to Furoxetil, Ampicillin/Cloxacillin, and Amoxicillin/Clavulanate antibiotics. Therefore there is a need to adopt adequate measures for the regular cleaning and disinfection of all surfaces in the toilets, including toilet door handles, while also maintaining good personal hygienic practices to prevent the transfer and spread of pathogens from these fomites. In addition, further research needs to be carried out using cultivation-independent techniques based on sequencing of the 16 S rRNA genes [30, 1] to investigate the diversity and distribution of bacterial species and other microorganisms on other surfaces in the public toilets on Baze University campus.

Conflict of interest: No conflict of interest declared.

Acknowledgement: Bilkisu Muhammed Auwal performed the experiments and wrote the draft. Olatunbosun O. Alonge supervised the project and revised the manuscript extensively with comments and inputs from Mercy I. Aboh.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1906 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.6>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 42-48

ELIMINATION OF MOTHER-TO-CHILD TRANSMISSION OF HIV (eMTCT) IN WESTERN NIGERIA: HOW FAR HAVE WE GONE?

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ABSTRACT

Background: HIV pandemic has continued to be a huge challenge in Nigeria, with the problem of stigmatization reducing the chances of early determination of the HIV status of pregnant women, which may increase the chances of transmission to the child from the mother. Hypotheses tested were the influence of maternal antiretroviral therapy (ART) use and infant's feeding option on baby's final early infant diagnosis (EID) outcome. The study was aimed at determining the trend as well as diagnosis of HIV infection in exposed infants. It will also determine among infants the factors associated with the transmission of the infection from their mothers.

Methods: This study was a prospective cohort study of HIV-exposed infants conducted in Ekiti State, South Western Nigeria, between June 2015 and June 2017. Dried Blood Spots (DBS) were analyzed using polymerase chain reaction technique. All data were statistically analyzed, using statistical package for the social sciences (SPSS) and statistical test of significance was performed with Chi-Square test.

Results: A total of 200 infants were included in the study, 91 (45.5%) female and 109 (54.5%) male. Three (1.5%) babies were confirmed positive after cessation of all exposures. Maternal antiretroviral therapy (ART) use has significant effect on baby early infant diagnosis (EID) outcome ($\chi^2 = 65.40$, $df = 2$, $P = 0.001$). Infant feeding option has significant effect on baby early infant diagnosis (EID) outcome ($\chi^2 = 132.67$, $df = 2$, $P = 0.001$). Baby's mode of delivery have higher association with the final EID outcome of the baby (OR: 1.018, 95% CI: 0.998 – 1.038).

Conclusion: ART administration to both HIV-infected mothers and their babies has demonstrated an effective mechanism in the elimination of mother-to-child transmission (eMTCT), as this is evident in the very low positivity outcome. However, the degree to which Cuba, Armenia, Belarus, and Thailand have eliminated HIV transmission from mother-to-baby is achievable in Nigeria through provision of universal access to health care.

Key words: infant, mother, dried blood spot, polymerase chain reaction

ÉLIMINATION DE LA TRANSMISSION DU VIH (eCTM) DE MÈRE À L'ENFANT DANS L'OUEST DU NIGÉRIA: COMBIEN EN SOMMES-NOUS ALORS?

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RÉSUMÉ

Contexte: Au Nigéria, la pandémie de VIH continue de poser un problème énorme, le problème de la stigmatisation réduisant les chances de déterminer rapidement le statut VIH des femmes enceintes, ce qui peut augmenter les risques de transmission de la mère à l'enfant.

Les hypothèses testées étaient l'influence de l'utilisation du traitement antirétroviral par la mère et de l'option d'alimentation du nourrisson sur le résultat final du diagnostic précoce du nourrisson. L'étude visait à déterminer la tendance ainsi que le diagnostic d'infection par le VIH chez les nourrissons exposés. Il déterminera également chez les nourrissons les facteurs associés à la transmission de l'infection par leur mère.

Méthodes: Cette étude était une étude de cohorte prospective menée entre juin 2015 et juin 2017 dans l'État d'Ekiti, dans le sud-ouest du Nigéria, auprès de nourrissons exposés au VIH. Les taches de sang séché ont été analysées à l'aide de la technique de réaction en chaîne de la polymérase. Toutes les données ont été analysées statistiquement, en utilisant un logiciel statistique pour les sciences sociales (SPSS) et un test statistique de signification a été réalisé avec un test de Chi-Square.

Résultats: Un total de 200 nourrissons ont été inclus dans l'étude, 91 (45,5%) femmes et 109 (54,5%) hommes. Trois bébés (1,5%) ont été confirmés positifs après la cessation de toutes les expositions. L'utilisation du traitement antirétroviral par la mère a un effet significatif sur l'issue du diagnostic précoce chez le nourrisson ($ID^2 = 65,40$, $df = 2$, $p = 0,001$). L'option d'alimentation du nourrisson a un effet significatif sur l'issue du diagnostic précoce du nourrisson ($ID^2 = 132,67$, $df = 2$, $p = 0,001$). Le mode d'accouchement du bébé est davantage associé au résultat final de l'EID du bébé (OR: 1,018, IC 95%: 0,998 - 1,038). Conclusion: l'administration d'un traitement antirétroviral aux mères infectées par le VIH et à leurs bébés a démontré un mécanisme efficace pour éliminer la transmission mère-enfant (eMTCT), ce qui est évident dans le résultat de très faible positivité. Cependant, il est possible d'atteindre au Nigéria le degré auquel Cuba, l'Arménie, le Bélarus et la Thaïlande ont éliminé la transmission du VIH de la mère à l'enfant grâce à un accès universel aux soins de santé.

Mots clés: nourrisson, mère, tache de sang séché, réaction en chaîne de la polymérase

INTRODUCTION

Prevention of mother-to-child transmission of HIV (PMTCT) is the most successful HIV prevention intervention globally (1). Elimination of mother-to-child transmission of HIV (eMTCT) is key to the global effort to combat sexually transmitted infections (STIs) and to end AIDS by the year 2030. Cuba was the first country validated by the World Health Organization (WHO) in 2015 to have successfully eliminated mother-to-child transmission (MTCT) of HIV and syphilis. Countries such as Thailand, Armenia, Belarus and Republic of Moldova have gone on to join Cuba in 2016 (2). The countries ensured early access to pre-natal care, HIV & syphilis testing for pregnant women and their partners and treatment for women who test positive, as well as their babies. They provided reproductive health information, engaged communities and conducted outreach to marginalized populations, in a manner consistent with basic human rights and gender equality, showing a perfect integration of maternal and child health with sexual & reproduction health and HIV services (2).

Mother-to-child transmission (MTCT) of HIV resulted in approximately 370,000 infant infections worldwide in 2009 (3). The same year, estimated 2.5 million children worldwide were living with HIV, mostly a consequence of MTCT and more than 90% of these children are in Sub-Saharan Africa (3). Mother-to-child transmission of HIV can occur in utero (in-utero), during delivery (intrapartum) or after birth (postnatally) through breastfeeding. Strategies to reduce MTCT focus on these periods of exposure and include the use of antiretrovirals (ARVs), caesarean section before the onset of labour or rupture of membranes and even may involve complete avoidance of breastfeeding (4). The combination of these interventions when effectively followed, reduce the risk of MTCT to as low as 1-2% (5). In the absence of intervention, 30-45% of all infants born to HIV positive mothers will be infected and 10-20% will be infected through breastfeeding (6).

In infants born from HIV-infected women, maternal anti-HIV antibodies cross the placenta and persist in infant blood for up to 18 months. These antibodies usually represent exposure to maternal HIV rather than the true infants less than 18 months by antibody-based tests. Only virological tests can be used for an accurate diagnosis of HIV before 18 months of age (7). A dried blood spot (DBS) is the sampling method used for HIV-exposed infants. Infants can be tested from six weeks of age and sample **collection is from finger, toe or heel depending on the age and weight of the baby** (8).

A research work published in 2014 on the impact of HIV PMTCT reported 7.0% of the children to be positive, with highest prevalence found among children in age-group 6-18 months (16.1%) (9). An overall prevalence of 16.98% of postnatal HIV was observed in a study on PMTCT published in 2010 (10). In Tanzania, an overall MTCT prevalence rate of 6.3% was reported, with infants on exclusive breastfeeding at the time of first DBS PCR was 86.5% (11). Another research in eastern Cameroon, published in 2013, reported that 50% of the infants were exclusively breastfed and based on the first PCR tests data, an overall prevalence of 11.6% was reported (12). In 2010, a study carried out in Malawi showed that 13.8% of the children born to HIV-positive mothers in 2009 were themselves HIV-positive as infants (13). In China, 4.4% mean HIV-positive rate of exposed infants was reported (14). Meanwhile, only 1.5% & 1.3% of babies within 18-month PMTCT program were confirmed positive in year 2013 & 2014 respectively after all exposure cessation in Western Nigeria (15). This study is therefore designed to determine the diagnosis and trend of HIV infection in exposed infants and the factors associated with mother-to-child transmission (MTCT) among the infants.

RESEARCH HYPOTHESIS

- Maternal antiretroviral has no significant effect on baby final early infant diagnosis outcome.

- Infant's feeding option has no significant effect on

METHODOLOGY

This study was a prospective cohort study of all HIV-exposed infants enrolled at health facilities in Ekiti State from June 2015 and June 2017. The study population was HIV-exposed infants whose second (i.e. final) early infant diagnosis (EID) result has been determined by HIV nucleic acid testing polymerase chain reaction (PCR) and baby's final outcome (alive/dead) is known. Relevant data such as age, sex, ARV therapy for mother and baby, infants feeding choices, place of delivery, duration of HAART use, HIV DNA results, among others were analysed. Results of samples analysed were received as dried blood spots (DBS) collected from each infant and analysed at the PCR Laboratory at State Specialist Hospital Akure Ondo State with an automated real-time amplification and detection of DNA using a qualitative DNA polymerase chain reaction (PCR) analyser by (Roche Molecular Diagnostics, Basel, Switzerland). HIV testing was also done at the Federal Teaching Hospital Ido Ekiti, State Specialist Hospital Ikere Ekiti & Ekiti State University Teaching Hospital Ado Ekiti.

The data analysis was done using statistical package for the social sciences (SPSS) for windows version 23.0 software (SPSS Inc; Chicago, IL, USA). Frequency counts were

final early infant diagnosis outcome.

generated for all variables and statistical test of significance was performed with chi-square test. Significance was fixed at $P < 0.05$ and highly significant when $P < 0.01$.

RESULTS

A total of 200 infants were included in the study with mean age (in months) 13.80 ± 1.20 , 91 (45.5%) female and 109 (54.5%) male. The research outcomes showed that majority of the children were breastfed exclusively, with the main mode of delivery being spontaneous vaginal delivery (SVD), 173 (86.5%) while one hundred and seven (53.5%) of the mothers had antiretroviral therapy started during pregnancy. Nearly 60% of the mothers used recommended first line regimen Tenofovir/Lamivudine/Efavirenz while most of the babies took single dose nevirapine.

Three (1.5%) babies were confirmed positive after cessation of all exposures, largely breastmilk. Maternal antiretroviral therapy (ART) use was found to have a significant effect on baby early infant diagnosis (EID) outcome ($\chi^2 = 65.40$, $df = 2$, $P = 0.001$) while infant feeding option also had significant effect on baby early infant diagnosis (EID) outcome ($\chi^2 = 132.67$, $df = 2$, $P = 0.001$). Baby's mode of delivery was also found to have higher association with the final EID outcome of the baby (OR: 1.018, 95% CI: 0.998 – 1.038).

The full results are captured in tables:

TABLE 1: CHARACTERISTICS OF INFANTS AND MOTHERS

CHARACTERISTICS	Frequency	%
BABY AGE (MONTHS)		
12	22	11.0
13	73	36.5
14	54	27.0
15	31	15.5
16	15	7.5
17	5	2.5
SEX		
Male	109	54.5%
Female	91	45.5%
MODE OF DELIVERY		
Spontaneous Vaginal Delivery (SVD)	173	86.5%
Caesarean Section (C/S)	27	13.5%

PLACE OF DELIVERY		
Mission	7	3.5
Primary Healthcare Centre	23	11.5
Private Clinic	12	6.0
Secondary Healthcare Facility	26	13.0
Tertiary Healthcare Facility	132	66.0
MATERNAL ENROLMENT TO CARE		
Yes	196	98.0
No	4	2.0
MATERNAL ENROLMENT/ART		
HAART started before pregnancy	89	44.5
HAART started during pregnancy	107	53.5
No HAART taken	4	2.0
MATERNAL OUTCOME		
Alive	197	98.5
Dead	3	1.5
TYPE OF ARV USED BY MOTHER		
Zidovudine/Lamivudine/Nevirapine	78	39.0
Tenofovir/Lamivudine/Efavirenz	118	59.0
None	4	2.0
INFANT ARVs (NEVIRAPINE TAKEN)		
Yes	199	99.5
No	1	0.5
INFANT EVER BREASTFED		
Yes	196	98.0
No	4	2.0
BABY FEEDING OPTION		
Exclusive Breastfeeding (EBF)	194	97.0
Breastmilk Supplement (EBS)	4	2.0
Mixed Feeding (MF)	2	1.0
FIRST EID DONE		

Yes	200	100
No	0	0
FIRST EID RESULT		
Negative	197	98.5
Positive	3	1.5
SECOND EID DONE		
Yes	200	100
No	0	0
SECOND EID RESULT		
Negative	197	98.5
Positive	3	1.5
RAPID TEST RESULT AT SECOND EID		
Negative	197	98.5
Positive	3	1.5
BABY FINAL OUTCOME		
Alive	200	100
Dead	0	0

TABLE 2 - CHI SQUARE RESULT SHOWING EFFECT ON BABY EARLY INFANT DIAGNOSIS (EID) OUTCOME

VARIABLES (HYPOTHESES)	χ^2	df	Critical value	Decision	P-Value
Maternal Antiretroviral Therapy (ART) use does not have significant effect on baby early infant diagnosis (EID) outcome	65.40	2	5.99	Rejected	0.001
Infant feeding option does not have significant effect on baby early infant diagnosis (EID) outcome	132.68	2	5.99	Rejected	0.001

The null hypothesis is rejected when the test statistic is greater than the tabled value or critical value.

TABLE 3 – ODDS RATIO (OR) TABLE

Variables	OR	95% CI
Estimated odds that mode of baby delivery have effect on baby final EID outcome	1.018	0.998 – 1.038

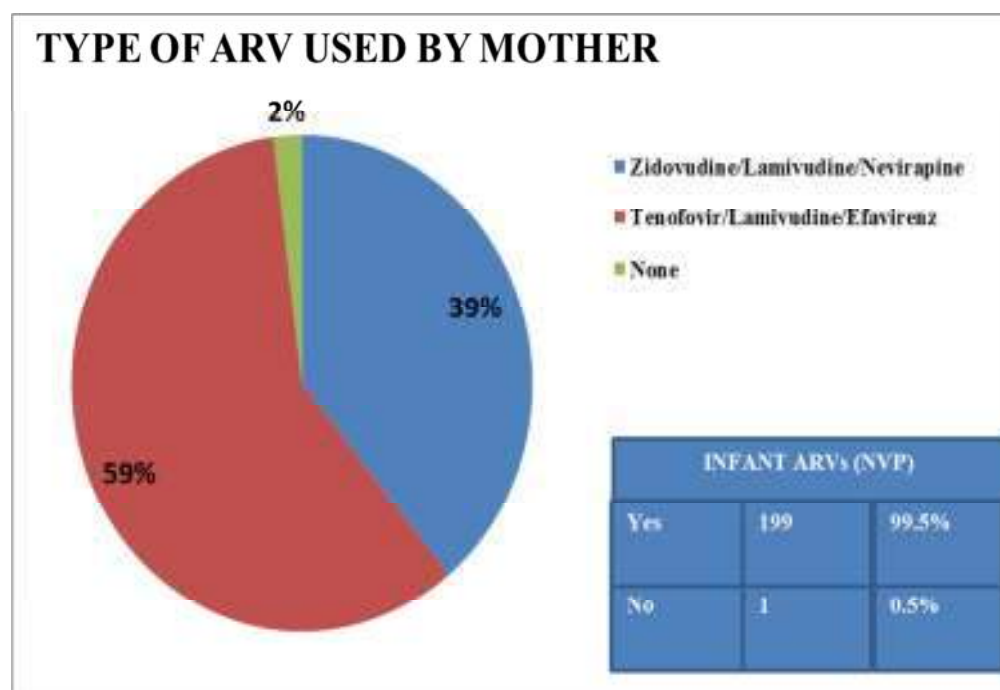


FIGURE SHOWING THE TYPE OF ARV USED BY MOTHER

DISCUSSION

The outcome of this study reveals a positivity rate of 1.5% after second EID test. The positivity rate is similar to previous research outcome in Western Nigeria where 1.5% & 1.3% of babies within 18-month PMTCT program were confirmed positive in year 2013 & 2014 respectively after all exposure cessation in Western Nigeria (15). It is however lower than those reported in various other studies such as 7.0% reported in 2014 & 16.98% reported in 2010 respectively (9, 10), 6.3% positivity reported in Tanzania (11), 11.6% reported in Eastern Cameroon in 2013 (12), 13.8% & 4.4% positivity rates reported in Malawi & China (13, 14). This prevalence outcome is a great improvement over most of the previous studies although very similar to the previous Western Nigeria study outcome. The low positivity rate might be attributed to enhanced spread of programmes now targeting the elimination of HIV infection to children through the mothers especially with utilization of

updated guidelines where most positive mothers are on antiretroviral (ARV) therapy either before pregnancy or commenced during pregnancy, with 98.0% of the mothers on ARV therapy and infants even born to mothers with HIV who are at high risk of acquiring HIV now receive dual prophylaxis for extended period showing strict adherence to the new national guidelines, with its main role in reducing HIV transmission. This outcome suggests therefore that the commencement of ARV prior or during pregnancy had a great impact on the babies' status outcome. Moreover, another contributory factor is the fact that the babies receive treatment as soon as possible after birth, mostly a single-dose nevirapine, from birth for a period of six weeks, after which the drug is replaced with cotrimoxazole. Infections to these few babies could be due to poor viral suppression which could not be ascertained in this study, as the HIV viral load, used to determine how well antiretroviral therapy (ART) is controlling the virus, was not included as part of the study objectives

as it was just beginning to be fully optimized during the course of this study. It is therefore recommended that mixed feeding especially within the first 6 months, should be highly discouraged.

The Chi Square analysis in table 2 shows that maternal antiretroviral therapy use ($\chi^2 = 65.40$, $df = 2$, $P = 0.001$) & infant feeding option ($\chi^2 = 132.68$, $df = 2$, $P = 0.001$) have significant effect on baby early infant diagnosis (EID) outcome, indicating that the antiretroviral therapy used before or during pregnancy and exclusive breastfeeding being the most common baby feeding option in this study cohort enhanced the baby eventual outcome after the second EID result. It is thus recommended that partial breastfeeding with complimentary food, should be in

place after 6 months to enhance HIV-free child survival.

CONCLUSION:

ART administration to both HIV-infected mothers and their babies has demonstrated an effective mechanism in the elimination of mother-to-child transmission (eMTCT), as this is evident in the very low positivity outcome. However, the degree to which Cuba, Armenia, Belarus, and Thailand have eliminated HIV transmission from mother-to-baby is achievable in Nigeria through provision of universal access to health care.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1907 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.7>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 49-53

SURVEILLANCE OF ANTI-HCV ANTIBODY AMONGST IN-SCHOOL YOUTH IN A NIGERIA UNIVERSITY

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ABSTRACT

Infection with Hepatitis C Virus (HCV) is a public health problem. Worldwide, there are about 170 million people infected with HCV. HCV is transmitted through sex and use of contaminated sharp objects during tattooing or intravenous drug abuse. These routes make youth to be more vulnerable. Transfusion and mother to child transmissions are also documented modes. This study was carried out to determine sero-prevalence of hepatitis C virus infection among in school youth at Achievers University, Owo in southwest Nigeria. Samples of blood were collected from 70 undergraduate students and sera harvested were tested for the presence of antibodies against hepatitis C virus by Enzyme Immunoassay Technique. Most participants fall within age range 21-25 (91.4%). The study showed that none of the subjects was positive for anti- HCV antibodies. Education and awareness level might have impacted positively on this outcome. Thus routine screening for HCV and sustained awareness creation activities to eradicate HCV and its attendant consequences from our society is of paramount importance.

Key words: HCV, prevalence, in-school youth, education.

SURVEILLANCE D'UN ANTICORPS ANTI-VHC CHEZ LES JEUNES À L'ÉCOLE DANS UNE UNIVERSITÉ DU NIGERIA

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ABSTRAIT

L'infection par le virus de l'hépatite C (VHC) est un problème de santé publique. À l'échelle mondiale, environ 170 millions de personnes sont infectées par le VHC. Le VHC se transmet par le sexe et l'utilisation d'objets tranchants contaminés au cours du tatouage ou de l'abus de drogues par voie intraveineuse. Ces itinéraires rendent les jeunes plus vulnérables. La transfusion et les transmissions mère-enfant sont également des modes documentés. Cette étude visait à déterminer la prévalence de la séropositivité au virus de l'hépatite C chez les jeunes scolarisés de l'Université Achievers, à Owo, dans le sud-ouest du Nigéria. Des échantillons de sang ont été prélevés chez 70 étudiants de premier cycle et les sérums prélevés ont été testés pour détecter la présence d'anticorps contre le virus de l'hépatite C par la technique d'immunoanalyse enzymatique. La plupart des participants sont âgés de 21 à 25 ans (91,4%). L'étude a montré qu'aucun des sujets n'était positif pour les anticorps anti-VHC. Le niveau d'éducation et de sensibilisation pourrait avoir eu un impact positif sur ce résultat. Le dépistage systématique du VHC et des activités de sensibilisation soutenue visant à éradiquer le VHC et les conséquences qui en découlent pour notre société sont de la plus haute importance.

Mots clés: VHC, prévalence, jeunes scolarisés, éducation

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INTRODUCTION

Viral hepatitis is a life threatening liver disease, caused majorly by hepatitis B and C viruses, and is a major public health problem; particularly in developing countries (1,2). Hepatitis C is an infectious disease affecting primarily the liver, caused by the hepatitis C virus (HCV). HCV is a single-stranded RNA virus of the flavivirus family, about 9.5 kb in length. HCV has a long lag time between onset of infection and clinical manifestation of liver disease which may be up to 20 years (3).

HCV has become a significant causative factor in the aetiology of chronic liver disease worldwide (4). Individuals with chronic infection of HCV have a high risk of liver cirrhosis and hepatocellular carcinoma. Serological markers for HCV are screened in blood banks and antenatal clinics routinely. The evaluation of the data of the prevalence of the HCV antibodies among patients gives an idea for the epidemiology of these infections in the community (5). The prevalence of HCV in a population can be predicated on risk factors associated with the transmission of infection such as blood transfusion, intravenous drug abuse, unsafe injections, sexual activity, shared needle, other body fluids such as semen, vaginal secretions and breast milk, from mother to child, needle stick injury, ear piercing, tattooing and scarifications, exposure to barbers razors, surgical procedures and vertical transmission. (6,7,8,9,10). Viral hepatitis during pregnancy is associated with high risk of maternal complication the virus can be transmitted through infected blood, mother to child in perinatal period. Perinatal transmission is the most common mode of HCV transmission worldwide (11). Viral infection is public health problem and is highly endemic in the sub-Saharan Africa (12, 13). Prevalence rates of anti-HCV antibodies have been determined for various nations of the world. Nigeria as one of the countries highly endemic for viral hepatitis was reported to have a prevalence rate of 3.6% through 12.3% (14, 24). Though HCV infections are known to occur in the general population, the mode of transmission makes youth rather more vulnerable.

The infection is often asymptomatic, but chronic infection can lead to scarring of the liver and ultimately to cirrhosis, which is generally apparent after many years. In some cases, those with cirrhosis will go on to develop liver failure, liver cancer, or life-threatening esophageal and gastric problems (25).

The aim of this work is to determine the sero-prevalence of hepatitis C viral infection among in school youths at Achievers University in Owo with a view to examine the effect of education on transmission of HCV.

MATERIALS AND METHODS

Study setting: This study was carried out at Achievers University, Idashen, Owo in southwestern Nigeria. The University is a private sector initiative established in 2007 and it houses students from all major tribes in Nigeria running undergraduate programs in various departments of the 2 faculties. Owo is situated halfway between the Nigerian towns of Ife and Benin. The primary occupation of Owo people is farming and trading. They are producers of cocoa, cotton and timber.

Study subjects: A total of 70 apparently healthy students of the university who consented to participate in the study were recruited. All the participants were offered pre-test and post-test counseling.

Procedure: Five milliliters (5ml) of venous blood were collected from each subject by venepuncture into vacuum plain tube. The specimens were centrifuged at 1200 revolution per minute (rpm) for 5 minutes to harvest serum into a microtube for the anti-HCV (IgG) testing with third generation Enzyme Immuno-Assay (EIA) method. Donor's serum was added to the microwell together with a second antibody conjugated with the enzyme horseradish peroxidase (the HRP-Conjugate) and directed against a different epitopes of HCV. During incubation, the specific immunocomplex formed in case of presence of anti-HCV in the sample, was captured on the solid phase which generates an optical signal that is proportional to the amount of anti-HCV antibodies present in the sample. The commercially prepared positive and negative controls were treated alongside the specimens. A cut-off value was determined and results were interpreted as positive and negative according to manufacturer's instructions.

Statistical analysis: The data generated were coded, entered, validated and analyzed using Statistical Package for Social Science (SPSS) version 20.0. The seroprevalence of HCV were expressed for the entire study group and documentation of participants' age, gender, knowledge and attitude was done.

RESULTS

A total of 70 healthy Achievers students within the age range of 15-30, were tested for HCV. The distribution of HCV in relation to age and gender was determined. Majority of the respondents were in the age range of 21-25 years. Out of 70 respondents, thirty nine were males (56%) and twenty one were females (44%). The sero-prevalence of HCV obtained from this study was zero (0%).

TABLE 1: AGE DISTRIBUTION AND PREVALENCE OF HCV AMONG IN SCHOOL YOUTH.

Age group	No examined (n)	Percentage (%)	Sero positivity of HCV
15-20	3	4.3	0
21-25	64	91.4	0
26-30	3	4.3	0
Total	70	100	0

TABLE 2: DISTRIBUTION OF HCV INFECTION BY GENDER

Gender	No examined (n)	Percentage (%)	Sero-positivity of HCV
Male	39	55.7	0
Female	31	44.3	0
Total	70	100	0

TABLE 4.3: RESPONDENT'S AWARENESS ON THE ROUTE OF TRANSMISSION OF HEPATITIS C

Major route of infection	Frequency (n)	Percentage (%)
Coitus	30	48.6
Blood transfusion	26	1.4
Use of infected material	14	50.0
Total	70	100

DISCUSSION

Seventy healthy students were examined for presence of marker of HCV infections. The age range of subjects was 15-30 years. There was no sero-positivity

to this viral infection among the subjects studied. No prevalence of HCV infection in this community was found and this is in contrast to reports from some countries in Western Pacific (3.9%), South East Asia (2.15%), America (1.17%), Europe (1.03%) and Eastern Mediterranean (26) (4.6%). It is still at variance with reports from some researchers about the entire Africa (5.3%) (26) and Egypt (20.0%) (15). In Enugu where 14.9% was reported (16); the 5.2% and 11.09% reported in Jos and Kaduna respectively (15) were all higher than what was reported in this study. The sero-prevalence of anti-HCV antibodies reported in this study differs from previous studies including the 13.3% reported for Anti-HCV in Keffi; Nigeria (17). The 9.2% found by Ogunro *et al.* 2007 (27) in Osun State; the 8.4% anti-HCV antibody seroprevalence rate reported in Lagos (28); the 3.0% found by Ezeani (29) in Southeastern, Nigeria; the 2.4% HCV infection rate found by Olokoba *et al* (21); the 1.1% reported by Buseri *et al* (22) for HCV in Osogbo; Nigeria are all alarming. However, our report is exactly the same with 0.0% HCV seroprevalence previously reported by Elfaki (30) in Sudan, another African country. The zero level prevalence reported in this study and that of Sudan may be due to high level of awareness and adherence to safety practices among the participants in the studies. No doubt, students of the Achievers University, Owo have enough information on transmission of HCV and their attitudes generally about transfusion transmissible infections reflect their level of awareness.

CONCLUSION

In conclusion, the results of this study have highlighted that HCV infection is not common in Owo. However, a prospective cohort study is suggested for newly admitted students till their year of graduation for HCV seroprevalence in order to affirm our observation.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No. 1
AJCEM/1908 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.8>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 54-59

SCREENING OF SELECTED MEDICINAL PLANTS FOR THEIR ANTIFUNGAL PROPERTIES

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ABSTRACT

Background: The rising incidence of fungal infections has created the need for the next generation of antifungal agents, as many of the currently available ones either have adverse effects, or are not active against emerging or re-emerging fungi, leading to the fast progression of resistant strains. **Objectives:** This study aims at evaluating the antifungal activities of some medicinal plants used traditionally for treating skin infections in Nigeria. **Methods:** In vitro antifungal activities of seven indigenous plants (*Leptadenia hastata*, *Lawsonia inermis*, *Hyptis suaveolens*, *Luffa cylindrica*, *Jatropha curcas*, *Pterocarpus erinaceus* and *Afromaxia laxiflora*) were screened against *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 13803, clinical strains of *Candida albicans*, *Candida tropicalis*, *Trichophyton rubrum*, *Microsporum canis* and *Epidermophyton floccosum* using agar dilution and micro broth dilution methods. Terbinafine and fluconazole were used as reference standards for comparison. **Results:** The results showed that the ethanol and ethyl acetate extracts of the plants produced better antifungal effects than the hexane and water extracts. *Luffa cylindrica* and *H. suaveolens* exhibited the strongest inhibitory activity against all the fungi tested with minimum inhibitory concentration values ranging between 250 and 1000 µg/mL. **Conclusion:** The plants screened could serve as leads for the development of new antifungal drugs.

Key words: Antifungal, agar dilution, micro broth dilution, minimum inhibitory concentration.

CRIBLAGE DE PLANTES MÉDICINALES SÉLECTIONNÉES POUR LEURS PROPRIÉTÉS ANTIFONGIQUES

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ABSTRAIT Contexte: L'incidence croissante des infections fongiques a créé la nécessité pour la prochaine génération d'agents antifongiques, comme beaucoup de ceux qui sont actuellement disponibles, soit avoir des effets indésirables, ou ne sont pas actifs contre les nouvelles ou les champignons qui refont surface, conduisant à la progression rapide de souches résistantes. **Objectifs:** Cette étude vise à évaluer les activités antifongiques de certaines plantes médicinales utilisées traditionnellement pour traiter les infections cutanées au Nigeria. **Méthodes:** l'activité in vitro antifongiques de sept plantes indigènes (*Leptadenia de hastée*, *Lawsonia inermis*, *Hyptis suaveolens*, *Luffa cylindrica*, *Jatropha curcas*, *Pterocarpus Erinaceus* et *Afromaxia laxiflora*) ont été projetés contre *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 13803, les souches cliniques de *Candida albicans*, *Candida tropicalis*, *Trichophyton rubrum*, *Microsporum canis* et *Epidermophyton floccosum* en utilisant des méthodes de dilution en gélose et de dilution au micro-bouillon. La terbinafine et le fluconazole ont été utilisés comme étalons de référence pour la comparaison. **Résultats:** Les résultats montrent que les extraits d'éthanol et d'acétate d'éthyle des plantes produisent de meilleurs effets antifongiques que les extraits d'hexane et d'eau. *Luffa cylindrica* et *H. suaveolens* ont présenté la plus forte activité inhibitrice contre tous les champignons testés avec des concentrations minimales inhibitrices comprises entre 250 et 1000 µg / mL. **Conclusion:** Les plantes examinées pourraient servir de fil conducteur à la mise au point de nouveaux médicaments antifongiques. **Mots clés:** Antifongique, dilution en gélose, dilution au micro-bouillon, concentration minimale inhibitrice.

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INTRODUCTION

Plants have constantly played an important role in the research and development of novel antimicrobial compounds [1]. Reports from The World Health Organization show that, eighty percent of people dwelling in remote and urban regions in developing countries depend on medicinal plants for their initial health care [2].

Nigeria is home to varieties of medicinal plants many of which are used in traditional practice for the cure of skin infections like fungal infections. Fungal skin infections are common in most tribal dwellers in rural and some urban settlements where good environmental sanitation, access to portable water and general hygienic practices are lacking due to the poor socioeconomic level of the inhabitants.

The incidence of fungal infections among individuals in developing countries is on the increase (Abad *et al.*, 2007). According to recent findings, resistance of some fungi to available antifungal drugs is fast becoming a major threat, especially among persons living with HIV or those on chemotherapy and drugs that suppress the immune system [3]. It is a known fact that the presently existing antifungal drugs are toxic and consequently have undesirable side effects, thus are becoming ineffective against fungi that have been in existence and upcoming ones [4].

In many countries, indigenous flora has played an important role for many generations in the treatment of infections. Based on this, it has been recognized that scientific validation of plants used to such treat infections is a viable approach in the discovery of new, effective drugs against the diseases (Taylor *et al.*, 2001). Therefore, there is the need for continuous research into medicinal plants within our environment for novel antifungal compounds. In the current study, the antifungal action of hexane, ethyl acetate, ethanol and water extracts of seven medicinal plants were investigated against dermatophytes and opportunistic fungi.

MATERIALS AND METHODS

Chemicals and media

Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were obtained from Oxoid, Germany. Dimethyl sulphoxide (DMSO), fluconazole (Cat No. F8929), Terbinafine HCl (T8826), the organic solvents *i.e.*, hexane, ethyl acetate, ethanol were obtained from Sigma Aldrich Laboratories, Germany.

Collection of plants and identification

The selection of the plants used in this research was on the basis of their ethnobotanical evidence of use for antimicrobial skin infections as documented in published literature. Fresh plants were collected from different places in Abuja. The plants were Identified and authenticated at the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria. Voucher specimens were deposited at the herbarium for reference purposes. The plants include leaves of *Leptadenia hastata* (Pers.), *Lawsonia inermis*, *Hyptis suaveolens*, *Luffa cylindrica*, *Jatropha curcas*, *Pterocarpus erinaceus* and *Afromaxia laxiflora*.

Preparation of plant extracts

Fresh plants were collected and dried in air under a shade for approximately one week. The dried leaves of the plants were then grinded in a manual mill. The powdered leaves were extracted separately by cold maceration in the various solvents (hexane, ethyl acetate, ethanol and water) for 48 h. The extracts were first sieved through a muslin cloth, and then filtered through a funnel with Whatman No. 1 filter paper. Concentration of the filtrate was done using a rotary evaporator and dried using a water bath at 70 °C. The extracts were weighed and stored at a temperature of 4 °C until when needed.

Test fungi

The fungi used for the study include *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 13803, clinical strains of *Candida albicans*, *Candida tropicalis*, *Trichophyton rubrum*, *Microsporum canis* and *Epidermophyton floccosum* obtained from Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

Preparation/ standardization of fungi

The yeast (*Candida* sp.) was standardized by inoculating sterile normal saline solution with a 48 h pure culture by adjustment of turbidity to match 0.5 Mc Farland standard. Standardization of the dermatophytes included harvesting fungal spores from a 7 day old culture on SDA slant. Ten milliliters of sterile normal saline containing 3% w/v Tween 80 was used to disperse the spores with the aid of sterilized glass beads [5]. Standardization of the spore suspension to 1.0×10^6 spores/mL was achieved with a UV spectrophotometer (Spectronic 20D; Milton Roy Company, Pacisa, Madrid, Spain) at 530 nm (OD530) of the suspensions and adjusted to a transmittance of 70-72 %. The standardized fungal suspensions was quantified was by spreading 100 μ L on Sabouraud dextrose agar plate. The plates were incubated at 37 °C for 24 h for yeast and 30 °C for 72 h for dermatophytes [6].

Antifungal assays

The antifungal activities of the extracts were achieved using micro broth dilution method according to standard reference method [1]. The stock concentration of the extracts was 8 mg/ml in 2% DMSO. The testing method involved a two-fold serial dilution of the extracts in SDB with the first well having a concentration of 4 mg/ml after inoculation with equal volume of standardized fungal suspension in SDB. Fluconazole and terbinafine hydrochloride served as positive control while 2 % DMSO served as negative control. The plates were incubated at 37 °C for 24 h for yeast and 30 °C for 72 h for dermatophytes. Minimum inhibitory concentration was seen as the lowest concentration of the extracts that inhibited fungal growth (no visible growth) after incubation period elapsed.

RESULTS

The ethno botanical uses, local names, parts used, medicinal uses and chemical constituents of the selected plants are represented in Table 1. Results on the antifungal activities of the plants are represented in Table 2. The plants exhibited variable degrees of antifungal activity. Generally, the ethyl acetate and methanol extracts of the plants were more active than the water and hexane extracts. The hexane extracts of all the plants were inactive with minimum inhibitory concentration (MIC) value greater than 8000 µg/ml.

DISCUSSION

The results of this study show that the plants exhibited greater inhibitory action on the dermatophytes than the yeasts, with *T. rubrum* and *M. canis* as the most sensitive. Dermatophytes are a specialized group of fungi that causes a zoonotic skin infection of keratinized tissues, leading to skin eruptions which last for a long time [2]. The strong activity of the ethyl acetate extracts against a broad range of fungi suggests that, most antifungal principles of these plants are soluble in ethylacetate. This observation is not strange as previous reports from our laboratory shows that, there was an increased antifungal activity of the ethyl acetate extracts over the other solvent extracts like hexane, ethanol and water (Aboh *et al.*, 2014),

All the extracts of *Afromaxia laxiflora* were active against *M. canis* however, the ethyl acetate extract

produced the strongest inhibitory action with an MIC of 500 µg/mL. However, the ethyl acetate extract of *A. laxiflora* produced its highest inhibition against *T. rubrum* (250µg/mL). The antimicrobial potential of *A. laxiflora* has already been expounded in literature [8]. *H. suaveolens* ethyl acetate and ethanol extracts exhibited a broad spectrum of antifungal activity. However the ethyl acetate extracts (500- 1000 µg/mL) was more effective than ethanol extract (500- 2000 µg/ml). This agrees with a work of Nantitanon *et al* [21], who reported the antifungal effect of ethanolic extracts of *H. suaveolens* oil which exhibited strong inhibitory action on *T. mentagrophytes* at a concentration of 10 and 20 %. The hexane and water extracts of the plant showed inhibition of all the fungal strains tested.

The ethanol and ethyl acetate extracts of *J. curcas* were effective on all of the fungal strains tested however, the ethanolic extract of the plant was most active against *C. albicans* and *T. rubrum* with MIC of 500 µg/ml. The antifungal activities of *J. curcas* have been reported by several researchers [10, 22] for example, *J. curcas* latex was reported to have inhibitory action on *C. albicans* [22]. The broad antifungal activities of *J. curcas* against the yeast and dermatophytes observed in this study, is consistent with the reports by Mbakwem – Aniebo *et al* [23]. These authors reported that, *J. curcas* crude stem extracts possesses a broad spectrum of antifungal effect. The poor antifungal potential of the water extracts of *J. curcas* observed in our study is also in line with the study by Sarin *et al* [24], where it was noted that the ethanol extract of the plant was more active than the water extract.

The ethyl acetate of *L. inermis* was most active against *C. tropicalis* with an MIC of 250 µg/mL however it inhibited the growth of all the fungal strain tested with an MIC range of 250- 1000 µg/ml. According to Arun *et al* [12], the antimicrobial activities of the plant can be attributed to the presence of flavonoids and naphthoquinones. The inhibitory action of *L. inermis* against *C. albicans* agrees with a work by Farah *et al.* (2012), although the MIC value in this study was higher. This could be attributed to the difference in extracting solvent and location of the plant. It has been reported that, the botanical and/or biological source of a medicinal plant affects its constituents as well as its physicochemical and biological/microbiological properties (Zohra *et al.*, 2011; Prashant *et al.*, 2011).

TABLE 1: THE ETHNOBOTANICAL USES, LOCAL NAMES, PARTS USED, MEDICINAL USES AND CHEMICAL CONSTITUENTS OF THE SELECTED MEDICINAL PLANTS

Name of plants	Local names	Part used	Medicinal uses	Chemical constituents	References
<i>Afromaxia laxiflora</i> (Papilionaceae) Syn. <i>Pericopsis laxiflora</i>	Makarho (Hausa) Emi (Yoruba) Osisi (Igbo)	Leaves, stem bark	Analgesic, antiparasitic, Diuretic, antibacterial	Tannin, Alkaloid, Flavonoid, Terpenoid, Saponin and Phenols	[7,8]
<i>Hyptis suaveolens</i> (Lamiaceae)	Misin (Gwari) Iyeye (Yoruba), Ijikara (Igbo), Tsadar lamarudu (Hausa).	Leaves, twigs, roots	stimulant, carminative, sudorific, galactagogue, parasitic infections, colic, stomachache antispasmodic, antirheumatic and antisuorific baths antiinflammatory, antifertility agents, burns, wounds, and various skin infections.	Volatile oil, starch, proteins, tannins, saponins, fats, alkaloids, glycosides	[9]
<i>Jatropha curcas</i> L (Euphorbiaceae)	Botuje, Lapalapa (Yoruba) Olulu-idu (Igbo), Zugu (Hausa)	Seeds, latex, leaves	Skin diseases, rheumatism, syphilis	Tannins, saponins, flavonoids, steroids, alkaloids, cardiac glycoside, terpenoid, anthraquinone.	[10]
<i>Lawsonia inermis</i> L. (Lythraceae)	Lalli (Igbo), Laali, Lali (Yoruba) Lalle (Hausa)	Leaves, flowers, stem bark, roots	Antioxidant, antidiabetic, hepaprotective, hypoglycemic, antimicrobial, anti-cancer, wound healing.	Flavonoids, alkaloids, tannins, Quinones.	[11,12,13]
<i>Leptadenia hastata</i> (Pers.). Decne. (Asclepiadaceae)	Bima (Gwari), Yaádiyaá (Hausa), Iran-aji (Yoruba) isanaje (Igbó) (Yoruba)	Leaves, latex, roots, whole plant	Hypertension, catarrh, skin diseases, wound healing, prostate complaints, aphrodisiacs	Alkaloids, saponins, phenolic glycosides, tannins, flavonoids, proanthocyanidins and triterpenes	[14,15]
<i>Luffa cylindrica</i> (L.) M. Roem. (Curcubitaceae) Syn. <i>L. aegyptiaca</i> Mill., <i>Momordica cylindrica</i> L.	Kankan (Yoruba), Asisa (Igbo), Baska (Hausa)	Leaves, fruit, seeds		Alkaloids, flavonoids, sterols, glycosides	[16,17]
<i>Pterocarpus erinaceus</i> (Papilionaceae)	(banuhi (Fulani), Madubiya (Hausa), Osun dudu, Apepo, Agbelosun (Yoruba)	Leaves, root, stem bark	Fungal skin diseases e.g, athlete foot, ring worm and eczema, cough remedy, gastrointestinal upsets, chest pains, hemorrhoids, and antigonadotropic	Saponins, phenols, tannins, flavonoids	[18,19,20]

TABLE 2: MINIMUM INHIBITORY CONCENTRATION OF COLLECTED PLANT EXTRACTS AGAINST FUNGI
($\mu\text{G/ML}$)

S/N	Plants	Extracts	C.a 10231	C.a	C.t 13803	C.t	T.r	M.c	E.f
1.	<i>A. laxiflora</i>	He	-	-	-	-	-	4000	-
		Ea	1000	1000	2000	1000	250	1000	8000
		Et	8000	2000	2000	2000	1000	500	2000
		Wa	-	-	-	-	4000	2000	4000
2.	<i>H. suaveolens</i>	He	-	-	-	-	-	-	-
		Ea	1000	1000	500	500	500	1000	1000
		Et	1000	2000	1000	500	500	500	1000
		Wa	-	-	-	-	-	-	-
3.	<i>J. curcas</i>	He	-	-	-	-	-	4000	-
		Ea	500	1000	1000	1000	500	2000	4000
		Et	2000	1000	2000	1000	1000	1000	8000
		Wa	-	-	-	-	-	-	-
4.	<i>L. inermis</i>	He	-	-	-	-	-	-	-
		Ea	1000	1000	1000	250	1000	1000	500
		Et	1000	1000	500	500	500	500	500
		Wa	-	-	-	-	-	-	-
5.	<i>L. hastate</i>	He	-	-	-	-	-	-	-
		Ea	1000	1000	1000	500	1000	1000	1000
		Et	1000	1000	2000	1000	500	500	1000
		Wa	-	-	-	-	-	-	-
6.	<i>L. cylindrica</i>	He	-	-	-	-	-	-	-
		Ea	1000	1000	1000	500	250	1000	1000
		Et	1000	1000	1000	1000	500	500	1000
		Wa	-	-	-	-	-	-	-
7.	<i>P. erinaceus</i>	He	-	-	-	-	-	-	-
		Ea	1000	2000	1000	1000	1000	2000	500
		Et	2000	1000	4000	4000	2000	1000	500
		Wa	-	-	-	-	4000	4000	4000

MIC > 8000 $\mu\text{g/ml}$ was taken as inactive; He - Hexane; Ea - Ethyl acetate; Et- Ethanol, Wa- water; T. r - *Trichophyton rubrum*; E. f - *E. floccosum*; M.c- *Microsporium canis*; C. a 2876 - *C. albicans* ATCC 10231; C.a- *C. albicans* (clinical isolate); C.t 13803- *C. tropicalis* ATCC 13803; C.t- *C.tropicalis* (clinical isolate)

L. hastata is one of the food plants known to possess antimicrobial activity. Several researchers have documented its antimicrobial potential [15, 26]. In the present study the plant showed a broad antifungal activity. Results from the present study revealed the broad antifungal activity of the ethyl acetate and ethanol extracts of *L. cylindrica*. The ethyl acetate fraction of the crude plant was however more active with its strongest inhibitory action against *T. rubrum* with an MIC of 250 $\mu\text{g/ml}$. The hexane and water extracts however were inactive. This disagrees with a study by Ahmad and Khan [27]; the authors reported that the n- hexane extract of the leaves showed better antimicrobial activities than the methanolic and buthanolic fractions.

The antifungal effect of the extracts of *P. erinaceus* was more pronounced on the dermatophytes than the yeast with exception of the hexane extracts

which was ineffective against any of the fungi tested. The lowest MIC value (500 $\mu\text{g/ml}$) was produced by the ethyl acetate and ethanol extracts on *E. floccosum*.

CONCLUSION: The outcome of this study show that the plants investigated possess anti-fungal activities, thus justifying their use in folk medicine for the treatment of skin and other related infections.

CONFLICT OF INTEREST STATEMENT: We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS: The authors are thankful to the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria for the enabling environment to carry out this study.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No.1
AJCEM/1909 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.9>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 60-66

TUBE METHOD AND CONGO RED AGAR VERSUS TISSUE CULTURE PLATE METHOD FOR DETECTION OF BIOFILM PRODUCTION BY UROPATHOGENS ISOLATED FROM MIDSTREAM URINE: WHICH ONE COULD BE BETTER?

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ABSTRACT

Background: Bacteria tend to live in assemblies called biofilms that aid bacterial virulence. Biofilms contribute to the development of antibiotic resistant urinary tract infection. Therefore, detection of biofilm production by urinary pathogens can assist the physicians to initiate the proper antimicrobial treatment.

Methods: We conducted a prospective study that included patients with suspected urinary tract infection. Collected midstream urine samples were processed by standard microbiological techniques. Detection of biofilm production by the isolated uropathogens was conducted by tissue culture plate method (TCPM), tube method (TM) and Congo red agar (CRA).

Results: A total of 43 (29.7%) isolated uropathogens showed positive biofilm formation by TCPM which was considered the gold standard for biofilm detection. When compared with the TCPM, TM truly identified 40 biofilm producers and 83 non-biofilm producers showing sensitivity and specificity of 93.0% and 81.4% respectively. The CRA truly identified 38 biofilm producers and 77 non-biofilm producers with sensitivity and specificity of 88.4% and 75.5% respectively.

Conclusion: The TM was superior to CRA in biofilm detection and demonstrated better sensitivity and specificity results. Out of the investigated three phenotypic biofilm detection methods, the TCPM was the ideal method for detection of biofilm formation by uropathogens isolated from midstream urine samples. It can be used routinely in the microbiology laboratory with good specificity results and less subjectivity errors.

PROCÉDÉ DE TUBE ET PLAQUE DE CULTURE DE TISSU D'AGAR ROUGE CONGO POUR DÉTECTER LA PRODUCTION DE BIOFILM PAR DES UROPATHOGENES ISOLÉS À PARTIR D'UNE URINE À MOYENNE FLUX: QUI POURRAIT ÊTRE MIEUX?

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ABSTRAIT

Contexte: Les bactéries ont tendance à vivre dans des assemblages appelés biofilms qui facilitent la virulence bactérienne. Les biofilms contribuent au développement d'une infection des voies urinaires résistante aux antibiotiques. Nous avons détecté des uropathogènes produisant des biofilms isolés à partir de l'urine médiane et évalué trois méthodes phénotypiques in vitro pouvant être appliquées en laboratoire pour la détection de biofilms.

Méthodes: Nous avons mené une étude prospective incluant des patients chez qui une infection des voies urinaires était suspectée. Les échantillons d'urine collectés à mi-chemin ont été traités par des techniques microbiologiques standard. La détection de la production de biofilm par les uropathogènes isolés a été réalisée par la méthode de la culture sur plaque de culture tissulaire (TCPM), la méthode du tube (TM) et l'agar rouge congolais (CRA).

Résultats: Un total de 43 (29,7%) uropathogènes isolés a montré que la formation de biofilm était positive par le TCPM, qui était considéré comme la référence en matière de détection du biofilm. En comparaison avec le TCPM, TM a vraiment identifié 40 producteurs de biofilms et 83 producteurs non-biofilms présentant une sensibilité et une spécificité de 93% et 81,4% respectivement.

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L'ARC a vraiment identifié 38 producteurs de biofilms et 77 producteurs de non-biofilms avec une sensibilité et une spécificité de 88,4% et 75,5% respectivement.

Conclusion: La MT était supérieure à l'ARC pour la détection de biofilm et a démontré de meilleurs résultats de sensibilité et de spécificité. Parmi les trois méthodes de détection phénotypiques de biofilms étudiées, le TCPM était la méthode idéale pour détecter la formation de biofilm par des uropathogènes isolés à partir d'échantillons d'urine en cours de route. Il peut être utilisé en routine dans le laboratoire de microbiologie avec de bons résultats de spécificité et moins d'erreurs de subjectivité.

INTRODUCTION

Bacteria tend to live in a community-like assembly called biofilm. Development of bacterial biofilms occurs in a dynamic process that includes bacterial attachment to a particular surface, irreversible binding and formation of a hydrated matrix of polysaccharides and protein (1,2). Surfaces that favor biofilm development include inert surfaces as medical devices and dead tissues as dead bone fragments (1,3). Antibodies are generated in response to the antigens released by the bacteria located in the biofilm. However, these antibodies are unable to kill the bacteria embedded within the biofilm even among people with excellent immune responses (1,4).

Biofilm production aids bacterial virulence through numerous pathogenic mechanisms as it facilitates attachment to solid surfaces, evasion of phagocytosis and gene exchange between the biofilm's members generating more virulent strains. Moreover, biofilms can protect bacteria from antimicrobial agents resulting in resistant infections that carry a great clinical significance (1,5). The mechanisms by which biofilms escape the effects of antimicrobial agents include: inability of the agent to reach the bacteria present at the deep part of the biofilm, the slowly growing bacteria in the biofilm shows decrease susceptibility to the agents, and some of the bacteria exist in a programmed protected phenotype that is generated in response to surface attachment (6).

Antibiotic resistant urinary tract infection (UTI), either community or healthcare acquired, is a threatening clinical problem faced by treating physicians (7). Biofilms are commonly associated with indwelling devices as urinary catheters leading to resistant UTI. Furthermore, biofilms may attach to urinary tract anatomical structures resulting in chronic and recurrent UTI with increased morbidity and economic burden (5). Therefore, detection of biofilm production by urinary pathogens can assist the physicians to

initiate the proper antimicrobial treatment for UTI cases (2).

With the appearance of biofilm associated infections, various laboratory methods for detection of biofilms were developed. Phenotypic detection of biofilm production can be conducted by various techniques as tissue culture plate method (TCPM), tube method (TM) and Congo red agar (CRA) (8).

Previous studies that investigated biofilm formation by uropathogens usually focused on catheterized patients (9-11). This study was performed trying to detect biofilm producing uropathogens isolated from midstream urine and to evaluate three in vitro phenotypic methods (TCPM, TM and CRA) that can be applied in laboratory settings for biofilm detection.

MATERIALS and METHODS

Setting

A prospective study was performed at the Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University from January to October 2018. The study protocol was revised and accepted by our institutional review board.

Sample Collection

During the study period, midstream urine samples were collected from patients showing clinical manifestations of UTI at Mansoura University Hospitals. Urine specimens were transported to the laboratory and processed immediately.

Microbiological Processing

Received urine samples were initially examined by standard microbiological techniques. Urine samples were inoculated on CLED agar plates that were then incubated at 37°C for 24-48 hours. The uropathogens were identified by colonial morphology, Gram staining and biochemical reactions.

Detection of Biofilm Production

Biofilm production by isolated uropathogens in our study was detected by three phenotypic

methods which included TCPM, TM and CRA. Reference strains of *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus epidermidis* ATCC 31484 were also included as negative and positive control strains respectively. Biofilm production was graded into strong, moderate and non/weak. Strong and moderate results were interpreted as positive biofilm production, while, non/weak results were interpreted as negative biofilm production.

Tissue Culture Plate Method

We used TCPM as the gold standard test for detection of biofilm formation (1,8). A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose. The inoculated broth was then kept in the incubator at 37°C for 24 hours. Bacterial suspensions were further diluted 1:100 with fresh medium. Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells, were filled by 200 µl of the prepared bacterial suspension. Similarly, control organisms were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and to identify non-specific binding. After incubation at 37°C for 24 hours, the plate was gently tapped to remove the content of the wells followed by washing with 200 µl of phosphate buffer saline. The washing step was repeated four times to remove any free bacteria present in the wells. Sodium acetate (2%) were added to the wells and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water to remove any extra stain.

After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation. The OD values were calculated and biofilm production was graded into strong, moderate and non/weak (Table 1) as described in previous studies (2, 12-14).

TABLE 1: GRADING OF BIOFILM FORMATION BY TISSUE CULTURE PLATE METHOD

Optical densities values	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120- 0.240	Moderate	Moderate
> 0.240	Strong	Strong

Tube Method

A loopful of the isolated bacteria from overnight cultured media was inoculated in each glass tube containing 10 ml of trypticase soy broth with 1% glucose. The inoculated tubes were then incubated at 37°C. After incubation for 24 hours, tubes were emptied and washed with phosphate buffer saline and left to dry. Crystal violet (0.1%) was used to stain the dried tubes for 15 minutes. Excess stain was then removed by washing the tubes with deionized water. The tubes were then dried in inverted position and examined for biofilm production. Presence of a visible film lining the bottom and the wall of the tube indicated positive result for biofilm production while formation of a stained ring at the air-liquid interface was an evidence of a negative result (5,13,15).

Congo Red Agar Method

Congo red agar is a specially prepared medium composed of brain heart infusion (BHI) broth (37 g/l) supplemented with sucrose (50 g/l), agar No 1 (10 g/l) and Congo red (0.8 g/l). We prepared a concentrated aqueous solution of the Congo red stain that was then autoclaved at 121°C for 15 minutes. Finally it was added to the autoclaved BHI agar with sucrose at 55°C. Prepared CRA plates were inoculated with the isolated uropathogens and aerobically incubated at 37°C for 24 hours. Appearance of black dry crystalline colonies on the CRA plates indicated biofilm production while the colonies of biofilm non-producer remained pink or red colored (5,8,16).

Statistical Analysis

In the present study, TCPM was considered the gold standard method of biofilm detection based on the available literature. Accordingly, the data of TCPM were compared with those of TM and CRA. The data were presented as numbers and percentages. Parameters like sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) were calculated for each test by using Greenhalgh's formulas (17).

RESULTS

A total of 180 midstream urine samples from patients with suspected UTI were processed in our study. Out of the processed 180 urine samples, 145 (80.6%) samples were culture positive. Gram-negative bacteria accounted for 89.0% of the recovered isolates (129/145) while Gram-positive bacteria accounted for 11.0% (16/145). *Escherichia coli* was the commonest isolate encountered in our study (55.9 %)

followed by *Klebsiella pneumoniae* (13.1%) and *Pseudomonas aeruginosa* (10.3%). *Enterococcus faecalis* was the predominant Gram-positive isolate (8.3%) as shown in Table 2.

TABLE 2: SPECTRUM OF ISOLATED UROPATHOGENS

Bacterial isolate	Number	Percentage (%)
<i>Escherichia coli</i>	81	55.9%
<i>Klebsiella pneumoniae</i>	19	13.1%
<i>Pseudomonas aeruginosa</i>	15	10.3%
<i>Enterococcus faecalis</i>	12	8.3%
<i>Proteus mirabilis</i>	10	6.9%
<i>Acinetobacter baumannii</i>	4	2.8%
MSSA	3	2.1%
MRSA	1	0.7%
Total	145	100%

MSSA=Methicillin sensitive *Staphylococcus aureus*,
MRSA=Methicillin resistant *Staphylococcus aureus*

Out of 145, 43 (29.7%) isolates demonstrated positive biofilm formation by TCPM which is postulated to be the gold standard for biofilm detection. *Enterococcus faecalis* isolates showed the highest biofilm production (75.0%) followed by *Escherichia coli* (32.1%), *Klebsiella pneumoniae* (21.1%), *Pseudomonas aeruginosa* (20%) and *Proteus mirabilis* (10%). None of the isolated *Acinetobacter baumannii*, *Methicillin sensitive Staphylococcus aureus* or *Methicillin resistant Staphylococcus aureus* strains produced biofilm as shown in Table 3.

TABLE 3: BIOFILM PRODUCTION AMONG ISOLATED UROPATHOGENS BY TCPM

Bacterial isolate	Total isolates	Biofilm producers (%)
<i>Escherichia coli</i>	81	26 (32.1%)
<i>Klebsiella pneumoniae</i>	19	4 (21.1%)
<i>Pseudomonas aeruginosa</i>	15	3 (20.0%)
<i>Enterococcus faecalis</i>	12	9 (75.0%)
<i>Proteus mirabilis</i>	10	1 (10.0%)
<i>Acinetobacter baumannii</i>	4	0
MSSA	3	0
MRSA	1	0
Total	145	43 (29.7%)

MSSA=Methicillin sensitive *Staphylococcus aureus*,
MRSA=Methicillin resistant *Staphylococcus aureus*

In the current study, the TCPM, TM and CRA detected biofilm formation in 29.7% (43/145), 40.7% (59/145) and 43.4% (63/145) of the isolates respectively as demonstrated in Table 4. Eight isolates were found positive only by TM while 14

isolates were found positive only by CRA. None of the isolates were only TCPM positive.

When compared with the TCPM, TM truly identified 40 biofilm producers and 83 non-biofilm producers (Table 5), while, CRA truly identified 38 biofilm producers and 77 non-biofilm producers (Table 6).

TABLE 4: DETECTION OF BIOFILM PRODUCTION AMONG ISOLATED UROPATHOGENS BY DIFFERENT METHODS

Method	Total isolates	Biofilm producers (%)	Non-biofilm producers (%)
TCPM	145	43 (29.7%)	102 (70.3%)
TM	145	59 (40.7%)	86 (59.3%)
CRA	145	63 (43.4%)	82 (56.6%)

TCPM=Tissue culture plate method, TM=Tube method, CRA=Congo red agar

TABLE 5: COMPARISON OF TM WITH TCPM FOR BIOFILM DETECTION

TM	TCPM		Total
	Positive	Negative	
Positive	40	19	59
Negative	3	83	86
Total	43	102	145

TCPM=Tissue culture plate method, TM=Tube method

TABLE 6: COMPARISON OF CRA WITH TCPM FOR BIOFILM DETECTION

CRA	TCPM		Total
	Positive	Negative	
Positive	38	25	63
Negative	5	77	82
Total	43	102	145

TCPM=Tissue culture plate method, CRA=Congo red agar

The performance characteristics of TM and CRA when compared with TCPM, which is the gold standard for biofilm detection, were demonstrated in Table 7. The TM showed a sensitivity of 93.0%, specificity of 81.4%, PPV of 67.8% and NPV of 96.5% while the CRA had a sensitivity of 88.4%, specificity of 75.5%, PPV of 60.3% and NPV of 93.9%.

In the present study, the TCPM detected 13 (9.0%) isolates as strong biofilm producers and 30 (20.7%) as moderate biofilm producers. The TM detected strong and moderate biofilm formation in 10.3% and 30.3% of isolates respectively, while, CRA detected strong and moderate biofilm formation in 11.0% and 32.4% of isolates respectively as shown in Table 8.

TABLE 7: PERFORMANCE CHARACTERISTICS OF TM AND CRA FOR BIOFILM DETECTION WHEN COMPARED WITH TCPM

Method	Sensitivity	Specificity	Positive predictive value	Negative predictive value
TM	93.0%	81.4%	67.8%	96.5%
CRA	88.4%	75.5%	60.3%	93.9%

TM=Tube method, CRA=Congo red agar

TABLE 8: GRADING OF BIOFILM FORMATION BY DIFFERENT METHODS

Biofilm formation	TCPM	TM	CRA
Strong	13 (9.0%)	15 (10.3%)	16 (11.0%)
Moderate	30 (20.7%)	44 (30.3%)	47 (32.4%)
Non/weak	102 (70.3%)	86 (59.3%)	82 (56.6%)
Total	145 (100%)	145 (100%)	145 (100%)

TCPM=Tissue culture plate method, TM=Tube method, CRA=Congo red agar

DISCUSSION

Antimicrobial resistant bacteria continue to be a major challenge for treating physicians. The ability to produce biofilm is considered one of the main causes of antimicrobial resistance. Bacteria embedded in the biofilm can survive a higher concentration of antimicrobials up to 1500 folds than those needed to eliminate free bacteria (18). In the present study, we processed midstream urine samples and then investigated the ability of isolates to form biofilm by three in vitro phenotypic methods that can be used in most laboratory settings. A total of 145 bacterial isolates were recovered from the urine samples in our study. Gram-negative bacteria found to be the predominant uropathogens constituting 89% of the total isolates (129/145). *Escherichia coli* was the most prevalent organism isolated from the urine samples (55.9%) followed by *Klebsiella pneumoniae* (13.1%) and *Pseudomonas aeruginosa* (10.3%). Similarly, other studies reported *Escherichia coli* and *Klebsiella pneumoniae* as the predominant uropathogens (2,5,11,19). In our study, *Enterococcus faecalis* was the most prevalent Gram-positive bacteria (8.3%) that was consistent with the work of Noor et al., and Ruchi et al., who reported that *Enterococcus species* were the commonest Gram-positive isolates in their studies (5,20). On the contrary, Panda and his colleagues reported that *Staphylococcus species* were the predominant Gram-positive isolated uropathogens (2).

The recovered 145 bacterial isolates were further subjected to TCPM, TM and CRA methods for phenotypic detection of biofilm production. The TCPM, the gold standard method, detected biofilm formation in 43 out of 145 bacterial isolates (29.7%). The highest biofilm production was found among *Enterococcus faecalis* as 9 out of 12 isolates (75%) were biofilm producers. In accordance with our results, Ruchi and his

colleagues reported that 27% of isolated uropathogen showed biofilm formation by the TCPM and that 71.4% of *Enterococcus faecalis* isolates were biofilm producers (5). Our results were higher than those reported by Ira et al., who found that 53% of isolated *Enterococcus species* produced biofilm (21). In another study by Panda and his colleagues, TCPM detected biofilm production in 137 out of 300 (45.6%) isolated uropathogens which were higher than our findings (2).

In the present study, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* produced biofilm in 32.1%, 21.1% and 20% respectively. These results were considerably lower than those of Niveditha et al., who reported that *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* produced biofilm in 60%, 63% and 100% respectively (11). Higher results than ours were also reported by Abdallah and his colleagues who found that 44.4% of *Klebsiella species* and 50% of *Pseudomonas species* were biofilm producers (7). There is no clear explanation for such variations in these studies. Though, this might be attributed to the different methodology as Niveditha et al., analyzed only urine samples from catheterized patients while Abdallah et al., compared biofilm formation in midstream and catheterized urinary specimens. Therefore, the pattern of biofilm production in these studies could be different from our study in which we only analyzed midstream urine samples. In agreement with this explanation, Ruchi and his colleagues who also analyzed midstream urine samples reported that *Escherichia coli* and *Klebsiella pneumoniae* produced biofilm in 27.1% and 16.7% of the isolates respectively which were consistent with our results (5).

A total of 145 isolates were tested for biofilm production by TCPM, TM and CRA in the current study. We chose these in vitro methods

because they can be performed in most laboratory settings. The TCPM detected biofilm production in 43 isolates (29.7%), the TM detected biofilm production in 59 isolates (40.7%) while the CRA detected biofilm production in 63 isolates (43.4%). Parallel detection pattern was reported by Ruchi et al., who detected biofilm production in 27% of isolated uropathogens by TCPM, 37.9% by TM and 40.8% by CRA (5). Similar pattern was also reported by Turkyilmaz and his colleagues who studied biofilm production in *Staphylococcal species* and detected biofilm in 50.5% by TCPM, 55.5% by TM and 61.1% by CRA (22).

Out of the tested 145 urine samples in our study, 43 isolates were biofilm producers and 102 isolates were non-biofilm producers according to the results of TCPM. The TM truly identified 40 biofilm producers and 83 non-biofilm producers showing a sensitivity of 93% and specificity of 81.4%. Ruchi et al., reported slightly better sensitivity and specificity results for the TM (94.5% and 83% respectively) (5). In other studies conducted by Hassan et al., and Panda et al., the TM had sensitivity results of 73% and 81% respectively and specificity results of 92.5% and 95.1% respectively (2,8). Ira et al., reported that TM sensitivity and specificity were 61% and 68% respectively which were considerably lower than our results (21).

The CRA, in the present study, truly identified 38 biofilm producers and 77 non-biofilm producers demonstrating a sensitivity of 88.4% and specificity of 75.5% which were lower than those of TM. Ira et al., reported a CRA specificity of 77% which was close to our results (21). Studies conducted by Ruchi et al., Hassan et al., and Panda et al., demonstrated better specificity results for the CRA than ours (81%, 92% and 93.9% respectively) (2,5,8). Similarly, the CRA sensitivity reported by Ruchi and his colleagues was 94.5% that was better than the one reported by us (5). These variations in the reported sensitivity and specificity of TM and CRA can be explained by the subjective errors during interpretation of these phenotypic qualitative tests. Moreover, inter-batch variation of the used media can affect their results.

In the current study, the TCPM and TM detected strong biofilm formation in 9% and 10.3% of isolates respectively. These results were similar to those of Panda et al., who reported that 11% and 10.7% of the isolates demonstrated strong biofilm formation when tested by TCPM and TM respectively (2). Similarly, Mathur and his

colleagues reported that 14.4% and 11.8% of the isolates demonstrated strong biofilm formation when tested by TCPM and TM respectively (23). However, other studies reported considerably higher results as up to 52% of the isolates showed strong biofilm formation by TCPM and TM (24, 25). The CRA, in our study, detected strong and moderate biofilm formation in 11% and 32.4% of isolates respectively that was higher than the results reported by other studies (2, 23, 24). Overall, we found that TM and CRA correlated well with TCPM regarding strong biofilm detection but not for moderate and non/weak biofilm detection. This could be accredited to the subjective assessment used in TM and CRA in comparison to the objective grading scheme used in TCPM.

The CRA is a simple qualitative screening method for biofilm detection with an advantage of remaining viable colonies that can be beneficial for further studies (5). In the present study, CRA was more rapid and easier than other phenotypic tests. However, the CRA demonstrated lower sensitivity and specificity results than those of TM. The TCPM remains to be the gold standard phenotypic test for detection of biofilm production and it was the most specific test in the present study. It was also an easy test to perform in the laboratory and it detected the biofilm production in both qualitative and quantitative ways. Moreover, the interpretation of the TCPM results is conducted by ELISA reader which eliminates the subjective errors seen with other phenotypic tests.

CONCLUSION

Biofilm producing bacteria are important etiological agents of UTI in non-catheterized patients. Incomplete clearance of infection caused by biofilm production can lead to chronic UTI with a worse outcome. Therefore, detection of biofilm formation in such cases is important as it allows for better antimicrobial choice by the treating doctors. The TM was superior to CRA in biofilm detection and demonstrated better sensitivity and specificity results. Out of the investigated three phenotypic biofilm detection methods, the TCPM was the ideal method for detection of biofilm formation by uropathogens isolated from midstream urine samples. It can be used routinely in the microbiology laboratory for biofilm detection with good specificity results and less subjectivity errors.

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CASE REPORT

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X JANUARY 2019 VOL20 No. 1
AJCEM/1910 <http://www.ajol.info/journals/ajcem>
COPYRIGHT 2018 <https://dx.doi.org/10.4314/ajcem.v20i1.10>
<http://dx.doi.org/10.4314/ajcem.v17i4.1>
AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 67-71

A TWO YEAR OLD INFECTED WITH *DICROCOELIUM DENDRITICUM*: A CASE REPORT

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ABSTRACT

Dicrocoelium dendriticum infection is rare in human and generally non-fatal unless infection of liver is severe. The disease could lead to biliary colic, digestive disturbances that include bloating and diarrhea. In heavy infections, bile ducts and the biliary epithelium may become enlarged with the generation of fibrous tissues around the ducts leading to hepatomegaly or inflammation of the liver (cirrhosis). This is a case presentation of a two-year old male child who is infected with Human Immune deficiency virus (HIV). BMI was 10.3kg/m², which is below the fifth percentile. Child lost 1.5kg on retrospective review of case file and another 0.5kg after presentation with loss of appetite, cough and fever. Patient was reportedly fed with liver on several occasion. Stool examination revealed many *Dicrocoelium dendriticum* and *Ascaris lumbricoides* ova. Albendazole treatment was instituted and after three months, body weight improved to 10.5kg. It becomes important to screen underweight children for helminthiasis, particularly HIV/AIDS patients whose HIV treatment plan might be of priority to the physician.

Keywords: *Dicrocoelium dendriticum*, Child, *Ascaris lumbricoides*, HIV/AIDS, Albendazole.

RAPPORT DE CAS D'INFECTION À *DICROCOELIUM DENDRITICUM*, AVEC UN BEBE DE DEUX ANS

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ABSTRAIT

L'infection à *Dicrocoelium dendriticum* est rare chez l'homme et n'est généralement pas mortelle, sauf si l'infection du foie est grave. La maladie pourrait entraîner des coliques biliaires et des troubles digestifs tels que ballonnements et diarrhée. Dans les infections graves, les voies biliaires et l'épithélium biliaire peuvent s'agrandir avec la formation de tissus fibreux autour des voies conduisant à une hépatomégalie ou à une inflammation du foie (cirrhose). Présentation d'un cas d'un enfant de sexe masculin de deux ans infecté par le virus de l'immunodéficience humaine (VIH). L'IMC était de 10,3 kg / m², ce qui est inférieur au cinquième centile. L'enfant a perdu 1,5 kg lors de l'examen rétrospectif du dossier et 0,5 kg après présentation avec perte d'appétit, toux et fièvre. Le patient aurait été nourri de foie à plusieurs reprises. L'examen des selles a révélé de nombreux ovules de *Dicrocoelium dendriticum* et d'*Ascaris lumbricoides*. Un traitement à base d'albendazole a été instauré et au bout de trois mois, le poids corporel s'est amélioré pour atteindre 10,5 kg. Il devient important de dépister l'helminthiase chez les enfants de poids insuffisant, en particulier les patients atteints du VIH / SIDA dont le plan de traitement du VIH pourrait être une priorité pour le médecin. Mots-clés: *Dicrocoelium dendriticum*, Enfant, *Ascaris lumbricoides*, VIH / SIDA, Albendazole

INTRODUCTION

Dicrocoelium dendriticum (liver fluke) is a trematode that causes the disease dicrocoeliasis which is a disease of the liver. The disease is rare in humans and fairly common in grazing animals throughout the

world. This disease can be a serious problem in animals as they can accumulate in large numbers in the bile duct eventually leading to cirrhosis. In humans, it is generally non- fatal unless infection of liver is severe.

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However, due to the unique morphology of the trematode- long and narrow, infections are confined to the more distal parts of the bile ducts and produces mild symptoms as a result. These symptoms could be biliary colic, general digestive disturbances which include bloating and diarrhea. However, in heavy infections, bile ducts and the biliary epithelium may become enlarged with the generation of fibrous tissues around the ducts leading to hepatomegaly or inflammation of the liver (cirrhosis) (1).

Ruminants such as cows and sheep including deer and rabbits (2) are usually the definitive host, but humans and other herbivores can also serve as definitive hosts through the ingestion of infected ants which is found in grass, herbs, raw fruit, vegetables or even drinking water (3). The main reservoirs for *D. dendriticum* are sheep, cows, land snails and ants and have also been found in goats, pigs.

The diagnosis of *D. dendriticum* involves the identification of the parasite eggs in faeces of human or animals. In humans, the eggs in stool may be as result of ingestion of infected animal liver and may not indicate dicrocoeliasis. Therefore, examining bile or duodenal fluid for eggs is a more accurate diagnostic technique.

Ascaris lumbricoides is the largest nematode (roundworm) parasitizing the human intestine. Adult female is 20 to 35 cm long, 3-6 mm wide; adult male: 15 to 30 cm long, 2-4 mm wide. Uteri of females may contain up to 27 million eggs at a time, with 200,000 being laid per day. Fertilized eggs are oval to round in shape and are 45-75 µm long and 35-50 µm (0.0014-0.0020 in) wide with a thick outer shell. Unfertilized eggs measure 88-94 µm (0.0035-0.0037 in) long and 44 µm (0.0017 in) wide.

It is the most common parasitic worm in humans. This organism is responsible for the disease ascariasis (loffer's syndrome), a type of helminthiasis and one of the groups of neglected tropical diseases. An estimated 1 billion people are infected with *A. lumbricoides* worldwide (4). While infection occurs throughout most of the world, *A. lumbricoides* infection is most common in sub-Saharan Africa, the Americas, China, and East Asia (5).

While the vast majority of these cases are asymptomatic, infected persons may present with pulmonary or potentially severe gastrointestinal complaints. Symptoms may include bloody sputum, cough, fever, abdominal discomfort, intestinal ulcer, and passing worms.— Accompanying symptoms include pulmonary infiltration, eosinophilia, and

radiographic opacities (6). Significant increases in fertility are observed in infected women (7). The rate of complications secondary to ascariasis ranges from 11-67%, with intestinal and biliary tract obstruction representing the most common serious sequelae, estimated 730,000 cases of bowel obstruction annually, 11,000 of which are fatal (8). In one series of pregnant patients in Bangladesh, biliary ascariasis was responsible for a plurality (28%) of non-obstetric etiologies of acute abdomen (9).

Ascariasis predominates in areas of poor sanitation and where raw human faeces are used as fertilizer. It is associated with malnutrition, iron-deficiency anemia, and impairments of growth and cognition. Children, because of their habits (eg, directly or indirectly consuming soil), are more commonly and more heavily infected than adults. Male children are thought to be infected more frequently, owing to a greater propensity to eat soil. Neonates may be infected by transplacental infection. Frequently, families may be infected and re-infected in group fashion due to shared food and water sources as well as hygiene practices.

Preventing ascariasis or any faecal-borne disease requires educated hygienic habits/culture and effective faecal treatment systems. The eggs are one of the most difficult pathogens to kill and the eggs commonly survive 1-3 years. *A. lumbricoides* lives in the intestine where it lays eggs. Infection occurs when the eggs are eaten. The eggs may get onto vegetables when improperly processed human feces of infected people are used as fertilizer for food crops. Infection may occur when food is handled without removing or killing the eggs on the hands, clothes, hair, raw vegetables/fruit, or cooked food that is (re)infected by handlers, containers, etc.

Case Presentation

A two year old male child who is infected with human immune deficiency virus (HIV). Patient was delivered through vaginal delivery at term at a TBA centre. He was breast fed at birth and breastfeeding was stopped at 2 months of age. There was no PMTCT intervention either to mother or child. Father is a 33 year old technical school graduate of unknown HIV status. Mother is a 29 year old technical school graduate, recently diagnosed of HIV positive. The patient is the only child of the parents and was placed on HAART, daily vitamin B complex, cotrimoxazole and immune booster. The child is in school.

He weighed 9.5kg at presentation (at 25th month of age) with a loss of 1.5 kg body weight at earlier visit at 18th month of age and a further drop of 0.5kg

weight at the next clinic visit three months from date of presentation. Body temperature was normal at 36.5°C, pulse was 128 beats per minute, while respiration was 30 breaths/min. Height was 96 centimeters which increased steadily from time of registration at two months of birth (57cm). Body mass index was 10.3kg/m² which falls below the fifth percentile indicating that the child is underweight. Head circumference also increased from 41cm at presentation to 48cm at 2 years. His CD4 count gradually improved from 961 to 1795 at 20th month of life then declined a little at presentation at 1542. The haemoglobin level, creatinine, Alanine aminotransferase, urea, glucose were at normal ranges. Viral load was undetectable throughout.

The 1.5kg loss in body weight was with poor appetite, cough and fever at 18th month of life. However, there was no parasitic investigation nor any parasitic treatment plan for the patient. The patient was reportedly not been given antiparasitic medicine by the parents and had been fed with cooked liver on several occasions.

The patient's stool was soft formed. Microscopic examination for ova and parasites revealed many *Dicrocoelium dendriticum* and *Ascaris lumbricoides* eggs. The patient was placed on albendazole. After three months, the body weight of the patient improved drastically to 10.5 kg.

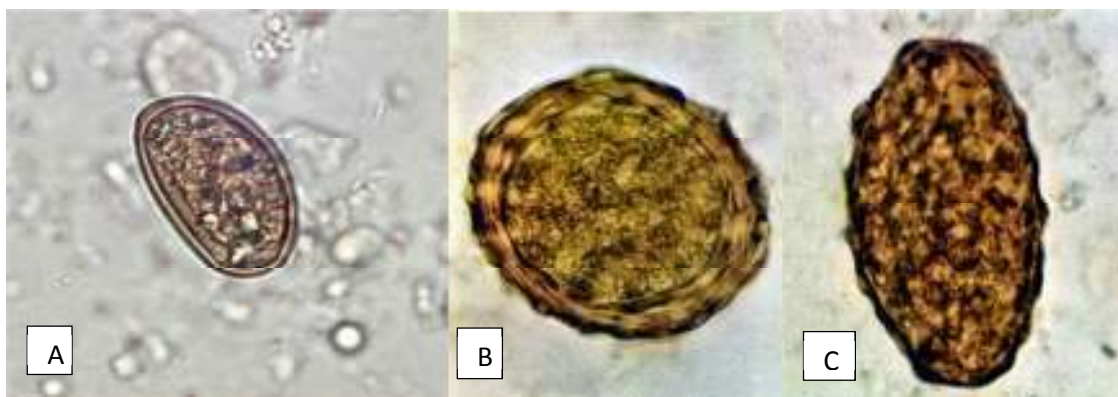


Figure1: A: a wet mount slide of *Dicrocoelium dendriticum* egg in an unstained stool. B: fertile *Ascaris* egg in faeces; C: infertile *Ascaris* egg.

DISCUSSION

The clinical features of *A. lumbricoides* infection are variable between asymptomatic and symptomatic disease depending upon the parasitic burden. The symptomatic disease is largely restricted to individuals with a high worm load (5). The symptoms are related either to larval migration or to the adult worm intestinal stage. Pulmonary manifestations of ascariasis are due to the larval migration through the lungs. Heavy infection with *A. lumbricoides* is frequently manifested by abdominal discomfort, anorexia, nausea and diarrhoea. Heavily infected children suffer from protein malnutrition and vitamin A deficiency (10,11).

Infection of *Ascaris* begins with the ingestion of embryonated (infective) eggs in faeces-contaminated soil or foodstuffs. Once ingested, eggs hatch, usually in the small intestine, releasing small larvae that penetrate the intestinal wall. Larvae migrate to the pulmonary vascular beds and then to the alveoli via the portal veins usually 1-2 weeks after infection, during which time they may cause pulmonary symptoms (e.g. cough, wheezing). The management

of the majority of cases of uncomplicated ascariasis can be done successfully with antiparasitic drugs (12). Meanwhile, complicated cases of ascariasis can be managed by surgical intervention. In our case, albendazole treatment was instituted with a positive response.

Although previously identified in North America, Asia and Africa, most reports of human infection by *D. dendriticum* originate in Europe and the Middle East, where the lancet fluke is a common parasite in sheep and cattle. In Nigeria, *D. dendriticum* infections have been reported in slaughtered- cattles in Bauchi (13). Most human cases represent spurious infections, reflecting the gastrointestinal passage of parasite eggs by consumption of infected animal liver.

The caregiver of the child confirms recent ingestion of animal liver, several times that year. We suspect that he passed *Dicrocoelium* eggs in his stool after possibly inadvertent consumption of infected liver, in keeping with a pseudoparasitic infection. True human infection is acquired by ingesting the field ant, which can occur after drinking contaminated water, or eating salads or raw vegetables containing infected

ants. The present patient reported no history of recent ant ingestion.

Symptoms of true human infection may include constipation, chronic diarrhea, vomiting and abdominal pain (14,15) as well as hepatomegaly and peripheral eosinophilia (15,16); and these were not demonstrated by our patient.

Patients infected with HIV and *D. dendriticum* have been reported in the literature (17,18); there is no indication that our patient's immunosuppressive condition had any influence on his clinical presentation as shown by his undetectable HIV viral load and high CD4 T lymphocyte count. His condition improved after successful treatment with albendazole, indicating that his condition was largely related to parasitic infection. Generally, there is no

need to initiate antiparasitic treatment in patients who pass *Dicrocoelium* eggs after ingesting contaminated liver, but we decided to treat the present patient with albendazole and confirm the absence of eggs in the stool, because of co-infection with *A. lumbricoides* as well as his immunosuppressive condition and the concern about his age and interference with physiologic development.

Conclusion

The present report underlines the importance of screening the stool of patients especially children with low body weight for age for underlying, often unsuspected parasitic infections, particularly HIV/AIDS patients whose HIV treatment plan might be of priority to the physician.

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ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY ISBN 1595-689X 2019 VOL20 No.

AJCEM/1811

<http://www.ajol.info/journals/ajcem>

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AFR. J. CLN. EXPER. MICROBIOL. 20 (1): 72-82

HIGH PREVALENCE OF EXTENDED-SPECTRUM β -LACTAMASE (BLACTX-M-15) AND NEW DELHI METALLO-B-LACTAMASE-1 (NDM-1) GENES AMONG HIGH-LEVEL CARBAPENEM RESISTANCE KLEBSIELLA PNEUMONIA: AN ALARM FOR OUR HEALTH SYSTEM

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ABSTRACT

Background: The extended-spectrum β -lactamase (ESBL) carbapenems-resistant *Klebsiella* isolates are considered one of the most significant challenging in the treatment of patients in hospitals. The aim of this study was to determine the prevalence of important carbapenem resistance genes ESBL subtypes and between *K. pneumoniae* from patients at hospital in Tehran, Iran.

Methods: Fifty-four isolates of *K. pneumoniae* were isolated from Shariatee Hospital in Tehran from February 2013 to July 2016. Antibiotic testing was done by using the standard disk diffusion method and E-test MIC. The confirmation of carbapenemase activity was performed using an MHT and a new method called the carbapenem inactivation method test (CIM). Finally, a polymerase chain reaction (PCR) and sequencing of related genes was performed.

Results: Our PCR data demonstrate that blaCTX-M group's 40 (81.4%) genes were the most prevalent in our hospital followed by group genes blaCTX-M-3 (18.51%) and blaCTX-M-2 (20.38%). The distribution of the CTX-M group revealed that blaCTX-M-15 23 (42.6%) was the dominant subtype. The coexistence of multiple genes included blaTEM, CTX-M and blaSHV, and CTX-M. The presence of blaNDM1, blaOXA-48, and blaKPC were identified in the carbapenem-resistant isolates, 22 (40.7%), 10 (18.5%), and 7 (12.9%) respectively.

Conclusion: Our research showed that a CIM test for the first time in Iran is possible and has a high facility for the fast identification of carbapenem-resistant *Klebsiella* (CRK). We are encountered with the emergence of CTX-M, OXA-48, KPC, and NDM1 harboring CRK strains in our hospitals. Therefore, the treatment of patients infected with these isolates will be an important future concern in our clinical settings.

Running Head: Resistance genes among carbapenem-resistant *Klebsiella pneumonia*

Keywords: New Delhi metallo-beta-lactamase-1, *Klebsiella pneumoniae*, Carbapenem, Extended-spectrum β -lactamase

HAUTE PREVALENCE DES GENES DE LA β -LACTAMASE A SPECTRE ETENDU (BLACTX-M-15) ET DU NOUVEAU DELHI METALLO-B-LACTAMASE-1 (NDM-1) PARMI LA RESISTANCE AU CARBAPENEM DE HAUT NIVEAU KLEBSIELLA PNEUMONIA: UN ALARME POUR NOTRE SYSTEME DE SANTE

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ABSTRAIT

Contexte: Les isolats de *Klebsiella* résistants aux carbapénèmes à β -lactamase à spectre étendu (BLSE) sont considérés comme l'un des défis les plus importants dans le traitement des patients en milieu hospitalier. Le but de cette étude était de déterminer la prévalence d'importants sous-types de BLSE de gènes de résistance au carbapénème et entre *K. pneumoniae* chez des patients hospitalisés à Téhéran, en Iran

Méthodes: Cinquante-quatre isolats de *K. pneumoniae* ont été isolés de l'hôpital Shariatee de Téhéran de février 2013 à juillet 2016. Le test des antibiotiques a été réalisé à l'aide de la méthode de diffusion sur disque standard et du test EIC MIC. La confirmation de l'activité de la carbapénémase a été réalisée à l'aide d'un MHT et d'une nouvelle méthode appelée test de la méthode d'inactivation du carbapénème (CIM). Enfin, une réaction en chaîne de la polymérase (PCR) et le séquençage de gènes apparentés ont été réalisés.

Résultats: Nos données de PCR démontrent que les 40 gènes du groupe blaCTX-M (81,4%) étaient les plus prévalents dans notre hôpital, suivis des gènes du groupe blaCTX-M-3 (18,51%) et blaCTX-M-2 (20,38%). La distribution du groupe CTX-M a révélé que blaCTX-M-15 23 (42,6%) était le sous-type dominant. La coexistence de plusieurs gènes comprenait blaTEM, CTX-M 14 et blaSHV, et CTX-M). La présence de blaNDM1, de blaOXA-48 et de blaKPC a été identifiée dans les isolats résistants au carbapénème, 22 (40,7%), 10 (18,5%) et 7 (12,9%) respectivement.

Conclusion: nos recherches ont montré qu'un test CIM pour la première fois en Iran est possible et dispose d'une grande facilité d'identification rapide de *Klebsiella* (CRK) résistant au carbapénème. Nous sommes confrontés à l'émergence de souches CTX-M, OXA-48, KPC et NDM1 hébergeant des souches CRK dans nos hôpitaux. Par conséquent, le traitement des patients infectés par ces isolats constituera une préoccupation future importante dans nos environnements cliniques.

Running Head: Gènes de résistance parmi la pneumonie à *Klebsiella* résistante au carbapénème

Mots-clés: métallo-bêta-lactamase-1 de New Delhi, *Klebsiella pneumoniae*, carbapénème, β -lactamase à spectre étendu

INTRODUCTION

In recent years, the use of third-generation cephalosporin (TGC) has led to the promotion of a selection of β -lactamase producer bacteria that performs a hydrolysis of TGC, especially the oxyimino-cephalosporins [1,31]. These enzymes, capable of hydrolyzing the newer β -lactams, are referred to as extended-spectrum β -lactamases (ESBLs) [1,2]. At hospital sites, ESBL *K. pneumoniae*-resistant isolates are becoming an emerging public health concern. The ESBL-producing isolates of *Klebsiella* spp. (ESBL-K) have been involved in several outbreaks of nosocomial infections throughout Asia [3], including Iran [4,32], the United States of America [5], the Far East, and Europe [6]. Recently, blaCTX-M-related genes have appeared as the leading type of ESBLs in many parts of the world, including Asia, Europe [7], the United States [8], and South America [9]. On the basis of amino acid sequence, more than 60 variants that include CTX-M-45, CTX-M-25, CTX-M-9, CTX-M-8, CTX-M-2, and CTX-M-1 [10]. These *Klebsiella* ESBL-producing isolates may have TEM, SHV, and CTX-M- β -lactamases [10]. ESBL-K strains are prevalent in Tehran hospitals, but relatively little data are available about β -lactamase genes. Moreover, carbapenem antibiotics are useful for treating infections caused by ESBL-producing gram-negative

bacteria. In recent years, carbapenem resistance is considered one of the most significant challenges in the treatment of patients in hospitals. The rapid spread of the carbapenem-hydrolyzing β -lactamase KPC between *Klebsiella* isolates CR in the Tehran region has turned alarming. Treatment of infection caused by this pathogen has become a matter of significant challenge in hospitals in Iran due to its high levels of resistance to virtually all classes of antibiotics. Therefore, the identification of KPC resistance is important for the appropriate choice of antibiotic treatment in addition to infection control measures to thwart the distribution of resistant *Klebsiella* strains in hospital settings. The aim of this study was to determine the prevalence of ESBL subtypes and important carbapenem resistance genes (KPC/OXA-48/NDM1) among *Klebsiella* cultured from patients at a hospital in Tehran, Iran.

MATERIALS AND METHODS

Bacterial strains

The present work is a descriptive cross-sectional study carried out from August 2013 to July 2016. Fifty-four high-level resistant isolates of *K. pneumoniae* were obtained from Shariatee Hospital in Tehran between February 2013 and July 2016. The *K.*

pneumoniae 7881 strain containing the blaSHV and blaTEM genes was used as a control. The samples yielding the isolates were obtained from different wards, including the ICU and ICU General, Neurology, Internal ICU, Post HSCT, Post-hematopoietic Stem Cell Transplantation, BAL (Bronchoalveolar lavage), Blood, Hematology, Oncology, Emergency, and Gland wards. Antibiotic susceptibility was checked as recommended by the Clinical and Laboratory Standards Institute (CLSI) procedure [11]: Ceftriaxone (CRO: 30 µg), Ceftazidime (CAZ: 30 µg), Cefotaxime (CTX: 30 µg), Imipenem (IMP: 10µg), Meropenem, Ampicillin-sulbactam, (ZOX: 30 µg), Gentamicin (GM: 10 µg), Amikacin (AN: 30 µg), Ciprofloxacin (CIP: 5 µg) (BBL), and *K. pneumoniae* ATCC 700603 were applied as the control in all tests. The Minimum Inhibitory Concentrations (MICs) of Imipenem (IMP: 10 µg) (MAST, Merseyside, UK) against isolates showing reduced susceptibility to this carbapenem was determined using the E-test MIC assay. Antibiotic susceptibility testing was performed as recommended by the Clinical Laboratory Standards Institute (CLSI) guideline and, using disks, *Escherichia coli* 25922 was applied as a control. The MICs for Cp, IMP, MP, CAZ, and CRO were determined by the E-test method according to CLSI guidelines. Isolates exhibiting MIC ≥4 µg were screened for the production of ESBL.

Modified Hodge Testing (MHT)

The confirmation of carbapenemase activity was done using an MHT as previously stated [11]. The *K. pneumoniae*, a positive control MHT Positive *Klebsiella pneumoniae* ATCC1705, and MHT Negative *Klebsiella pneumoniae* ATCC1706 were used as a positive control. In plates, the presence of a distorted or clover leaf-shaped inhibition zone was considered as positive for carbapenemase-producing isolates, as recommended by the document M100-S24 of the Clinical and Laboratory Standards Institute methods [11,12].

Carbapenem Inactivation Method Test (CIM)

The CIM was performed as previously stated with brief changes [3]. The isolates were cultured on MHA (Mueller-Hinton agar) plates and a full 12-µl inoculation loop of each strain was immersed in 420 µl of sterile distilled water, and an active susceptibility meropenem disc (MEM) was homogenized in the solution. After two hours incubation at 35°C, the disk was detached and placed on an MHA plate inoculated with a suspension of OD595 1.25 (correlates with a McFarland value of 0.5) standard of susceptible *E. coli* indicator strain ATCC

25922 with a sterile cotton swab. Finally, incubation of the plate was done at 35°C for 24 h. The results of the test were surveyed after overnight incubation: The inhibition zone in plates around each disk was measured. Plates with inhibition circles <10 mm in diameter were considered to indicate CIM positivity [13-15].

Polymerase chain reaction amplification (PCR) and DNA sequence analysis

The total genomic DNA from the clinical isolates was extracted by the boiling method: four colonies of each isolate in 600 ml of distilled water for 12 min. and centrifuged at 11,000 rpm for 12 min. The supernatant was used for the PCR test. PCR amplification was performed using the specific primers for the blaKPC family, Uni-KPC-F (5'-ATGTCACGTATCGCCGTCT-3') and Uni-KPC-R (5'-TTACTGCCCGTTGACGCCC-3') genes as previously described(16). PCR amplification and primers for the detection of blaCTX-M, blaTEM, blaSHV, blaKPC, blaOXA-48-like, blaNDM, blaVIM, and blaIMP genes was carried out as previously described by Hosseinzadeh et al. and Fursova et al [17,18,33,34]. The sequences were aligned and compared using the online BLAST software, (BLAST <http://www.ncbi.nlm.nih.gov>).

RESULTS

Of the eight available antibiotics, the most effective was colistin (100% of the strains were susceptible). The results of the antimicrobial susceptibility test are shown in Table 1. Distribution of collected samples included BAL 22 (40.7%), Wound 5 (9.2%), Urine 9 (16.6%), Blood 9 (16.6%), Plural 3 (5.5%), Sputum 4 (7.4%), Abscess 1 (1.8%), and Abdominal 1 (1.8%) (Fig.4). *Klebsiella* spp. was detected in 25.9% (n=14) isolates in the <50 years age group and 70.0% (40) isolates in the ≥ 50 years age group, but this difference was not statistically significant (P > 0.05). Thirty-one (57.4%) males and 23 (42.5%) females were infected with *Klebsiella* (Fig.3). Isolates with positive results of the carbapenem inactivation method test (CIM) and modified Hodge testing (MHT) were considered carbapenem-resistant. MIC results for five antibiotics Cp, IMP, MP, CAZ, and CRO showed a high level of resistance (MIC≥4). The phenotypic confirmatory test showed that among the 54 isolates, 54 (100%) were ESBL producers (14). The PCR test of the ESBL-producing isolates showed that TEM 24 (44.4%) isolates were positive for the blaTEM gene, 40 (80.1%) positive for the blaCTX-M groups, and 30 (55.6%) isolates had the blaSHV gene. Moreover, the SHV types were characterized as SHV-27, SHV-11, and SHV-1.

TABLE 1: THE DETAILED RESULTS OF CARBAPENEM-RESISTANT ISOLATES

Phenotypic Tests			MIC value (µg/ml)					Hospital unit	Isolation date Date	Age	Sex	Site of Isolation	Strain	Gene		N
MHT	CIM	DDST	CR O	CA Z	M P	IMP	CP							ESBL	CP Genes	
Positive	Positive	Positive	12	192	24	32	8	Internal ICU	December/2016	26	M	BAL	kleb pne	blaCTX-M15	bla _{NDM-1}	1
Positive	Positive	Positive	16	192	32	32	24	ICU General	November/2016	71	F	BAL	Kle b spp.	blaCTX-M15	bla _{NDM-1}	2
Positive	Positive	Positive	32	192	32	32	12	Neurology	November/2016	14	M	BAL	kleb pne	blaCTX-M15	bla _{NDM-1}	3
Positive	Positive	Positive	32	256	32	32	32	Neurology	November/2016	51	F	Wound	Kle b spp.	blaCTX-M15	bla _{NDM-1}	4
Positive	Positive	Positive	24	256	32	24	24	Urology	December/2016	59	M	Urine	kleb pne	blaCTX-M15	bla _{kpc}	5
Positive	Positive	Positive	32	256	24	32	32	Oncology	December/2016	62	M	BAL	Kle b spp.	blaCTX-M15	bla _{kpc}	6
Positive	Positive	Positive	16	256	32	32	12	Internal ICU	December/2016	90	M	Wound	kleb pne	blaCTX-M15	-	7
Positive	Positive	Positive	32	192	32	32	12	Internal ICU	December/2016	69	F	BAL	kleb pne	blaCTX-M15	-	8
Positive	Positive	Positive	24	128	32	32	24	CCU (Heart)	December/2016	75	M	BAL	Kle b spp.	blaCTX-M15	-	9
Positive	Positive	Positive	32	192	32	32	24	Urology	November/2016	86	M	BAL	kleb pne	blaCTX-M2	bla _{NDM-1}	10
Positive	Positive	Positive	24	256	32	24	32	Glands	November/2016	75	F	BAL	Kle b spp.	blaCTX-M15	bla _{kpc}	11
Positive	Positive	Positive	32	256	32	32	32	General Internal	December/2016	90	F	Blood	Kle b oza	blaCTX-M15	bla _{kpc}	12
Positive	Positive	Positive	32	256	32	32	32	ICU General	December/2016	72	M	BAL	Kle b spp.	blaCTX-M15	bla _{NDM-1}	13
Positive	Positive	Positive	32	256	24	32	32	Oncology	December/2016	53	M	Wound	Kle b spp.	blaCTX-M15	bla _{kpc}	14
Positive	Positive	Positive	24	192	32	32	24	General Internal	December/2016	76	M	Sputume	Kle b spp.	blaCTX-M15	bla _{OXA-48}	15
Positive	Positive	Positive	32	256	24	32	32	Oncology	December/2016	65	M	Wound	Kle b spp.	TEM,SHV	-	16
Positive	Positive	Positive	32	192	32	24	12	Neurology	December/2016	68	M	BAL	Kle b spp.	blaCTX-M3	bla _{NDM-1}	17
Positive	Positive	Positive	24	256	32	32	12	General Internal	December/2016	86	M	Sputume/trach	kleb pne	blaCTX-M15	bla _{OXA-48}	18
Positive	Positive	Positive	32	256	32	32	24	ICU General	December/2016	64	M	Blood	Kle b spp.	blaCTX-M3	bla _{NDM-1}	19
Positive	Positive	Positive	32	256	32	32	24	ICU General	August/2015	59	F	BAL	kleb pne	blaCTX-M15	-	20 G
Positive	Positive	Positive	24	256	32	24	32	Oncology	October/2015	55	F	Urine	Kle b spp.	TEM,SHV	-	21
Positive	Positive	Positive	24	192	32	32	32	Neurology surgery	September/2015	37	F	Blood	Kle b spp.	blaCTX-M2	bla _{OXA-48}	22
Positive	Positive	Positive	32	256	32	24	32	Oncology	September/2015	16	F	Abscess	kleb pne	blaCTX-M15	-	23
Positive	Positive	Positive	24	256	32	32	32	General	October/2015	64	F	Urine	kleb	blaCTX	bla	24

e	e	ve						Internal	2015				pne	-M3	ND M-1	
Positive	Positive	Positive	24	192	24	24	24	General Internal	October/ 2015	70	F	BAL	kleb pne	blaCTX -M15	bla ND M-1	25
Positive	Positive	Positive	24	256	32	32	32	ICU nerves	October/ 2015	32	F	Urine	kleb pne	blaCTX -M3	bla OXA -48	26
Positive	Positive	Positive	32	256	32	32	12	ICU General	October/ 2015	70	F	BAL	Kle b spp.	TEM,S HV	bla ND M-1	27
Positive	Positive	Positive	32	256	32	32	12	ICU General	October/ 2015	88	F	BALV	kleb pne	blaCTX -M15	bla OXA -48	28
Positive	Positive	Positive	24	192	32	24	12	Emergen cy Blood	Septem ber/ 2015	64	M	Blood	kleb pne	blaCTX -M2	-	29
Positive	Positive	Positive	12	256	24	32	24	ICU General	Decemb er/ 2015	65	M	BAL	kleb pne	TEM,S HV	bla ND M-1	30
Positive	Positive	Positive	24	192	32	24	32	ICU General	August /2015	50	M	BAL	Kle b spp.	blaCTX -M3	bla OXA -48	31
Negative	Negative	Positive	12	192	24	32	24	ICU General	Decemb er/2015	72	F	BAL	Kle b spp.	blaCTX -M3	-	32
Positive	Positive	Positive	24	256	24	12	32	surgery room	Septem ber/ 2015	46	M	Abdomina l F	kleb pne	TEM,S HV	bla ND M-1	33
Positive	Positive	Positive	24	192	24	12	32	Lung	Decemb er/2015	78	F	BAL	Kle b spp.	TEM,S HV	bla OXA -48	34
Positive	Positive	Positive	24	256	32	24	32	ICU General	August /2015	49	M	BAL	kleb pne	blaCTX -M2	-	35
Positive	Positive	Positive	24	256	32	32	32	Blood	October/ 2015	59	M	Blood	kleb pne	blaCTX -M15	bla ND M-1	36
Positive	Positive	Positive	24	192	24	32	32	General Internal	Septem ber/ 2015	64	F	Wound	Kle b spp.	blaCTX -M15	bla ND M-1	37
Positive	Positive	Positive	32	256	24	32	24	ICU General	Septem ber/ 2015	39	M	Urine	Kle b spp.	blaCTX -M3	bla kpc	38
Positive	Positive	Positive	32	256	24	32	32	ICU General	May /2015	56	M	Plural	kleb pne	blaCTX -M3	bla OXA -48	39
Positive	Positive	Positive	32	256	24	32	32	General Internal	May /2015	80	M	Sputum	Kle b spp.	blaCTX -M2	bla ND M-1	40
Positive	Positive	Positive	32	192	24	32	32	General Internal	May/ 2015	58	F	Sputum	Kle b spp.	blaCTX -M3	bla ND M-1	41
Positive	Positive	Positive	32	256	32	32	32	Hematol ogy	May /2015	69	M	Blood	kleb pne	blaCTX -M2	bla ND M-1	42
Positive	Positive	Positive	16	256	32	32	24	ICU General	April /2016	59	M	Blood	kleb pne	blaCTX -M2	-	43
Positive	Positive	Positive	32	256	32	24	32	Oncolog y	March /2016	77	M	Urine	kleb pne	blaCTX -M3	bla kpc	44
Positive	Positive	Positive	32	256	32	32	12	ICU (Heart)	April /2016	30	M	BAL	Kle b spp.	blaCTX -M2	bla ND M-1	45
Positive	Positive	Positive	32	256	32	32	24	Oncolog y	April /2016	30	F	Urine	kleb pne	TEM,S HV	bla OXA -48	46
Positive	Positive	Positive	32	192	32	32	32	Emergen cy (Blood)	April /2016	43	M	Plural	kleb pne	TEM, SHV	bla ND M-1	47
Positive	Positive	Positive	32	256	32	32	32	ICU General	April /2016	46	F	BAL	kleb pne	blaCTX -M15	-	48
Positive	Positive	Positive	32	256	32	32	32	Emergen cy (Blood)	April /2016	43	M	Plural	kleb pne	TEM,S HV	-	49
Positive	Positive	Positive	32	256	24	32	32	Emergen cy	March /2016	43	M	Blood	kleb pne	blaCTX -M2	-	50

								(Blood)								
Positive	Positive	Positive	16	256	32	32	32	POST HSCT	April /2016	50	F	BAL	Kle b spp.	TEM,S HV	-	51
Positive	Positive	Positive	32	256	24	32	32	POST HSCT	April /2016	33	F	Urine	kleb pne	blaCTX -M2	blaND M-1	52
Positive	Positive	Positive	32	192	32	24	24	ICU (Heart)	April /2016	30	M	Urine	kleb pne	blaCTX -M2	bla OXA -48	53
Positive	Positive	Positive	32	256	32	32	32	ICU	March /2016	72	F	Blood	kleb pne	blaCTX -M15	blaND M-1	54

Abbreviations: POST HSCT; Post-hematopoietic stem cell transplantation, BAL; Broncho alveolar lavage, MIC = Minimum inhibitory concentration; IMP = Imipenem; MHT = Modified hodge test; DDST = Double disk synergy test; ICU = Intensive care unit; Cp= Ciprofloxacin;IMP= Imipenem; MP= Meropenem; CAZ= Ceftazidime; CRO=Ceftriaxone ; CIM= Carbapenem Inactivation method; N= Isolates number; F=Female;M=Male; Cp Genes= Carbapenem Genes;

Coexistence of multiple gene-encoding ESBLs identified among the isolates included bla_{TEM}, CTX-M bla_{SHV}, and bla_{TEM}, SHV (Fig. 1). Our PCR data demonstrated that among the *K. pneumoniae* clinical strains, bla_{CTX-M15} 23 (42.6%) gene were the most prevalent in our hospital followed by group genes bla_{CTX-M2} 11 (20.38%) and bla_{CTX-M3} 10 (18.51). The distribution of CTX-M groups revealed that bla_{CTX-M15} was the dominant subtype. In the present study, all 44 CTX-M-harboring Klebsiella isolates studied were found to be resistant to cefotaxime and ceftazidime by using the standard disk diffusion method and E-test MIC, according to the CLSI guidelines. Of the 55 high level resistance Klebsiella included in the study, 40 (74.0%) were found, by the PCR reaction test, to carry a carbapenemase-encoding gene, and all PCR-positives (100%) were shown to produce carbapenemase by the CIM except one isolate (Table 2). The results of MHT in all ESBL isolates were positive. bla_{NDM1}, bla_{OXA-48} and bla_{KPC} were identified in carbapenem-resistant isolates, 22 (%40.7), 10 (%18.5), and 7 (%12.9) respectively.

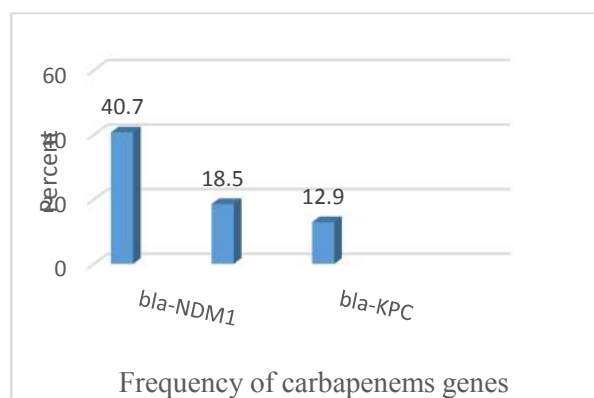


Fig1: Distribution of important Carbapenems resistance genes (KPC, OXA-48, and NDM1) among *Klebsiella* cultured from patients



Fig2: The MHT performed on a Muller Hinton Agar plate. (1) MH positive result

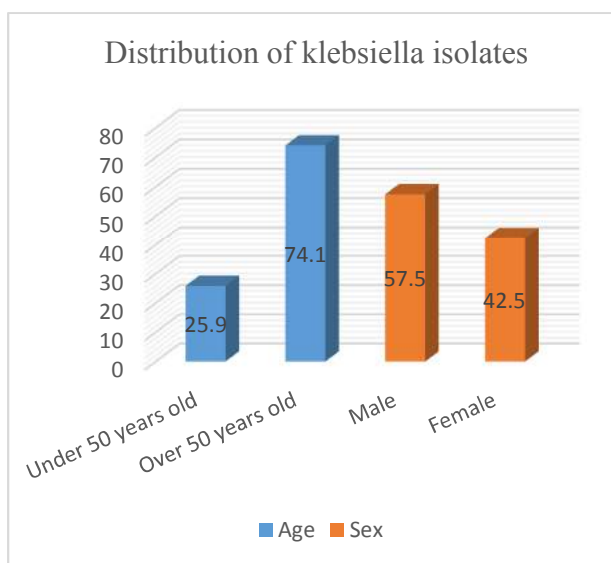


Fig3: Distribution of *Klebsiella* isolates based on Sex and Age

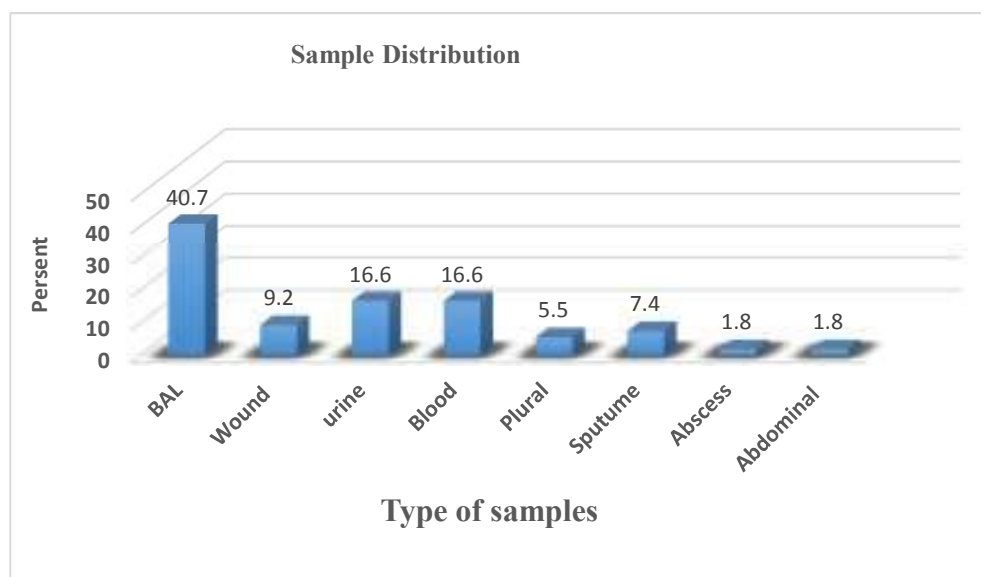


Fig4: Distribution of Klebsiella positive samples

DISCUSSION

ESBL carbapenem-resistant *Klebsiella* (CRK) strains have become globally disseminated in recent years, causing concern over the control of hospital infections [14]. In this situation, with increasing high-level resistant isolates, early detection of CPK strains is critical. A study by van der Zwaluw et al. [15] showed that CIM is a new method having high specificity and sensitivity for detecting carbapenemase producers. The modified Hodge test and CIM methods were used in our research. Results demonstrated that, of the 54 isolates, only one was CIM-negative (2.0%), while all the other isolates emerged positive in the modified Hodge test. The application of the CIM test for the detection of carbapenem-resistant isolates in our study showed it is capable of detecting carbapenemase production. Using PCR, 40 (74%) of these isolates were found to contain a carbapenemase-encoding gene. These results of CIM are consistent with other findings stated by some other researches [14,15-19]. With respect to our research, CIM for the first time in Iran was found to be very efficient and low-cost in the recognition of carbapenemase-producing *K. pneumoniae* isolates. In the clinical setting, the distribution of ESBL-producing *K. pneumoniae* was considered an important therapeutic and epidemiological concern. During the past decade, CTX-M15, CTX-M14, and CTX-M2 have been the most prevalent CTX-M enzyme in different European countries and Iran [20,21]. The present study further showed that the CTX-M_I group had a high (92.7%) prevalence in clinical ESBL-producing isolates, and the most common subtype was CTX-M_{I5}. In a study

by Agamy et al. in Saudi Arabia, a high number of isolates (97.3%) carried the bla_{SHV} gene [22]. Moreover, Ghafourian et al. [23], in contrast to our results in Iran, reported that 94% of *K. pneumoniae* strains harbored the bla_{SHV} genotypes. In our study, TEM-1 and SHV-1 subtypes were the majority of subtypes of TEM and SHV in *K. spp.* strains. Further, it characterized all the *K. pneumoniae* isolates from the Labbafi Nejad and Zahedan hospitals that carried the SHV-11, TEM-1 subtypes, SHV-5, and SHV-12 were reported to be the dominant ESBLs in Iran [24,25]. In agreement with our study, Dedeic-Ljubovic et al. confirmed the presence of CTX-M-15 in KPC isolates, which belongs to the CTX-M-1 group [20], in one of the most important hospitals in Bosnia-Herzegovina. Researchers in South America introduced CTX-M-2 and CTX-M-8 enzymes as the predominant ESBL types [26]. Similar to our finding, in United States, CTX-M-15 was most common genotype but the CTX-M-4 and CTX-M-2 groups were rarely detected [26]. In contrast, with a low frequency of the CTX-M genotype among KP in the United States, our region showed a wide range of strains (high prevalence of carbapenem-resistant strains harbored bla_{CTX-M}). Of 55 *K. pneumoniae* isolates, 22 (40.7) were bla_{NDM-1} positive. Our findings and previous studies indicate that the frequency of bla_{NDM-1} harboring *K. pneumoniae* strains in Iranian hospitals show an increasing trend. A research by Zowawi et al. showed that the most common carbapenemase were 16 isolates of NDM and 35 isolates of OXA-48 types [27]. In our finding, the bla_{OXA-48}-like gene was identified in 10 (18.5) isolates; while two isolates were positive in MHT and

CIM. Phenotype OXA-48-positive in KPC strains has been stated in several regions, including France, Russia, Turkey, Saudi Arabia, Taiwan, and China, and can be a serious concern in hospital settings [17]. In our study, a small number of carbapenem-resistant isolates lacked the targeted genes. The reasons for this may be the reduced permeability of the outer membrane, presence of other genes, AmpC betalactamases, and an extended-spectrum beta-lactamases (ESBLs) [17,28,29]. In the present study, we detected the bla_{KPC} gene in just seven (12.9) CR isolates that were susceptible to tigecycline and polymyxin B. Similar to our findings, other KPC-harboring isolates revealed a resistance to the majority of antimicrobial agents [30]. In summary, we have described for the first time the high coexistence of bla_{KPC}/NDM1/OXA-48 and bla_{CTX-M}-producing *Klebsiella* spp. in KP isolates in Tehran, and characterized the CIM method as being efficient for the detection of CRK strains in hospital settings in Iran.

Conclusion: Our research confirmed that the CIM test is practicable in our laboratory and has a high capability of fast detection of CRK. We are faced with the emergence of CTX-M, OXA-48, KPC, and NDM1 harboring CRK strains in our hospitals. All isolates showed a high level of resistance (ESBL-positive/carbapenem-positive), indicating a situation that was considered to pose a future threat and highlighting the necessity of further surveillance in a hospital setting. So, with the rapid emergence of multi-drug resistance isolates over time, the prevention of the spread of the CPK and the initiation of appropriate antimicrobial therapy will be a matter of serious concern in our clinical settings.

Acknowledgment: We thank all personnel of the Shariati Hospital's microbiology laboratory for their cooperation. This study was supported by the Tehran University of Medical Sciences, Grant No. 32152-31-02-95.

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