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African Journal of Microbiology Research

August 2019
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

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Vol. 13(26), pp. 407-413, August, 2019
DOI: 10.5897/AJMR2019.9084
Article Number: E3F64FA61643
ISSN: 1996-0808

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African Journal of Microbiology Research

Full Length Research Paper

Prevalence of urinary tract infection and antibiotic resistance pattern in pregnant women, Najran region, Saudi Arabia

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Received 25 February, 2019; Accepted August 5, 2019

Urinary Tract Infection (UTI) is one of the commonest infectious disease in pregnancy, and in pregnancy we have very limited number of antibiotics to treat the UTI. This study was conducted on 151 patients who attended the gynecology clinic during the study period. Nineteen UTI proven cases of UTI were studied for prevalence of microorganism and sensitivity pattern against different antibiotics. Among the bacteria isolated, *Escherichia coli* (73.68%) and *Staphylococcus aureus* (10.52%) were the most prevalent Gram negative and Gram positive bacteria respectively. To know the resistance pattern of microorganism we used commercially available discs of different antibiotics. Gram negative bacteria showed more resistance as compared to Gram positive one. It is observed that the most effective antibiotic for Gram negative isolates is Ceftriaxone (87.5%), followed by Amoxicillin + Clavulanic acid (81.25%), Amikacin (75%), Cefuroxime (75%), Cefixime (68.75%) and Mezlocillin (62.5%). For the Gram positive bacteria, Ceftriaxone, Amikacin and Amoxicillin + Clavulanic acid were the most effective antimicrobials (100%). Multidrug resistance Gram negative bacteria were also tested for Extended-spectrum Beta Lactamase (ESBL), 35.71% of *E. coli* isolates were ESBL producer.

Key words: Urinary tract infection, pregnancy, drug resistance, uropathogens, extended spectrum β-lactamase.

INTRODUCTION

Urinary Tract Infections (UTIs) are common and in the clinical practice it is the second most community-acquired infection worldwide (Awaness et al., 2000; Marcus et al., 2005). UTIs affects all age groups, but due to absence of prostatic secretion, short urethra, easy contamination of urinary tract by fecal bacteria and pregnancy, women are more prone than men (Jido, 2014; Awaness et al., 2000).

UTI is one of the most common type infections during pregnancy and after anemia second most common disease (Santos et al., 2011). Due to the physiological change, plasma volume increases and urine concentration decreases during pregnancy. Around 70%

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pregnant women may suffer from glucosuria, which leads to the bacterial growth and UTI (Morgan, 2004; Lucas and Cunningharm, 1993). Additionally, 2-13% of pregnancies complaint for asymptomatic bacteriuria and, if not cured, may lead to adverse fetal and maternal consequences including symptomatic UTIs (Bahadi et al., 2010; Amiri et al., 2015). UTIs can cause certain complications, prolonged hospital stays and significant morbidity (Matuszkiewicz-Rowińska et al., 2015; Smaill and Vazquez, 2015).

The ability of uropathogens (that is, *Escherichia coli* and *Klebsiella pneumoniae*) to produce large quantity of Extended-spectrum beta lactamase (ESBL), makes UTI challenging to treat (Zorc et al; 2005; Stürenburg and Mack, 2003). In addition, ESBL may cause serious problems, particularly in those patients who have functional structural anomalies of the urinary tract, diabetic patients undergone a kidney transplant and patients with polycystic kidney disease (Zorc et al., 2005).

Usually in clinical practice the treatment for UTIs is given empirically, so earlier data of uropathogens prevalence as well as susceptibility for the particular region or setup is necessary (Haider et al., 2010). Emerging trend of bacterial resistance towards different antimicrobials, is due to the excessive application of antibiotics (Dias Neto et al., 2003; Farrell et al., 2003). Therefore, it is necessary to assess the antimicrobial susceptibility to accomplish the most suitable and effective therapy (Farajnia et al., 2009). Furthermore, for screening of ESBL, the sensitivity also depends upon the antimicrobial agents used for testing. However, in Saudi Arabia only few studies are conducted and reported that prevalence of ESBL varies from 11-36% in different regions (Nicolas-Chanoine et al., 2008). The surveillance of antimicrobial resistance is obligatory to decide the susceptibility pattern of uropathogens for the selection of empirical antibiotics.

MATERIALS AND METHODS

Study setting

This prospective cross-sectional study was conducted at Najran University Hospital, Najran region of Saudi Arabia from August 2018 to January 2019.

Study participants

The study was carried out on the patients consulting the Outpatient Department (OPD) and Inpatient Department (IPD) of the hospital.

Inclusion and exclusion criteria

All the pregnant women irrespective to their age, parity and gestational age were included, while women with underlying renal pathology, chronic renal disease, patients who are already on antibiotics for other diseases, mentally retarded, unconscious patients, drug addicts and patients unable to comply were excluded from the study.

Ethical approval

The research was conducted in accordance with the Declaration of Helsinki. The study protocol was reviewed and approved by Najran University Ethical Committee (12-06-16 EC). An informed consent was also signed by the patients for participating in the study.

Isolation and identification of UTI isolates

Patients were instructed to collect the Midstream Sample Urine (MSU) and cultured in the pathological laboratory of Najran University hospital for the isolation of the uropathogens, urine sample was streaked on MacConkey's agar plates (Saudi Prepared Media Laboratory, Riyadh, Saudi Arabia) and incubated at 37°C for 24 h. Next day or after 24 h individual colonies were selected and identified on the basis of their biochemical and morphological characteristics (Akhtar et al., 2014).

For identification of gram negative bacteria

Motility test, Gram-staining and capsule staining (Anthony's method) were performed to check the morphological characteristics, to see the growth pattern of bacteria, different medias like Mac-Conkey's agar no.3, Eosine Methylene Blue agar were used, and for the observation of biochemical characteristics, sugar fermentation (glucose, lactose, maltose, manitol, xylose and sucrose) TSI, IMVIC (indole, MR, VP, citrate) and nitrate test were done (Al Yousef et al., 2016).

For identification of gram positive bacteria

To check the morphological characteristics, gram-staining and capsule staining (Anthony's method) were performed. To check the growth pattern, different media including MacConkey's agar no.3, Nutrient agar, Brain Heart infusion agar, Manitol Salt agar and blood agar base (Oxoid) supplemented with 5% sheep blood were used. For biochemical characteristics, sugar fermentation, oxidase, catalase, novabiocin, optochin, bactracin and bile esculin sensitivity test were performed (Akhtar et al., 2014; Al Yousef et al., 2016).

Maintenance of clinical uropathogens

All the stock cultures were preserved in vials by growing the UTI isolates in 3 ml nutrient broth and next day overlapping with 3 ml 40% glycerol. Vials (tightly sealed) were used to freeze the isolates at 2-8°C.

Determination of antibiotic resistance profile

Disc diffusion method is used for screening of antibiotic resistance. For this purpose, with the help of wire loop UTI isolate lawn were made on nutrient agar plate, then the discs of commercially available antibiotics were placed on that culture plate and incubated at 37°C for 24 h. After 24 h the zone of inhibition around the discs were observed (Al Yousef et al., 2016). Various antibiotics of different classes, such as Ampicillin, Amoxicilin, Amoxicilllin + Clavunic acid, Mezlocillin, Cefuroxime, Ceftazidime, Cefixime, Cefipime, Ceftriaxone, Imipenem (cell wall synthesis inhibitors); Tetracycline, Gentamicin, Amikacin, Clindamycin, Erythromycin, Nitrofurantoin, Tobramycin (protein synthesis inhibitors); Trimethoprim, Co-trimoxazole, Ciprofloxacin, Norfloxacin (inhibits bacterial DNA synthesis), were used to assess the susceptibility profile of uropathogens. ESBL production was assessed using the

CLSI recommendations for ESBL screening and phenotypic confirmation tests (CLSI USA; 2018).

Statistical analysis

The statistical relation between age and antibiotic sensitivity was analysed by two tailed chi-square test, wherein Pearson Chi-Square value and likelihood ratio were calculated using Statistical Package for Social science (SPSS), Version 16 for windows. Differences were considered significant if $p<0.05$.

RESULTS

For this observational study, a total of 200 pregnant women were selected for the study. However, only 151 pregnant women were examined based on their symptoms for UTI.

Demographic characteristics of study subjects

In this study, we observed that mean age of the patients was 28.52 ± 5.96 years. The age group 26-30 years shows highest number (47.36%) positive cases among the pregnant women, there is a statistically significant difference ($p< 0.001$) when compared to other age groups (Table 1). Further, correlation between the different age group and sensitivity as well as resistance was computed using chi-square test. Chi-square test revealed no significant association between different age groups and antibiotic specificity (Table 5) with a p value > 0.05 and Chi-Square value of 3.590.

Different UTI isolates

Urine culture reports revealed that only 19 patients were found to have a positive culture result (presence of UTI). Amongst positive culture results, 14 patients (73.68%) were found to be positive for *E. coli*, followed by 2 patients (10.52%) each for *Staphylococcus aureus* and *Pseudomonas*, and 1 patient (5.26%) for *Staphylococcus haemolyticus* (Figure 1).

Antimicrobial susceptibility profile

In our study it is observed that most effective antibiotic for Gram negative isolates is Ceftriaxone (87.5%) followed by Amoxicillin + Clavulanic acid (81.25%), Amikacin (75%), Cefuroxime (75%), Cefixime (68.75%) and Mezlocillin (62.5%) and among the gram positive isolates Ceftriaxone, Amikacin and Amoxicillin + Clavulanic acid are most effective (100%) (Table 2). It is also observed that antibiotic efficacy varies from 26.31 to 89.47% (Table 3).

ESBL prevalence

The Gram-negative bacteria showing multidrug resistance were also tested for ESBL, and we observed that only 35.71% of *E. coli* isolates were ESBL producer. Another Gram negative bacteria like *Pseudomonas aeruginosa* was not found to be ESBL producer (Table 4).

DISCUSSION

During pregnancy asymptomatic bacteriuria is a commonest infection, which usually leads to the development of symptomatic UTI and put the mother and fetus on risk (Akhtar et al., 2014; Al-Aali and Yousef, 2015; Haider et al., 2010). We observed that overall prevalence of UTI among the pregnant women is 12.5% which is almost similar to a study conducted in different parts of Saudi Arabia (Al-Sibai et al., 1989).

The age group of 26-30 years indicated highest percentage (47.36%) of UTI infection, as this age group is sexually more active, leading to higher incidence of UTIs. This result is co-related with another study conducted by Al-Aali and Yousef, in Taif region of Saudi Arabia (Al-Aali and Yousef, 2015) and Imade et al, in Benin City, Nigeria (Imade et al., 2010). For the symptomatic bacteriuria during pregnancy, advanced maternal age is one of the important risk factor and women likely to have had many children before current pregnancy and this may be the multifactorial risk factor for acquiring the UTI during pregnancy (Akinloye et al., 2006).

It is also observed that the frequency of Gram negative isolates is more as compare to the Gram positive isolates, and the most predominant microorganism is *E. coli (*73.68%*)* followed by *Pseudomonas* (10.52 %). This result correlates with the other studies conducted in different part of Saudi Arabia and other countries as well (Saeed and Tariq, 2011; Al-Aali and Yousef, 2015). Actually the pattern may be differing from place to place and time to time, but in most of the studies conducted in different regions of the world, it is observed that *E. coli* is the most common microorganism causing UTI in pregnancy or clinical practice.

For the treatment of UTIs antibiotics are commonly used, though the antibiotic resistance is reported by conducting different studies all over the world, especially in developing countries (Lamikanra and Okeke, 1997; Gaspari et al., 2005). Our results were in accordance to another study conducted in Dessie area, North-East Ethiopia, which reported that *E. coli*, *pseudomonas* and *proteus* species were most common pathogens and the *E. coli* were almost resistant to Ampicillin, Tetracycline, and Co-trimoxazole (Abejew et al., 2014).

Due to the increasing resistance of antibiotics, treatment of UTI is challenging, especially in pregnancy because we have limited options of antibiotics to treat the

Table 1. Distribution of study subjects across age groups.

Age group	UTI Present n= 19 (%)	UTI Absent n=134 (%)	Total UTI presence (%)
20-25	4 (21.05)	24 (17.91)	21.05
26-30*	9 (47.36)	28 (20.89)	47.36
31-35	4 (21.05)	47 (35.07)	21.05
36-40	2 (10.52)	35 (26.11)	10.52

**P*= 0.001 statistically significant.

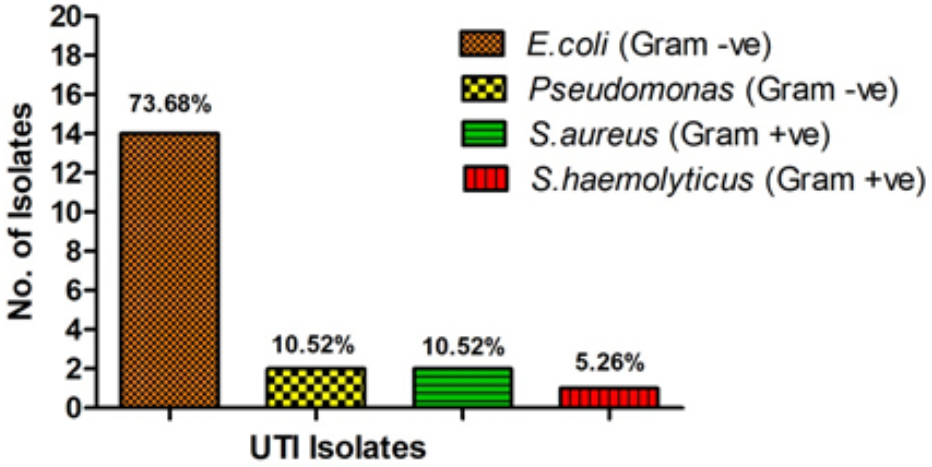

Figure 1. Percentage of gram negative and gram positive bacteria isolated from UTI.

Table 2. Percentage effectiveness of different antibiotics against gram-positive and gram-negative isolates.

Antibiotics	Effectiveness against gram-positive isolates (n=3)			Effectiveness against gram-negative isolates (n=16)		
	Sensitive	Resistant	Gram +ve efficacy (%)	Sensitive	Resistant	Gram -ve efficacy (%)
Ampicillin	1	2	33.33	4	12	25
Amoxicillin	1	2	33.33	6	10	37.5
Tetracycline	1	2	33.33	7	9	43.75
Co-trimoxazole	1	2	33.33	5	11	31.25
Gentamicin	2	1	66.66	5	11	31.25
Amikacin	3	0	100	12	4	75
Ciprofloxacin	2	1	66.66	8	8	50
Clindamycin	2	1	66.66	6	10	37.5
Cefipime	2	1	66.66	7	9	43.75
Cefuroxime	2	1	66.66	12	4	75
Cefixime	2	1	66.66	11	5	68.75
Ceftriaxone	3	0	100	14	2	87.5
Imipenem	1	2	33.33	7	9	43.75
Nitrofurantoin	1	2	33.33	5	11	31.25
Amoxicillin+ Clavulanic acid	3	0	100	13	3	81.25
Norfloxacin	1	2	33.33	9	7	56.25
Tobramycin	1	2	33.33	6	10	37.5
Mezlocillin	1	2	33.33	10	6	62.5
Erythromycin	2	1	66.66	-	-	-

Table 3. Sensitivity profile, resistance profile and % efficacy of various antibiotics against uropathogens isolates. (n=19).

Antibiotics	Disc code	Sensitive	Resistant	Total efficacy (%)
Ampicillin	A_{MP}	5	14	26.31
Amoxicillin	A_{MX}	7	12	36.84
Tetracycline	T_{TC}	8	11	42.1
Co-trimoxazole	C_{TM}	6	13	31.57
Gentamicin	G_{M11}	7	12	36.84
Amikacin	A_{MK}	15	4	78.94
Ciprofloxacin	C_{IP}	10	9	52.63
Clindamycin	C_{LD}	8	11	42.10
Cefipime	C_{PM}	9	10	47.36
Cefuroxime	C_{EF}	14	5	73.68
Cefixime	C_{FM}	13	6	68.42
Ceftriaxone	C_{EX}	17	2	89.47
Imipenem	I_{MP}	8	11	42.10
Nitrofurantoin	N_{IT}	6	13	31.57
Amoxicillin + Clavulanic acid	A_{MCL}	16	3	84.21
Norfloxacin	N_{0R}	10	9	52.63
Tobramycin	T_{BM}	7	12	36.84
Mezlocillin	M_{ZN}	11	8	57.89
Erythromycin	E_{RY}	10	9	52.63

Table 4. Frequency (%) of ESBL producing Gram-negative bacteria.

Gram-negative bacteria isolates	Total number of isolates	Number and % of isolates with ESBL	
		Present (+)	Absent (-)
Escherichia Coli	14	5 (35.71%)	9 (64.28%)
Pseudomonas aeruginosa	2	0 (0%)	2 (100%)

Table 5. The age group wise categorization of sensitive and resistant cases.

Age group	Characteristics	
	Sensitive	Resistant
16-20	5	1
21-25	40	3
26-30	43	9
31-35	29	3
36-40	11	2
41-45	4	0
Total	133	18[#]

#p>0.05 for association between age and development of resistance.

disease. In our study, we also determined the antimicrobial sensitivity pattern for all the isolates by disc diffusion method with the help of commercially available disc of different antibiotics. Majority of the isolates (Gram negative and Gram positive) were found to be resistant against most of the antibiotics, while antibiotic efficacy varied from 26.31 to 89.47%, moreover Gram negative isolates showed more resistance than gram positive one. We observed the most effective antibiotic is Ceftriaxone (87.5%) followed by Amoxicillin + Clavulanic acid (81.25%)

and Amikacin (75%), the findings were consistent with the study conducted by Saeed and Tariq (2011).

Interestingly, resistance to Ampicillin, Amoxicillin, Co-trimoxazole, Tobramycin, Nitrofurantoin and Erythromycin was found to be more common in our study. It can be correlated between use and frequency of antibiotics and the kind of resistance strain in humans (Akhtar et al., 2014). Over the past few years, ESBL producing strains are continuously increasing among the different clinical isolates and in different part of the world, it varies from 1.5% in Denmark to 69% in India (Hansen et al., 2012; Pathak et al., 2014). In our study we found that only 35.71% of *E. coli* is ESBL producer which is almost similar to another study conducted in Riyadh and New Delhi (Alqasim et al., 2018; Akhtar et al., 2014). Other studies conducted in 2002 and 2004 observed that the prevalence of ESBL was 5.6 and 10.3% respectively (Kader and Kumar, 2005). The recent reports show that the prevalence of ESBL in UTIs is increasing continuously in Saudi Arabia, leaving very few options for the physicians for effective treatment of UTIs.

Finally, we suggest that for the empirical use of antibiotic in pregnancy or in clinical practice, local study and prevalence should be conducted rather than to follow the universal guidelines, because the prevalence and resistance pattern of microorganism against different antibiotics varies from time to time and place to place.

Conclusion

In conclusion, regional clinical data regarding the prevalence and efficacy of antibiotics should be taken into consideration along with the treatment guidelines. Our findings urge the need for routine screening for susceptibility of uropathogens in different populations, especially with pregnant women with UTIs, as it may have serious consequences on both mother and fetus. Therefore, screening of all antenatal women visiting the gynecology clinics should be made mandatory, and positive cases must be followed up closely after treatment to avoid recurrence. Adopting this strategy will significantly aid the clinicians in the rational use of antibiotic therapy, prevent overuse and/or misuse of antibiotics, and reduce the emergence of bacterial resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are thankful to the Najran University Hospital, Najran, Saudi Arabia for providing the facilities to conduct this research.

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Vol. 13(26), pp. 414-420, August, 2019
DOI: 10.5897/AJMR2019.9147
Article Number: D15F2D761651
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Detection of multidrug-resistant enterobacteria simultaneously producing extended-spectrum β-lactamases of the PER and GES types isolated at Saint Camille Hospital Center, Ouagadougou, Burkina Faso

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Received 16 May, 2019; Accepted 22 July, 2019

Resistance to a wide variety of common antibiotics is observed among clinical strains designated as extended-spectrum β-lactamase (ESBL) producers. These produce enzymatic proteins that effectively inactivate cephalosporins and aztreonam and are a serious global health problem that complicates treatment strategies. Many studies report a high prevalence of ESBL producers among Gram-negative bacilli. The purpose of this work was to identify resistance genes in enterobacterial strains. Gram-negative bacilli resistant to at least one third-generation cephalosporin, aztreonam or showing a synergy image between amoxicillin + clavulanic acid and a third generation cephalosporin were isolated during an antibiogram. Antibiotic resistance was detected for the following antibiotics: Ceftriaxone, Cefotaxime, Ceftazidime and Aztreonam. Classical polymerase chain reaction (PCR) analyzes of *Pseudomonas* extended resistance (PER) and *Guiana* extended-spectrum (GES) β-lactamase genes were performed using specific primers in 60 ESBL-producing isolates. Among 250 strains of Gram negative bacilli collected, 60 strains (24%) showed resistance to antibiotics used. Stool samples are a major source of ESBL producers. The highest prevalence of resistant strains (35%) was observed in *Escherichia coli*. The GES and PER genes were simultaneously detected at a proportion of 13.33%. This study represents the first detection of PER and GES genes in multidrug-resistant enterobacteria in Burkina Faso.

Key words: Broad-spectrum beta-lactamases (ESBLs), Pseudomonas extended resistance (PER) gene, Guiana extended-spectrum (GES) gene, polymerase chain reaction (PCR).

INTRODUCTION

Enterobacteria, which are mainly responsible for community and nosocomial bacterial infections, have developed mechanisms of resistance to the fatal action of the antibiotics used against them. This resistance can be explained by the excessive and unregulated use of antibiotics, where they are prescribed excessively (Monnet, 2000). This problem is not solved even by the discovery of new, more effective antibiotics and the multi-

resistance is increasing becoming common (Ouedraogo, 2017).

The majority of Broad-spectrum beta-lactamases (ESBLs) are the result of genetic mutations of natural β-lactamases (Pana and Zaoutis, 2018). They are very active against penicillins and moderately active against first generation cephalosporins. The genetic mutations at the origin of ESBLs broaden the spectrum of these enzymes and also affect third-generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone, etc.) and monobactams (aztreonam). Virtually all Gram-negative bacteria have a chromosomal gene that codes for a céphalosporinase (Munita and Arias, 2016). ESBLs are mainly found in the *Enterobacteriaceae*, mainly *Escherichia coli* and genus *Klebsiella*, more rarely *Serratia*, *Citrobacter*, *Enterobacter*, *Morganella*, *Proteus*, *Salmonella*, *Shigella* or non-fermentative Gram-negative bacteria such as *Pseudomonas* spp., *Acinetobacter* spp. and others. The majority of ESBLs are derived from TEM and SHV enzymes, but new ESBLs have been described such as cefotaximase (CTX-M), oxacillinase (OXA), *Pseudomonas* Extended Resistance (PER), Vietnam extended-spectrum β-lactamase (VEB), Guiana extended-spectrum β-lactamase (GES), TEM Like Activity (TLA), Brazilian Extended Spectrum β-lactamases (BES), *Serratia fonticola* (SFO) and Fecal *E. coli* (FEC) (Cattoir, 2008). The rarer types such as SFO, TLA, PER, BES, GES, are found in *Acinetobacter baumanii, Serratia fonticola, Pseudomonas aeruginosa, Klebsiella pneumoniae* (Vodovar et al., 2013). ESBLs, because of their mode of distribution, constitute a major threat for West African countries where weak socio-economic conditions result in poor hygiene conditions, favoring the spread of resistance. It is therefore necessary to determine the presence of genes conferring these resistances. Studies in Burkina have already identified the presence of TEM, SHV and CTX-M genes, which are responsible for bacterial resistance in enterobacteria (Zongo et al., 2015). These genes, although widespread, are not the only ones responsible for bacterial resistance as determined in other countries (Cattoir, 2008). In a context of escalating antibiotic resistance in our country, we must not neglect the search for new ESBLs. This study was conducted with the aim of detecting the presence of PER and GES types of resistances together in enterobacterial strains at Saint Camille Hospital in Ouagadougou, Burkina Faso.

MATERIALS AND METHODS

Isolation and identification of strains

The bacterial strains in our study were gram-negative bacilli resistant to a cephalosporin of third-generation or monobactam. These strains were collected between September and October 2018 at the bacteriology Department of the Saint Camille hospital laboratory. Strains were isolated from urine, pus, stool and vulvar specimens, from internal or external patients at Saint Camille Hospital Center of Ouagadougou (HOSCO). The isolates were identified by API 20E tests (BioMérieux S.A., Marcy l'Etoile, France).

Antibiotic sensitivity test and ESBL detection

Antibiotic sensitivity was tested by the disk diffusion method. Antibiotics were tested on Petri dishes containing Muller Hinton agar. The antibiotic discs used were: aztreonam (30 µg) for monobactam, cefotaxime (30 µg), ceftriaxone (30 µg) and ceftazidime (30 µg) for third-generation cephalosporins. Isolates resistant to at least one antibiotic in clinical trials were collected, purified by single colony isolation and stored at -20°C for further analysis. In order to determine the production of ESBL, the antibiotic discs were deposited in such a way as to reveal the synergy action image representing a champagne plug characteristic of the ESBL profile through a synergy test between the third-generation cephalosporins (cefotaxime or ceftazidime) and amoxicillin + clavulanic acid (Jarlier et al., 1988).

Molecular characterization of isolates producing ESBL

Bacterial DNA extraction

The extraction was done by the boiling method. The conserved strains are reactivated by culture on the MH medium for 18-24 h. An isolated colony is taken from the Petri dishes and suspended in 200 µl of distilled water previously aliquoted in labeled eppendorf tubes. The suspension thus obtained is immersed in a water bath (100°C for 15 min) in order to release the genetic material. The suspension is then centrifuged at 12,000 rpm for 10 min and the supernatant containing the released DNA is transferred to a new eppendorf tube and stored at -20°C until use.

Detection of PER and GES genes

All isolates with antibiotic resistance or showing a synergy image were screened for the gene encoding the β-lactamase of the PER and GES famillies. The crude DNA extracts obtained after extraction was used for the detection of the gene and the Green Master Mix polymerase chain reaction (PCR) was used. The reaction volume was prepared for 25 µl as follows: 12.5 µl of Green master mix + 0.5 µl of primer F + 0.5 µl of primer R + 10.5 µl of distilled water + 1 µl of DNA extracted. PCR was performed with the Gene Amp System PCR 9700 thermocycler (Applied Biosystems, California, USA). The following primer sequences were used: GES-1 F: ATGCGCTTCATTCACGCAC and GES-1 R: CTATTTGTCCGTGCTCAGG; PER-1 F: ATGAATGTCATTATAAAAGC and PER-1 R: AATTTGGGCTTAGGGCAGAA.

The amplification was carried out according to the following program: Initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 60 s, annealing at 50°C for 60 s, extension at 68°C for 1 min, to finish with a final extension at 68°C for 5 min

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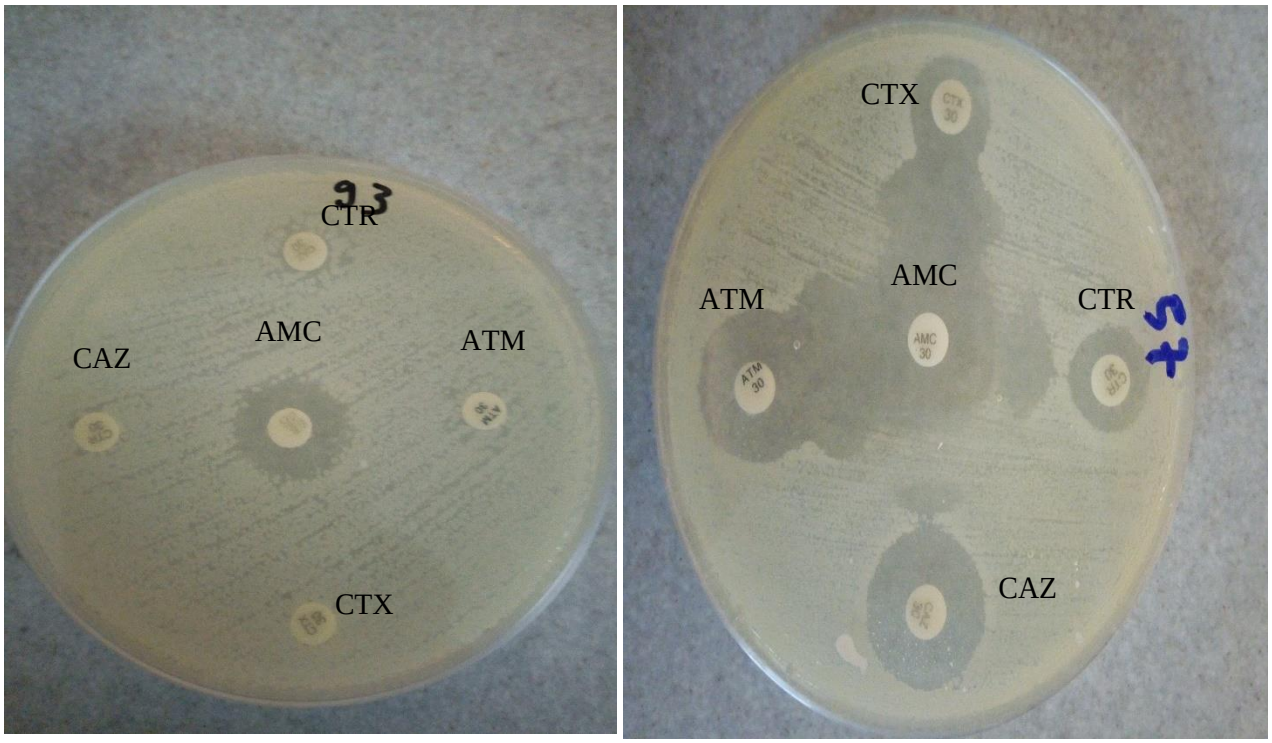

(A) (B)

Figure 1. (a) Petri dish with a strain of enterobacteria resistant to CTR, CAZ, CTX, ATM. **(b)** Petri dish representing a synergistic image characteristic of an ESBL.

this cycle was repeated 30 times.

Agarose gel electrophoresis

Electrophoretic migration at 120 volts for 30 min was performed on a 1% agarose gel in the presence of ethidium bromide. A molecular weight marker of 1000 Pb was used as a reference. The resulting bands were observed under UV light from the GENE FLASH apparatus.

RESULTS

Bacterial strains and antibiotic susceptibility testing

In this study, 24% (60/250) *Enterobacteriaceae* strains resistant to at least one third generation cephalosporin or aztreonam and / or showing a synergy image were detected (Figure 1). Among the 60 strains of resistant enterobacteria, the following species were detected (Table 1). Most of the resistant strains came from cytobacteriological examinations of the urine (50%). Most of the resistant strains were from out patients at Saint Camille Hospital with 51.64%. The age of patients harboring the strains with resistance ranged from 22 days to 95 years with an average age of 36.66 years. The sex ratio H / F was 0.71. The distribution of the strains according to their resistance or to the presence of a synergy image is represented in Table 2. The resistance rates observed were: 65% for cefotaxime, 63.33% for ceftriaxone, 50% for ceftazidime and 60% aztreonam.

PER and GES gene amplification and electrophoresis

The search for the PER gene by classical PCR with specific primers showed that 9 strains (15%) harbor this gene. About 1000 bp bands were observed after migration and visualization of the PCR products (Figure 2). 13.33% of the multidrug-resistant enterobacterial strains produced ESBLs of PER and GES types simultaneously and they were all from patients over 30 years of age, whereof 75% were men. Isolates that produce ESBL-PER and ESBL-GES at the same time are much more common in stool with 62.5% and come from external patients at the HOSCO. The distribution of genes according to bacterial species is shown in Table 3.

DISCUSSION

The objective of this study was to detect Gram-negative bacteria that simultaneously produce ESBL PER and GES types at Saint Camille Hospital in Ouagadougou. We observed the ESBL phenotype in five bacterial

Table 1. Frequency of bacterial strains.

Species	Number	Percentage
Escherichia coli	21	35
Klebsiella pneumoniae	18	30
Enterobacter cloacae	6	10
Enterobacter aerogenes	1	1.67
Citrobacter freundii	3	5
Citrobacter brakii	1	1.67
Citrobacter youngae	1	1.67
Proteus mirabilis	4	6.66
Salmonella arizona	1	1.67
Serratia marescens	4	6,66
Total	60	100

Table 2. The distribution of strains according to their resistance or the presence of a synergy image.

Strain	ATM resistant	CAZ resistant	CTX resistant	CTR resistant	Synergy image
Escherichia coli	13	12	15	15	4
Klebsiella pneumoniae	12	8	11	12	0
Enterobacter cloacae	6	4	4	4	0
Enterobacter aerogenes	0	1	0	0	0
Citrobacter freundii	1	2	3	1	0
Citrobacter brakii	0	0	0	1	0
Citrobacter youngae	0	0	0	0	1
Proteus mirabilis	0	0	2	2	2
Salmonella arizona	1	0	1	0	0
Serratia marescens	3	3	3	3	0
Total	36	30	39	38	7

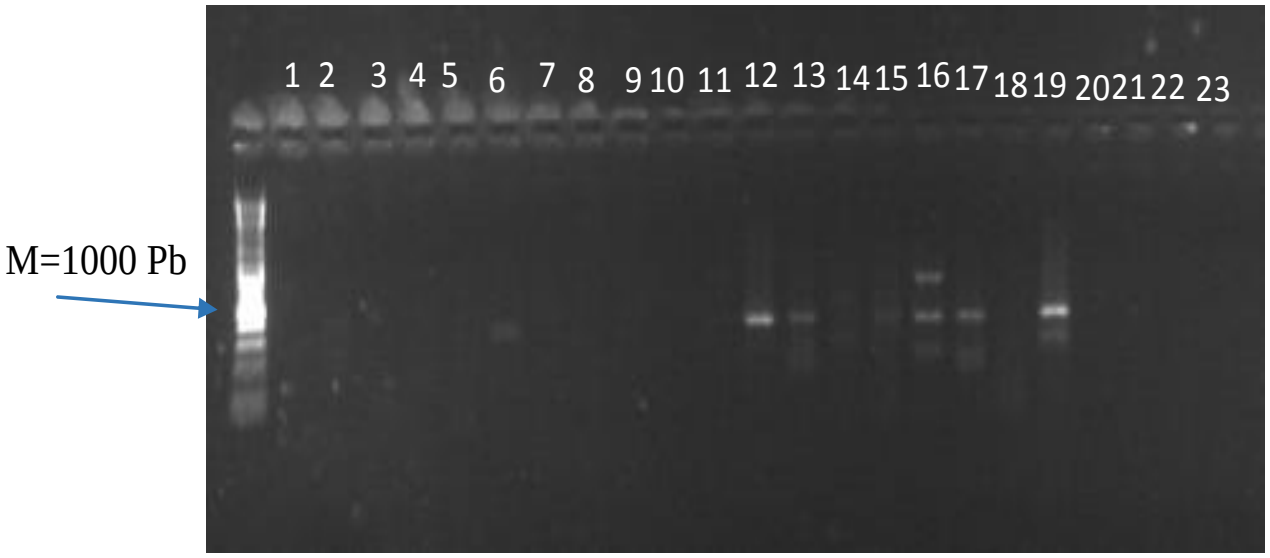

Figure 2. PCR agarose gel from PER and GES. Legend: 1 to 13 correspond to the PER gene; 14 = negative control; 15 to 23 correspond to the gene GES, M = Molecular Weight Marker (GeneRuler 1Kb DNA Ladder).

Table 3. Gene distributions according to bacterial species.

Species	GES and PER gene
E. coli	2
Enterobacter cloacae	3
Salmonella arizona	1
Citrobacter freundii	1
Serratia marescens	1
Total	8

genera. The prevalence of resistant strains was 24% of isolated bacteria comprising of 25% *E. coli*, 37.5% *Enterobacter cloacae*, 12.5%, *Citrobacter freundii*; 12.5 % *Salmonella arizona* and 12,5% *Serratia marescens*. All the bacteria studied showed a high level of resistance to the antibiotics used. Several multiresistant strains were observed in this study. This multidrug resistance can be explained by the fact that the genes responsible for these resistances can be carried by the same plasmid, by the coexistence of several resistance mechanisms, or by the production of several enzymatic types (Harbottle et al., 2006; Boerlin and Reid-Smith, 2008; Muylaert and Mainil, 2012). The resistance rates observed are very high for some antibiotics [cefotaxime (65%), ceftriaxone (63.33%), ceftazidime (50%) and aztreonam (60%)] compared to those observed in developed countries but are approaching resistance rates reported in developing countries (Philippon and Arlet, 2006). This high level of resistance is a consequence of the acquisition of antibiotic resistance factors that is generally secondary to the misuse and uncontrolled use of these antibiotics (Boerlin and Reid-Smith, 2008; Muylaert and Mainil, 2012). The absence of routine surveillance in most countries and especially in the West African region does not allow for a good estimate of ESBL proportions among the strains isolated during infections. Other studies have reported the high incidence of resistance as in Ghana where half of the enterobacteria (49.4%) isolated from the various infections diagnosed at Korle-Bu Hospital were producing ESBL (Feglo et al., 2013). In Togo 66% of E. coli strains isolated from urinary tract infections had the ESBL phenotype (Kubo et al., 2014). These results demonstrate the emergence and dissemination of antibiotic-resistant germs, generally because of the poor living and hygiene conditions observed in developing countries (Ouedraogo, 2017). The results on antibiotic susceptibility [cefotaxime (65%), ceftriaxone (63.33%), ceftazidime (50%) and aztreonam (60%)] confirm the results of earlier work on the high prevalence of enterobacteria resistance to β-lactams in developing countries, which is higher than those in developed countries. In this study, the majority of ESBL producing strains (51.67%) were isolated from out-patients at Saint Camille Hospital. This can be explained by the lack of strict rules for the acquisition of antibiotics in developing countries which means that everyone can have access to even broad-spectrum antibiotics without any medical prescription (Nugent and Okeke, 2010). In all West African countries, antibiotics are sold, like many other medicines, in popular markets and these drugs are known as street drugs (Sirinavin and Dowell 2004; Herindrainy et al., 2011). Self-medication and ignorance promote sharing of antibiotics between individuals based on similar clinical signs.

The CTX, SHV and TEM genes involved in bacterial resistance are the most abundant and documented genes in West Africa (Pieboji, 2007; Zhuo et al., 2013; Metuor-dabire, 2014), but besides these genes, rare genes such as PER, GES, SFO, TOHO, VEB etc are also responsible for resistance. The results show for the first time in Burkina and West Africa the presence of PER and GES genes simultaneously in enterobacterial strains. Extended spectrum β-lactamase (ESBL) PER was detected for the first time in 1993 in a *P. aeruginosa* isolate from a Turkish patient in France (Nordmann et al.; 1993). This enzyme is weakly related to other ESBLs and confers resistance to penicillins, cefotaxime, ceftriaxone, ceftazidime and monobactam-aztreonam, but does not confer carbapenem and cephamycin resistance. Its activity is inhibited by clavulanic acid (Nordmann and Naas, 1994). The bla_{PER} gene is widespread in *Acinetobacter* spp., *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium in Turkey (Vahaboglu et al., 1996, 1997) and has also been detected in *Providencia rettgeri* in that country (Bahar et al., 2004). In addition, PER-1 has been identified in *Acinetobacter* sp. isolated in Korea (Yong et al., 2003). In Italy, PER-1 was detected in isolates of *P. aeruginosa*, *Alcaligenes faecalis* and *Proteus mirabilis* (Pereira et al., 2000; Pagani et al., 2004). In our study, the PER gene was found in *E. coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia marescens*, *Citrobacter freundii* and *Salmonella arizona* with a predominance in *Enterobacter cloacae*. The majority of our PER-producing strains were not only resistant to third-generation cephalosporins and monobactams, but also exhibited a multiresistant phenotype, which corroborates the results obtained in Italy by Pagani et al. (2004). In this study, we have demonstrated the presence of the GES gene, an enzyme weakly similar to another Ambler class, A β-lactamases (Ambler, 1980), in particular to ESBL localized in a plasmid, identified so far in *enterobacteriaceae*. As for class A ESBL, GES has been identified in *K. pneumoniae*, a species that remains the main reservoir of enzymes worldwide, for unknown reasons (Poirel et al., 2000). GES has 23 variants that are identified. In this study, we describe clinical isolates of *K. pneumoniae*, *E. coli*, *Enterobacter cloacae*, *Serratia marescens, Citrobacter youngae, Citrobacter freundii, Salmonella arizona* and *Citrobacter brakii* producing Ambler class A enzyme, GES. This enzyme has been reported in Europe

in *K. pneumoniae* (Poirel et al., 2000) and *Pseudomonas aeruginosa* (Dubois et al., 2002). In most ESBL, GES - 1 does not hydrolyze aztreonam (Naas et al., 2008). GES-2 hydrolyzes the carbapenems and is not sensitive to inhibitors, due to a 2-bp substitution, leading to a single change of Gly170Asn in the Ω loop of the catalytic site. GES - 9, which differs from GES - 1 by the change of Gly243Ser, does not hydrolyze carbapenems but expands monobactam activity (Poirel et al., 2005). Our results show that 62.5% of our strains possessing the GES gene hydrolyzes aztreonam, which could suggest that it is GES-9. 13.33% of the multidrug-resistant enterobacterial strains produced ESBLs of the PER and GES types simultaneously. This corroborates the results obtained by Metuor-Dabire et al. (2018), which found 11 multidrug-resistant ESBL-producing strains harboring both CTX-M and SHV as well as TEM and SHV (Mètuor-Dabiré et al., 2018).

Other studies have detected the presence of its genes in an isolated way in bacterial strains (Pereira et al., 2000; February et al., 2003), our study is the first to have highlighted the simultaneous presence of two types of ESBL, PER and GES.

Conclusion

The aim of this work was to detect extended spectrum β - lactamase-producing *Enterobacteriaceae* (ESBL) at Saint Camille Hospital in Ouagadougou. The isolated bacteria have high levels of resistance to the β-lactams tested. The prevalence of third generation cephalosporin resistant bacteria, aztreonam and / or synergy image is 24%. In view of these results it appears that ESBLs play an important role in bacterial resistance and this is accentuated by the appearance of mutants of these often more dangerous enzymes that compromise health care.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 13(26), pp. 421-429, August, 2019
DOI: 10.5897/AJMR2018.8819
Article Number: 61B445A61684
ISSN: 1996-0808

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Anti-dermatophytic activity of hexane extracts of *Azadirachta indica* A. Juss.

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Received 13 February, 2018; Accepted 3 August, 2018

Dermatophytes cause superficial fungal infections that pose public health problem to man and animals. Long term treatment with antifungal agents is required to control these infections. Various parts of *Azadirachta indica* are claimed to have significant medicinal value in treatment of infections especially ringworm. To determine the antifungal activity of the oil and fractions against dermatophytes isolated from clinical cases. Seeds of *A. indica* were collected, dried, grinded and extracted with hexane using soxhlet and cold maceration. Physicochemical analysis of the oil was carried out as described by Association of Official Analytical Chemists methods (AOAC, 1990). Fractionation of the oil was subjected using column chromatography and Infra-Red (I.R) analysis using spectrophotometer. Swab samples were collected from pupils in Kudan, Kaduna State with suspected cases of *Tinea corporis* (ringworm). The causative fungal organisms were isolated and identified by routine mycological and biochemical procedures. The antifungal activities of the extracts were evaluated by determining the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and diameter zones of inhibition. Hexane extract from soxhlet method of extraction showed higher diameter zone of inhibition against isolated dermatophytes *(Trichophyton mentagrophytes, Trichophyton rubrum* and *Microsporum canis*), ranging from 14.33 – 17.33 mm. The MIC and MFC values of the extract range from 3.13 to > 50% v/v and 50 to >50%v/v respectively. The oils and fractions recorded class of compounds which include alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. Hexane extract of seed of *A. indica* extracted by soxhlet and cold maceration methods had inhibitory activities against the causative agents of *T. corporis* (ringworm) when tested.

Key words: Dermatophytes, *Azadirachta indica*, antifungal, Fourier-transform infrared (FT-IR).

INTRODUCTION

Dermatophytes are fungi that require keratin for growth. These fungi can cause superficial infection of the skin, hair and nails. Dermatophytes are spread by direct contact from other people, animals and soil. Dermatophytes which comprise a group of closely related fungi made up of three genera; *Trichophyton, Microsporum,* and *Epidermatophytes,* have the ability to invade the stratum corneum of the epidermis and

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keratinized tissues derived from it, such as skin, hair, and nail of humans and other animals. They cause superficial fungal infections that pose public health problems to man and animals. Dermatophytes infections can be disfiguring and recurrent and generally need long-term treatment with antifungal agents (Tortorano et al., 2014).

The use of plants in the alleviation and cure of bodily ills goes as far back as the history of the human race itself. The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premise (Evans, 2002).

Use of herbal medicines in the developed world has continued to rise because they are rich source of novel drugs and their bioactive principles form the basis in medicine, pharmaceutical intermediates and lead compounds in synthetic drugs (Akula et al., 2003). More importantly in Africa, particularly West Africa, new drugs are often beyond the reach of the poor such that up to 80% of the population uses medicinal plants as remedy against infections and diseases (Thomford et al., 2015).

Various parts of neem tree are claimed to have significant medicinal importance: which include uses in leprosy, malaria, asthma, intestinal worms. Topical application of neem seed oil can cure dermatological diseases within 3-4 days (Akula et al., 2003). Neem oil used in cosmetic industries is a product of the neem tree and is employed in soap making (Edwards, 2015). Nimbolide, gedunin and nimbin (triterpenoids) are chemical compounds with antifungal activity from neem seed oil (Hashmat et al., 2012). The objective of this research is to determine the *in-vitro* antifungal activity of n-hexane extracts and fractions of *Azadirachta indica* against clinical isolates. The work aimed at a therapeutic alternative against dermatophytes and the functional groups responsible for such activity.

MATERIALS AND METHODS

Media used were: Sabouraud Dextrose Agar (Oxoid, Basingstoke, U.K.), Sabouraud Dextrose Liquid Medium (SDLM: Oxoid, Basingstoke, U.K.) and Sabouraud Dextrose Agar + Chloramphenicol + Cycloheximide (Cat No.21089.00 Deben Diagnostics Limited, U.K). The media were prepared according to the manufacturer's instruction.

Chemical and reagents used include: Tween 80 (Sigma Aidrich-Missouri, U.S.A.), Dimethylsulfoxide (DMSO: Sigma Aidrich-Missouri, U.S.A.), n-Hexane (Sigma Aidrich- Missouri, U.S.A.), Chloroform (Sigma Aidrich- Missouri, U.S.A.), Christensen's urea agar medium (Sigma Aidrich- Missouri, U.S.A.) and Lactophenol cotton blue (Sigma Aidrich- Missouri, U.S.A).

Antifungal used: Terbinafine powder (Cat No. F8929, Sigma Aidrich, U.S.A.).

Instrument

Spectrophotometer, Single-beam, Spectronic 20D; Milton Roy Company, Madrid, Spain.

Collection, extraction and fractionation

Plant seeds were obtained from National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria. Identification and authentication was done in the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria with a voucher specimen number 900151.

The seeds were washed, sun-dried and foreign materials removed by winnowing. The cleaned neem seeds were oven dried at 50°C and then grinded into powder using milling machine at National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State.

Oil extraction

Soxhlet extraction: Soxhlet extraction method was used to extract the oil from the processed seeds. One thousand five hundred (1500 g) of the neem seed powder was packed inside a muslin cloth and placed in a thimble of Soxhlet extractor. A round bottom flask containing n-hexane was fixed to the end of the extractor and a condenser was tightly fixed at the bottom end of the extractor. Extraction was done each time with n- Hexane. The flask was then heated at 60°C with the use of a heating mantle. The solvent was vaporized and condensed into the evaporator. The process continued for 4 h. Oil was recovered from the mixture (oil and solvent) by the use of rotary evaporating process. The oil was obtained and stored in a bottle for further processes (Awolu et al., 2011).

Cold maceration: In this process, 1500 g of the coarsely powdered crude plant seed was placed in a stoppered container (conical flask) with 1000 ml of n- hexane and was allowed to stand at room temperature for a period of 3 days with intermittent agitation (stirring) until the soluble matter dissolved. The mixture was then strained, the marc (the damp solid material) pressed, and the oil extract was clarified by filtration after standing. The oil was recovered from the mixture (oil and solvent) by the use of rotary evaporator.

Physicochemical analysis

The following physicochemical analysis which involved organoleptic properties, density, viscosity, saponification value, iodine value, acid value, peroxide value, and free fatty acid of the neem oil were carried out as described by Association of Official Analytical Chemists methods (AOAC, 1990).

Fractionation of oil extract

A portion (50 ml) of crude n-hexane oil extract was subjected to column chromatography using silica gel G as an absorbent. The column was successively eluted with hundred milliliters (100 ml) of n-hexane (100%), hexane: chloroform mixture (75:25 and 50:50%) and 100% chloroform.

Spectroscopic analysis of *A. indica* oil and its fractions

Infra-red (I.R) analysis

Infra-red (I.R) analysis of the absorption spectra of *A. indica* oil and the fractions that have activity against dermatophytes was conducted at National Institute for Chemical Technology (NARICT), Basawa, Zaria, Kaduna state of Nigeria. A Fourier- transform infrared (FTIR) spectrometer was used in which the sample was

placed. The spectrometer directed beams of IR at the sample and measured how much of the beam and at which frequencies the sample absorbs the infrared light. The molecular identities were determined through a reference database which houses thousands of spectra, so samples can be identified. The functional groups present in the oil and its fractions were determined by comparing the vibration frequencies in wave numbers of the samples spectrograph obtained from an FT-IR spectrophotometer (Coates, 2000).

Isolation and identification of dermatophytes

Thirty swab samples of *Tinea corporis* (ringworm) were collected from infected skins of pupils at Kudan, Kaduna State using sterile scalpels. Affected areas were cleansed with 70%v/v ethanol, allowed to dry and light scrapings from the edge of the lesions were taken using a blunt sterile scapel blade. The specimens were placed in well labelled clean white envelopes. Mycological analysis of the 30 specimens from the suspected infected sites of the participants was carried out in the Pharmaceutical Microbiology Laboratory at the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Kaduna State, Nigeria.

The specimens were inoculated into 10 ml Sabouraud Dextrose Liquid Medium (SDLM) and incubated for 48 h. Growth from the broth was then streaked on Sabouraud Dextrose Agar containing chloramphenicol and cycloheximide and incubated at 30°C for 14 days. Cultures were examined weekly for sporulation. Colonies of dermatophytes were later subculture on respective petri-dishes containing SDA and incubated at 30°C for 21 days. The isolates were identified by routine mycological and biochemical procedures (Urease Test, Corn meal test and Nutritional test) as modified by Refai et al. (2013).

Standardization of the innoculum

Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Candida albican and *Aspergillus niger* fungal spores were harvested from 7 day old Sabouraud dextrose agar (SDA) slant cultures by washing with 10 ml sterile normal saline containing 3% v/v Tween 80 with aid of sterile glass beads to help in dispersing the spores. Thereafter, the spore suspension were standardized to 1.0×10^5 spores / ml by using a single-beam spectrophotometer at 530 nm (OD530) adjusted to 70-72% transmittance for *T. mentagrophytes, T. rubrum* and *M. canis*. All adjusted suspensions were quantified by spreading 100 µl on Sabouraud dextrose agar plate and incubated at 30°C for 72 h for dermatophytes (Aberkane et al., 2002). Standardized isolates were maintained at 4°C (in the refrigerator) until required for use.

Determination of antifungal activity of *A. indica A. Juss*

The plates were allowed to dry at 30°C temperature in a sterilized incubator. Using the agar diffusion cup plate method, a sterile cork borer (6 mm) was used to bore wells in the agar plates. The bottoms of the wells were each sealed with a drop of molten SDA. Using micropipette, 0.1 ml each of the different graded concentrations of the extract (soxhlet and cold maceration) were dispensed into the wells marked 100 and 90%v/v and 10% DMSO (used in diluting the extracts and a negative control). These were allowed to diffuse into the agar at room temperature for an hour before incubation at 30°C for 72 h. The diameter of zones of inhibition of the test organisms were measured to the nearest millimetre using a well-calibrated meter ruler. The experiment was carried out in triplicate.

Table 1. Biochemical and mycological tests of dermatophytes.

Test	TM	MC	TR
Urease test	+	-	-
Corn meal	-	-	+
Nutritional test	-	+	-

TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum* , Urease test, + = Pink colour, Corn meal, + = Dark red colour, Nutritional test, + = Growth.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined by agar dilution method as modified by Serban et al. (2011). Ten millilitres of the graded concentration of the crude extracts was mixed with 10 ml of double strength SDA supplemented with 0.5%v/v Tween-80 and poured aseptically into sterile plates. The plates were allowed to set. Ten microlitres of the standardized organisms containing 10^6 $CFUmL^{-1}$ was inoculated on the equidistantly placed sterile filter paper disc. The plates were allowed to stand for one hour and then incubated at 30°C for 48 h. The same procedure was repeated using Terbinafine. The lowest concentration of the agent that inhibits the visible growth of the test organisms was taken as the MIC. The experiment was carried out in triplicate.

Determination of minimum fungicidal concentration (MFC)

The filter paper discs showing visible growth at two test concentrations below MIC, discs at MIC and discs at two concentrations above MIC were aseptically removed with the aid of a sterile forceps and transferred into 5 ml sterile SDLM supplemented with 0.3%v/v Tween 80 and incubated at 30°C for 48 h. Minimum fungicidal concentrations were determined as the lowest concentration resulting in no growth on subculture.

Statistical analysis

All the data obtained from the studies were expressed as mean ± standard deviation (SD).

RESULTS

Dermatophytes isolated with *T. corporis* infections

A total of 18 dermatophytes consisting of *Trichophyton species* (16 isolates) (Table 1).

T. mentagrophytes	10 Isolates
T. rubrum	6 Isolates
M. canis	2 Isolates

Extraction of oil from *A. indica* A. Juss seeds

The percentage yields of the oils from *A. indica* seeds using n-hexane and extraction methods are shown in Table 2. Soxhlet method of extraction yielded more oil than the cold maceration method; about 4% higher than

Table 2. Percentage yield of oil extracts using various solvents and methods of extraction.

Solvent	Methods	Yield (ml)	Percentage yield
Hexane	Soxhlet	220	13.33
	Maceration	154	09.33

Table 3. Physicochemical characteristics of the extracted seed oil.

	Extraction method	
Oil/ parameter	**Soxhlet**	**Cold Marceration**
Colour	Light brown	Dark brown
Smell	Pungent	Pungent
Taste	Bitter	Bitter
Viscosity (mm^2S^{-1}) /60	492.8	449.4
Relative density (g/ml)	0.917	0.914
Density (g/ml)	0.911	0.908
Refractive Index	1.4652	1.4647
Acid value (mgKOH/g)	6.311	7.013
Peroxide value (mEq KOH/g)	0.10	0.26
Iodine value (mgI_2/g)	154.18	172.58
Saponification value (mgKOH/g)	207.54	223.03
Free fatty acid (mgKOH/g)	4.982	8.648

cold maceration.

Physicochemical characteristics of *A. indica* seeds oil

The physicochemical and organoleptic characteristics of the oils obtained via the two methods of extractions (soxhlet and maceration) using n-Hexane is shown in Table 3. Hexane oil from maceration recorded higher acid, iodine, saponification and free fatty acid values compared to values for the other oil. Oil obtained by soxhlet method was generally lighter in colour; more viscous, denser with lower peroxide and acid values. Oil obtained by maceration was associated with lower viscosity higher acid, iodine, and free fatty acid values. The variations can be attributed to the different methods involved in the oil extraction as reported by Jessinta et al. (2014).

Antifungal activities of the oil fractions

A total of 8 fractions were collected from the elution of the n-hexane and chloroform of the two oil extracts. The results of their antifungal test are presented in Table 4. Antifungal activities of the fractions varied among the fractions. None of the fractions obtained from oil extracted by soxhlet method using Hexane as solvent had inhibitory activity against the test isolates. On the other hand, fractions from the oil obtained by maceration had activity only against the Trichophyton species (*T. mentagrophytes*).

Determination of the functional groups present in the oils and its fractions

The spectroscopic analysis (FT-IR) interpretation detecting functional groups of the oils and the fractions having antifungal activity is shown in Tables 5 and 6 respectively. Both the oils and fractions recorded class of compounds which include alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. However, there was a significant difference in FT-IR of the oil extract and its fractions, where the oil extract recorded the presence of aromatic compounds.

A strong and broad absorption band or frequency from 2500- 3500 cm^{-1} showed the presence of O – H stretch from carboxylic acid as seen in all the spectra, a strong absorption band from 1740- 1755 cm^{-1} revealed the presence of C = O bond for a five member cyclic ketone. A weak absorption band from 3400-3500 showed the presence of N – H stretch from an amides. The absorption frequency with a strong band from 1160-1210 cm^{-1} revealed the presence of O = C – O – C from an aliphatic esters. A medium absorption band 1450-1600 cm^{-1} showed the presence of C = C ring from an aromatic

Table 4. Antifungal susceptibility profiles of test fungal isolates to fractions of *A. indica* oil.

Test organisms / Solvent systems	Zone of inhibition (mm)		
	TM	MC	TR
A 1	0	0	0
A2	0	0	0
A3	0	0	0
A4	0	0	0
C1	0	0	0
C2	16.33 ± 0.58	0	0
C3	0	0	0
C4	12.33 ± 0.58	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates, cork borer- 6 mm, A = Extract from soxhlet, C = Extract from cold maceration, 1= 100% Hexane and 0% chloroform solvent system, 2 =75% Hexane and 25% chloroform solvent system, 3= 50% Hexane and 50% chloroform solvent system, 4 = 0% Hexane and 100% chloroform solvent system.

Table 5. Functional groups present in the oil fractions.

Sample	Absorption (cm^{-1})	Class of compound
C2	412. 78	Alkyl halide
	718.51	Alkenes
	1743.71	Ketone
	2862.46	Carboxylic acid
	2924.18	Alkyl
	3471.98	Amide
C4	384.81	Alkyl halide
	715.61	Alkenes
	1743.71	Ketone
	2865.35	Carboxylic acid
	2929	Alkyl
	3472.95	Amide

C2 = 75% Hexane and 25% chloroform solvent system, C4 = 0% Hexane and 100% chloroform solvent system.

Table 6. Functional groups present in the oil extract.

Sample	Absorption cm^{-1}	Class of compounds
HS	416.64	Alkyl halide
	1165.04	Aliphatic esters
	1458.25	Aromatic compounds
	1743.71	Ketone
	2924.18	Carboxylic acid
	3471.98	Amide
HCM	1165.04	Aliphatic ester
	1458.23	Aromatic compound
	1743.71	Ketone
	2924.18	Carboxylic acid
	3464.27	Amide

Key: HS = Oil obtained by n-Hexane using soxhlet, HCM = Oil obtained using n-Hexane using cold maceration.

Table 7. Susceptibility of the test fungi to the extracts of *A. indica* at 100 and 90% v/v.

Organism	Zone of inhibition (mm)					
	100%w/v		90%w/v			
	Soxhlet	Cold maceration	Soxhlet	Cold maceration	DMSO	TBF (32 µg/ml)
TM	17.33 ± 0.58	14.00 ± 0.00	14.67± 0.58	13.33±1.16	0	34.67 ± 0.58
MC	15.33 ± 0.58	11.67 ± 0.58	16.00±0.00	12.00±0.00	0	39.67 ± 0.58
TR	14.33 ± 0.58	14.00 ± 0.00	16.33±0.58	14.00±0.00	0	32.67 ± 0.58

*Values are mean inhibition zone (mm) ± S.D of triplicate cork borer- 6 mm. DMSO = 10% Dimethylsulfoxide, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum,* TBF = Terbinafine

Table 8. Minimum inhibitory/fungicidal concentrations (%v/v).

Parameter	M.I.C		M.F.C		TBF (µg/ml)
	Soxhlet	Cold maceration	Soxhlet	Cold maceration	
TM	50.00 ± 0.00	50.00 ± 0.00	50.00± 0.00	> 50.00 ± 0.00	< 2.00 ± 0.00
MC	3.13 ± 0.00	12.50 ± 0.00	12.50± 0.00	25.00± 0.00	4.00 ± 0.00
TR	50.00 ± 0.00	25.00 ± 0.00	> 50.00 ± 0.00	>50.00± 0.00	32.00 ± 0.00

*Values are mean inhibition zone (mm) ± S.D of triplicate determination.

compounds and frequency bands of 200-500 cm^{-1} revealed the presence of an alkyl halide.

Antifungal activities of n-hexane extract of *A. indica*

Table 7 shows the results of the susceptibility of the isolates to the undiluted and diluted with DMSO in a ratio of 90 to 10%v/v extracts of *A.indica*. Hexane extract from soxhlet method of extraction had shown higher diameters of zones of inhibition against the isolated dermatophytes. Activity was exerted mostly on *T. mentagrophytes* and *M. canis*. However, the levels of inhibition against the dermatophytes were far less compared those exerted by terbinafine. The level of inhibitory also changed following dilution of the n-hexane extracts with DMSO. The results of the susceptibility of the isolates from soxhlet showed higher activities against isolated dermatophytes.

Minimum Inhibitory Concentration and Minimum Fungicidal Concentration values

The MIC of n-hexane extract of *A. indica* is as shown in Table 8. The MIC against *T. mentagrophytes* and *T. rubrum* was 50%v/v by both extraction methods but *M. canis* was as low as 3.13%v/v (by soxhlet) and 12.5%v/v (by cold maceration). The MFC against *T. mentagrophytes* and *T. rubrum* is 50%v/v and above by both extraction methods while against *M. canis*, it was as low as 12.5%v/v (by Soxhlet and 25%v/v (by cold maceration).

DISCUSSION

Plants have been known to contain bioactive constituents with inhibitory substances against bacteria and fungi (Kadhim et al., 2016). Results from this study reveal a degree of antifungal activities of the plant seed oil which varied from one clinical isolate to another. The gradual increase in the diameter zones of inhibition with increase in concentration of the oil shows that the inhibitory action on the fungi clinical isolates is dependent on the amount of drug used (Table 8). Generally, oil obtained from soxhlet method of extraction had better antifungal activity on *T. rubrum, T. mentagrophytes*, *M. canis.* The zones of inhibition of the oil against all the clinical isolates tested at 100% v/v was comparable to the standard drugs (Terbinafine) used. The antifungal activity of the oil and its fraction can be linked to the presence of secondary metabolites which have been shown to possess bioactive properties. The seed oil which is usually extracted by steam or solvents from crushed seed consists mainly of triglycerides and large amounts of triterpenoids (Takase et al., 2015). The oil extracts and some fractions revealed an antifungal activity against dermatophytes, the classes of compounds present include, alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide and aromatic compounds (Supplementary Figures 1 and 2). The presence of ketone C = O functional group from a five membered cyclic ring and O = C – O – C functional group from an aliphatic esters revealed that gedunin, nimbin, nimbinin and nimbolide constituents are likely present in the oil extracts and its fractions. Thus, these functional groups of ketone, hydroxyl, carboxyl and

aliphatic ester may be responsible for the antidermatophytic activity of neem seed oil. The constituents nimbinin, gedunin, nimbin and nimbolide are terpenoid compounds and a work done by Nuzhat and Vidyasagar (2014) revealed that terpenoid compounds are responsible for antidermatophytic activity.

The predominance of *Trichophyton species* as the causative agent of *Tinea corporis* (Ringworm of the body) is not unexpected. Most studies found *T. rubrum* and *T. mentagrophytes* as the commonest etiological agents of dermatomycosis (Hayette and Sacheli, 2015). *Trichophyton* spp accounted for 76.2% of *T. corporis* in India (Harinath, 2016). *T. mentagrophytes* has been cited as the major causative agent for *T. corporis* and is known to account for as much as 47.6- 69.5% of all dermatophytic infections (Pranab et al., 2003).

The key discovery that has emerged from this research is that the oil extracted from *A. indica* A Juss seeds using both soxhlet and maceration methods of extraction inhibited the growth of clinical isolates of *dermatophytes (T. mentagrophytes*, *T. rubrum* and *M. canis)*. This study has established the *in-vitro* activity of *A. indica* seed oil against dermatophytes isolated from clinical sample. It has also shown that the oil extract can be used to carry out further studies (bioassay guided) so as to isolate lead compounds responsible for such activity and enable its redeployment as potent antidermatopytic agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Figures

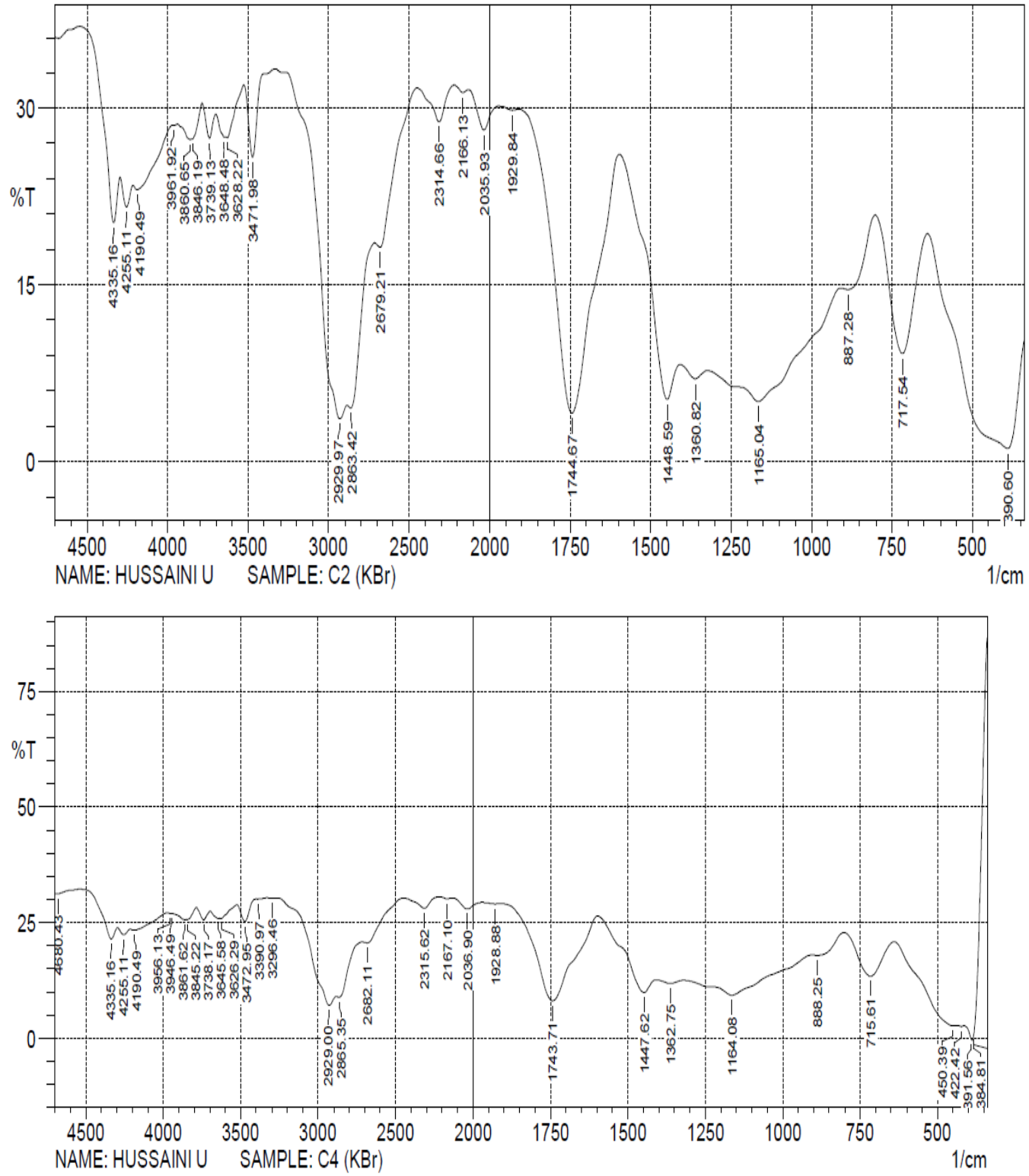

Figure 1. Infra red (IR) absorption spectra of oil fraction having antifungal activity against *T. mentagrophyte*.

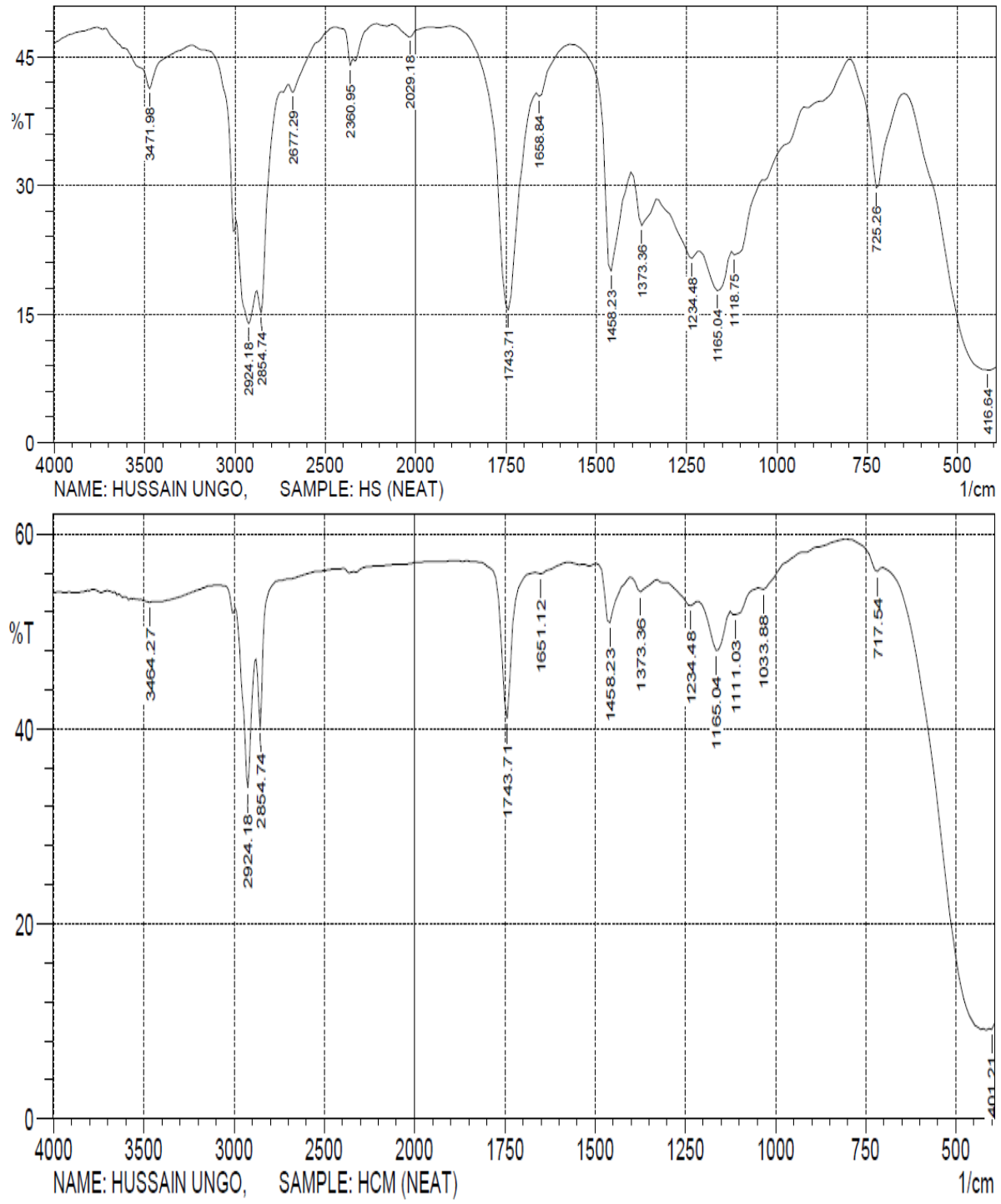

Figure 2. Infra red (IR) absorption spectra of oil extracted from Soxhlet (HS) and maceration (HCM) using hexane.

Vol. 13(26), pp. 430-437, August, 2019
DOI: 10.5897/AJMR2018.8929
Article Number: 730E99C61698
ISSN: 1996-0808

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Optimisation of biological wastewater treatment for yeast processing effluent using cultured bacteria: Application of response surface methodology

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Received 5 July, 2018; Accepted 14 August, 2018

In the present study, the interactive effects of temperature and cultured bacteria on the performance of a biological treatment system of wastewater from a local yeast producing plant were investigated. The main objective of this study was to optimize the operating parameters that reduce organic load and colour. Biological treatment was conducted using a Central Composite Design (CCD) and optimised using Response Surface Methodology (RSM) on Design Expert 7 software. Two dependent variables namely, Chemical Oxygen Demand (COD) removal and colour reduction were studied. COD removal efficiency of 26% and decolourization efficiency of 44% were recorded for the wastewater treatment. The optimised conditions for the biological treatment were found to be at 16.37 g/l of bacterial powder at 25°C for COD removal and colour reduction. The optimised parameters can be used for biological treatment of yeast plant effluent for removal of organic load and colour.

Key words: Cultured bacteria, wastewater, treatment, response surface methodology.

INTRODUCTION

The large quantity of aqueous waste generated by domestic activities and industries has become a significant environmental problem worldwide, due to its harmful nature (Thirugnanasambandham and Sivakumar, 2015). The escalating pollution levels in water bodies has been attributed to massive industrialization and booming population densities coupled by escalating urbanization (Chiuta et al., 2002; Moyo and Mtetwa, 2002; Chakona, 2005; Noukeu et al., 2016). Water bodies serve as recipients of untreated or partially untreated industrial wastewater which in turn alters the physical structures of such water sources. To address surface water pollution and protect eco-systems, wastewater needs to be treated in order to contribute to a cleaner environment (Noukeu et al., 2016). Effluent originating from baker's yeast production process leads to extensive soil and water pollution. The yeast processing industries produce very large amounts of wastewater characterized by high

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biological oxygen demand (BOD_5), Chemical Oxygen Demand (COD) and a dark brown color (Christoforakos and Lazaridis, 2018). Wastewater from baker's yeasts industries contain dissolved organic substances, namely dextrins, organic acids, resins, gums, trimethylglycine, coloured melanoidins and phenol substances. It is also characterised by insoluble organic substances or materials in suspension, high concentrations of total nitrogen, variable phosphorus content and sulphate (Sirbu and Begea, 2011). Some of these coloured compounds are melanoidins formed through a process known as Maillard reaction (Peña et al., 2003). Melanoidins impart a characteristic dark brown colour to water which blocks sunlight penetration thereby impairing the ability of aquatic flora to photosynthesize. In combination with other compounds, this quality of wastewater reduces soil alkalinity, with strong and objectionable odour that presents significant disposal or treatment problem (Agarwal et al., 2010). Elimination of pollutants and colour from the effluent is becoming increasingly important from environmental and aesthetic point of view.

Food-processing industries in Zimbabwe are under increasing pressure to reduce the impact of their wastewater discharge on the environment. With the advent of the Statutory Instrument 6 of 2007 in the Environmental Management Act of 2007 (the basic legislation requirement on pollution control in Zimbabwe), companies have been forced to establish pre-treatment facilities on site. Treatment systems are developed to reduce toxicity of effluent and minimize chances of pollution. Biological treatment systems involve the use of activated sludge, commercial seed granules or more recently mixed culture bacterial formulations for wastewater treatment. Since environmental protection has become a global issue, cleaner and greener technologies are warranted to abate industrial pollution (Zeinu and Sahu, 2015). Readily Fermenting Mixed (RFM) cultured bacteria is a ready-to-use bacterial powder formula made of wheat bran grain-like substance as a substrate, and used for accelerating organic wastewater degradation by commercial industries to reduce pollution of well, underground water sources, and sewage pits. However, the efficacy of these bacterial formulations has not been fully optimised in high strength wastewater treatment.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing the effects on the response of several independent variables (Hadavifar et al., 2010). Optimisation aims at enhancing effectiveness in any stage of response in an experiment for long term implementation of change. Optimisation as a tool enables the increase of efficiency in a process while costs of operation are kept low. A suitable value for the concerned variable has to be determined in order to obtain the maximum yield in an experiment. Myers and Montgomery (2012) suggest that RSM can be employed for optimisation of chemical reactions and in other industrial processes. One of the main objectives of RSM is the determination of the optimum settings of the control variables that result in a maximum (or a minimum) response over a certain region of interest (Korbahti and Tanyolac, 2008). Optimisation of biological wastewater treatment by the classical method involves changing any one of the independent variables while maintaining all others at a fixed level which is extremely time consuming and expensive for a large number of variables. To overcome this difficulty, experimental factorial design and response methodology can be employed to optimize the process.

This research was conducted to reduce COD and colour of the wastewater from a yeast producing plant using the RSM. This involved the optimisation of biological treatment of yeast processing wastewater using a cultured bacterial formulation.

METHODOLOGY

Experimental design

A sample of wastewater was collected from a local yeast processing plant. The wastewater was refrigerated at 4°C to minimize auto-decomposition. RSM in this study was done using Design Expert 7 software. A two-factor Central Composite Design (CCD) was used to design the experiments for determining the effect of variables on color and COD. A set of 13 experiments were produced employing CCD on Design Expert 7. Optimisation was used to assess the outcome of two variables; temperature and bacterial concentration on reduction of COD and colour of yeast processing wastewater.

Wastewater treatment

Batch experiments for biological wastewater treatment were performed in 500-ml serum bottles. Serum bottles were seeded with Readily Fermenting Mixed (RFM) cultured bacteria. The powdered bacterial culture was thoroughly mixed and filtered through a screen of pore size 1 mm before use. The selected parameters were adjusted as per the experimental design and run in a reciprocating water bath shaker incubator (ZWY-110X50, Zhicheng, China) at a prescribed temperature range shown in Table 1.

Analyses

COD was analyzed as per the closed reflux colorimetric method (APHA, 1998). In the COD method, the water sample is oxidized by digesting in a sealed reaction tube with sulphuric acid and potassium dichromate in the presence of a silver sulphate catalyst. Digestion of the samples is carried out in a COD reactor (Model HI 839800, HANNA Instruments, USA). COD measurement was carried out on a Multiparameter Bench photometer (Model 83214, HANNA Instruments, USA). The colour of the sample was measured in terms of the absorbance at $\lambda = 475$ nm using a UV–vis spectrophotometer (Sahu, 2017). The percent colour reduction was calculated using Equation 1:

$$\text{Percent decolourisation} = [A_b - A_a / (A_b)] \times 100\% \quad (1)$$

Table 1. Summary of the experimental design in the biological treatment of yeast processing effluent.

Study type:	Response surface
Initial design:	Central composite
Design model:	Quadratic
Experiments:	13

Factor	Low actual (Code)	High Actual (Code)
A: (RFM) bacterial formulation (g/l)	**4 (-1)**	**20 (+1)**
B: Temperature (%)	**25(-1)**	**45 (+1)**

Response	Observations
Y1: COD reduction (%)	**13**
Y2: Colour (%)	**13**

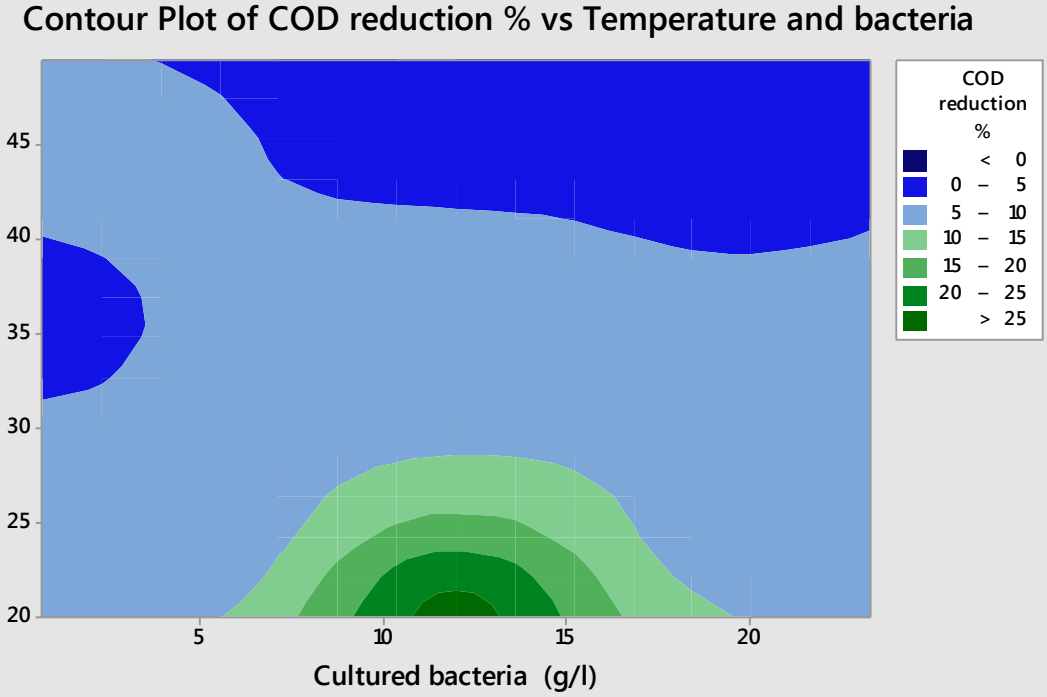

Figure 1. Contour plot of the % COD reduction at prescribed concentrations of RFM cultured bacteria (g/l) and temperature (°C) using Minitab 17.

Where A_b is absorbance of wastewater at 475 nm before degradation and A_a is absorbance at same wavelength after degradation.

Statistical analysis

Using the Design Expert Software 7, the responses from the data were analysed. To evaluate the goodness-of-fit of the mathematical models, analysis of variance (ANOVA) was used in a confidence interval of 95% (Veli et al., 2016). The ANOVA results for all responses were summarized. Contour plots were done using Minitab 17.

RESULTS

COD reduction

Figure 1 shows blue regions indicating lowest percentage of COD reduction while dark green regions indicate highest percentage of COD removal. The maximum COD reduction recorded in this study was 26.2%. The highest percent removal (26.2%) was obtained using cultured bacteria concentration of 12 g/l and temperature 20°C). The lowest activity of the cultured bacteria was noted at

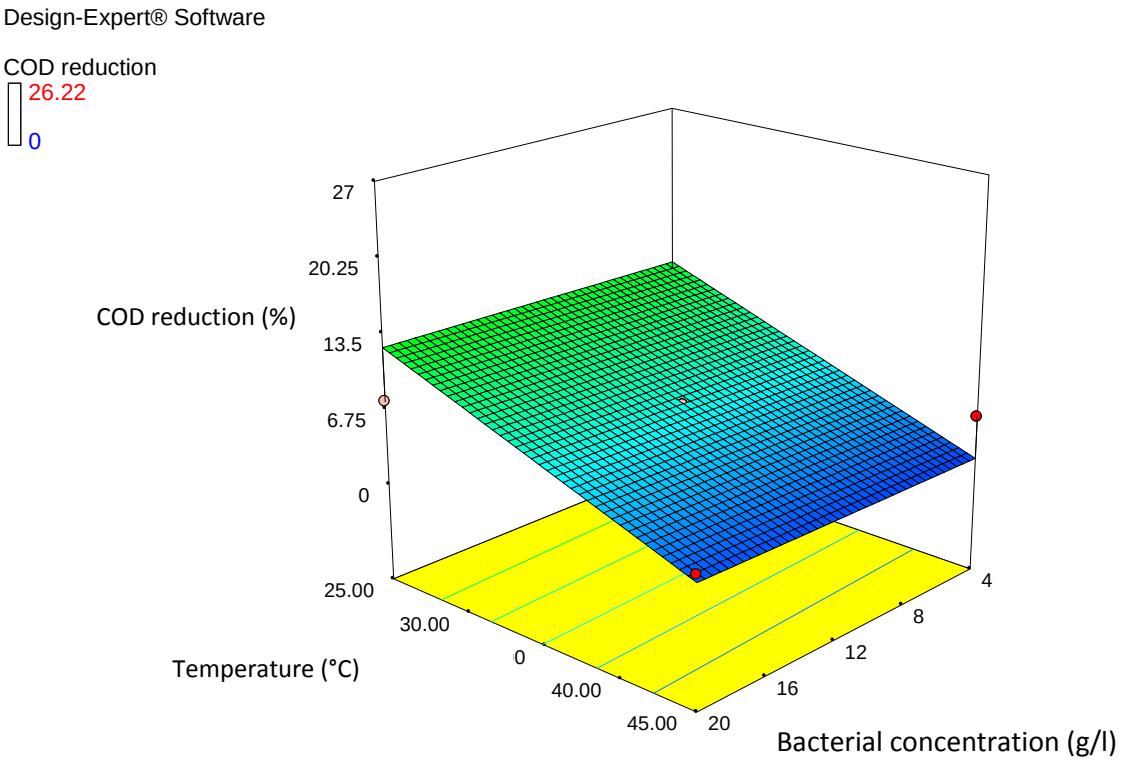

Figure 2. Three-dimensional surface plot showing the outcome in percent COD reduction of different bacterial culture concentration and temperature combinations using a linear model.

the combination of RFM concentration of 12 g/l and temperature 49.4°C where COD reduction was at its minimal.

RSM helps us to obtain graphical response surfaces and contour plots through computations from the developed model regression equations. A three-dimensional (3-D) representation of the outcome under various RFM culture concentrations and temperature effect is noted in Figure 2, with an increasingly higher reduction at lower temperatures and higher cultured bacteria concentrations.

For optimisation using Design Expert 7, the desired maximum COD reduction was set at a target of the maximum COD response. Desirabilities ranged from 0 to 1 for any given response. The program combines individual desirabilities into a single number and then searches for the greatest overall desirability. A value of 1 represents the ideal case. A zero indicates that one or more responses fall outside of the desirable limits. The highest desirability value as shown in Figure 3 was 0.634 at 16.37 g/l of cultured bacteria and 25°C with the lowest desirability value of 0.106.

The Model F-Value of 4.66 implies the model is significant. There is only a 3.71% chance that a "Model F-Value" this large could occur due to noise. Concluding from the analysis in Tables 2 and 3, values of "Prob > F" less than 0.0500 indicate model terms are significant.

Colour reduction

The main purpose of using RFM bacteria for treating industrial yeast processing wastewater was to reduce COD and to decolorize the wastewater. Baker's yeast production industry is one of the most polluting industries, which generates large volumes of high strength, bad smell, and dark brown color wastewater (Pirsaheb et al., 2015). Coincidentally, maximum experimental decolourization of 44% was obtained at 12 g/l cultured bacteria concentration and 20°C temperature as shown in Figure 4, which was similar to the parameter levels observed for the COD reduction. However, reduction of wastewater decolourisation was noted at temperature above 45°C. This might be attributed to heat effect rather than the RFM bacteria concentration and their enzymes. The enzymes as biological systems are denatured by heat. Therefore, complete removal of melanoidins that impart a dark colour to the wastewater was made difficult.

Figure 5 is the 3-D surface plot showing the relationship between bacteria culture formulation and temperature in colour reduction. Evidently, there is low colour reduction at extreme temperatures used in this study, that is, 45 and 25°C. The response surface plot has a clear peak which suggested that the optimum condition fell well inside the design boundary (Han et al., 2012). At optimum temperatures in the range of 35°C,

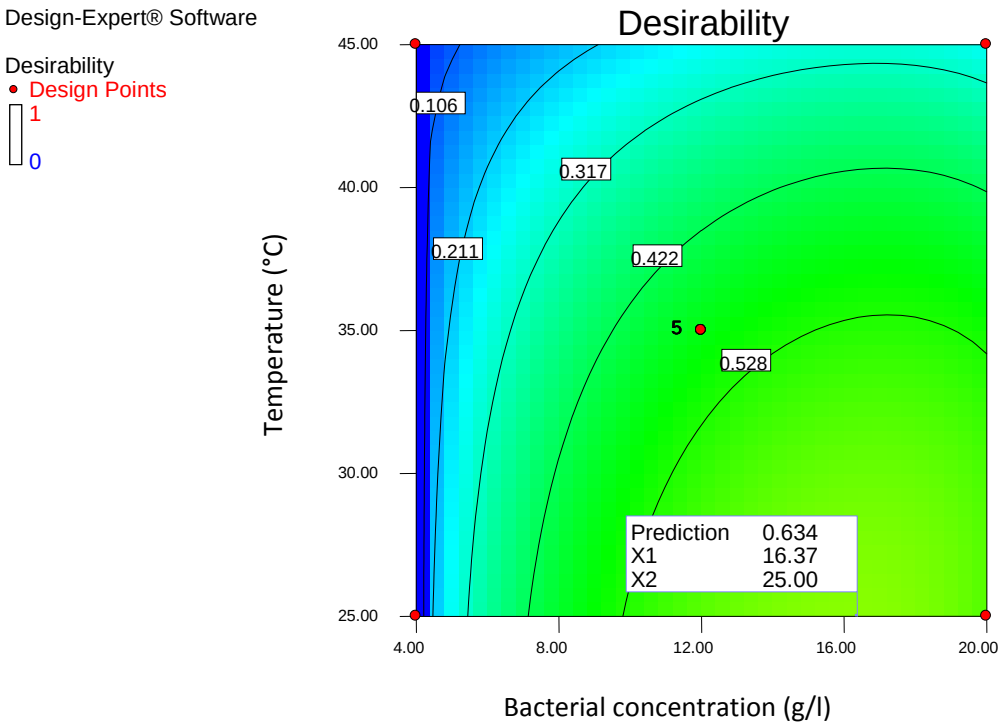

Figure 3. A summary of desirability showing the optimum temperature and RFM culture concentration for COD reduction.

Table 2. ANOVA table for linear model using design expert 7 for COD reduction.

Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	218.68	2	109.34	4.66	0.0371 sig.
A-Bacterial formulation	0.48	1	0.48	0.021	0.8889
B-Temperature	218.20	1	218.20	9.30	0.0122
Residual	234.51	10	23.45		
Lack of Fit	234.51	6	39.09		
Pure Error	0.000	4	0.000		
Corrected Total	453.19	12			

Table 3. ANOVA for Response Surface Quadratic Model using Design Expert 7 for colour reduction.

Source	Sum of squares	df	Mean Square	F Value	p-value Prob > F
Model	1987.42	5	397.48	4.95	0.0295 sig.
A-Bacterial formulation	31.36	1	31.36	*0.39*	*0.5518*
B-Temperature	484.00	1	484.00	*6.03*	*0.0438*
AB	64.00	1	64.00	*0.80*	*0.4016*
A^2	1216.70	1	1216.70	*15.16*	*0.0060*
B^2	333.60	1	333.60	*4.16*	*0.0809*
Residual	561.94	7	80.28		

maximum reduction in colour is obtained, giving a typical concave 3-D graph shown in Figure 5.

Desirability values range from values of 0 to 1 since it is a probability of occurrence. The aim is to select values

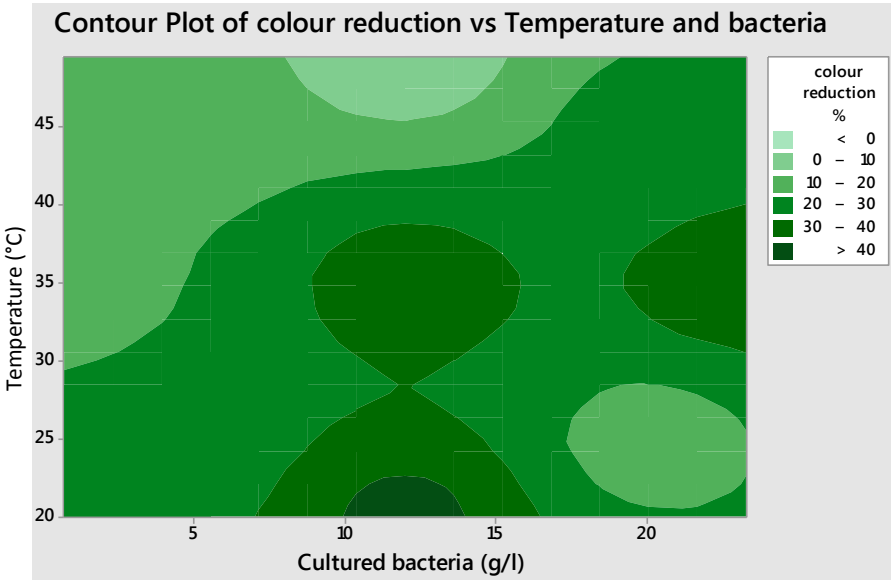

Figure 4. Contour plot showing colour variations to highlight difference in colour reduction at different RFM concentration (g/l), temperature (°C) combinations.

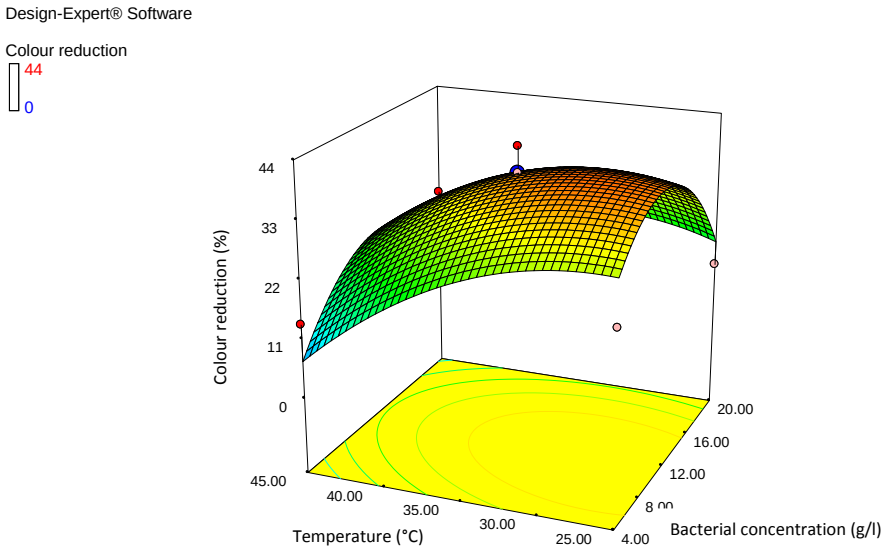

Figure 5. Three-dimensional surface plot showing the outcome in percentage colour reduction at different bacterial concentrations (g/l) and temperature (˚C) combinations.

with the highest desirability for optimisation. Design-Expert 7 software sorts the results from most desirable to least. Therefore, in Figure 6 the highest prediction value of desired colour reduction that can be obtained is 0.634 at 16.37 g/l and 25°C.

The Model F-Value of 4.95 implies the model is significant. There is only a 2.95% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, B and A^2 are significant model terms.

DISCUSSION

COD reduction after treatment of yeast processing wastewater

The maximum COD reduction recorded in this study was 26% (Figure 1). This was very minimal reduction, showing that biological treatment cannot be used as a sole wastewater treatment system. Elsewhere, it has been recorded that conventional anaerobic and aerobic treatment could accomplish degradation of the

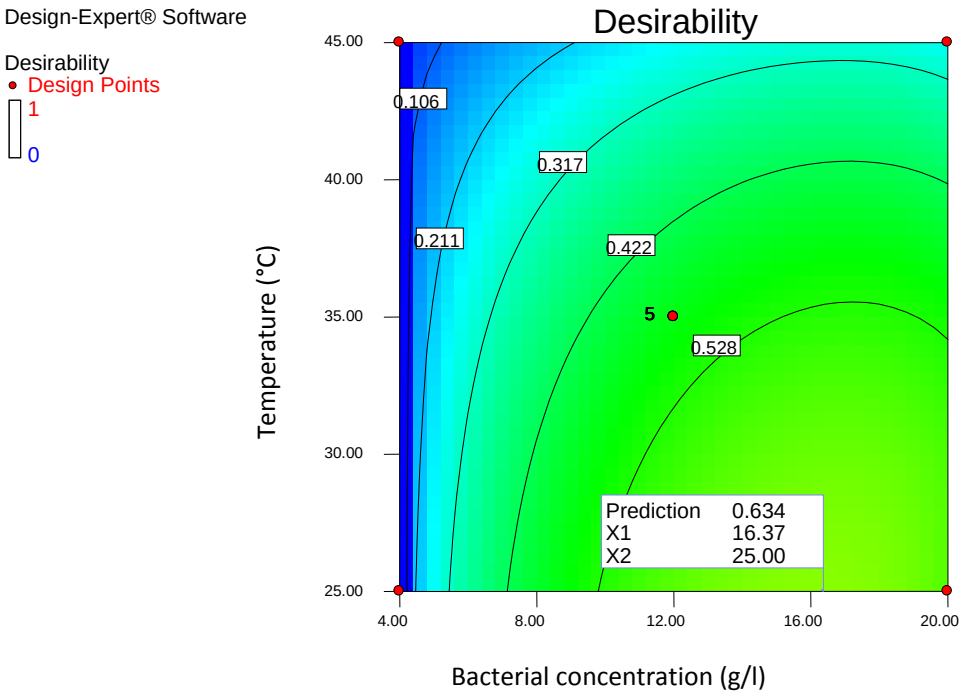

Figure 6. Desirability values for optimising the bacterial concentration (g/l) and temperature (°C) and their effect on percentage colour reduction.

melanoidins up to only about 6-7% (Agarwal et al., 2010; Onyango et al., 2012). Yeast processing wastewater contains melanoidins which are known to be lethal to many microorganisms because of their antioxidant properties (Kumar, 1997). Absence of COD reduction at 49.4°C with RFM culture of 12 g/l can be a result of recalcitrant melanoidins which limit growth of bacteria. As shown in Figure 2, COD removal efficiency increases with increase in RFM dose, since metabolic activities leading to pollutant removal are increased. However, high temperatures are known to encourage repolymerization of melanoidins and in the process inactivating bacteria. Generally, biotreatment of melanoidins containing wastewater has not given impressive results and post treatment is necessary. In a related study, post-treatment using coagulation step and ozonation impacted on the biologically quality of the wastewater effluent, resulting in the reduction of COD by 30–49% (Zub, 2007).

Colour reduction after treatment of yeast processing wastewater

The decolourization pattern indicated that the degradation of melanoidins was highly temperature dependent. In Figure 4, a contour plot showed the highest colour reduction of 44%. Melanoidins removal by micro-organisms can occur through enzymatic degradation, utilizing the pigments as carbon and nitrogen sources as noted elsewhere (Bezuneh, 2016). Various forms of intracellular and extracellular enzymes such as laccases, manganese peroxidases, lignin peroxidase, sugar oxidases such as sorbose oxidase have been reported to show melanoidins degradation activity (Couto et al., 2005; Freitas et al., 2009).

There was a notable increase in intensity of the wastewater colour when COD removal was at its lowest. The reduction in decolourization might be due to the fact that melanoidins stability varies with pH and temperature as additional parameters. This might be due to the fact that at higher temperatures some bacterial proteins are denatured; therefore removal of melanoidins which are responsible for dark colour in yeast processing wastewater is made difficult. After conventional biological treatment, most of the organic load is removed but nevertheless, the dark brown colour still persists and it can even increase due to repolymerization of coloured compounds (Jiranuntipona et al., 2009).

Process optimisation for COD reduction

In an effort to optimize this process, the Design Expert 7 software provided selection of the desired goal for each factor and response from the menu with 5 possible goals (maximizing, minimizing, target within range, none (for responses only) and set to an exact value (factors only). A minimum and maximum level is to be provided for each

parameter included in the Optimisation. The maximum COD reduction was obtained at a temperature of 25°C and 16.37 g/l of bacterial culture. The obtained desirability value of 0.634 demonstrates that the estimated regression function may represent the experimental model and desired conditions satisfactorily.

Conclusion

It has been shown that biological treatment of wastewater from a yeast producing plant is a suitable process for the removal of organic load and colour from wastewater, especially when the operating parameters are optimized as confirmed. However, due to the recalcitrant nature of the complex compounds in the effluent the treatment efficiencies are generally low.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the local yeast manufacturing plant personnel for their support and allowing this research to proceed using their wastewater. The authors also appreciate Midlands State University for the financial and technical assistance to carry out this project.

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Vol. 13(26), pp. 438-445, August, 2019
DOI: 10.5897/AJMR2019.9150
Article Number: BD8668661735
ISSN: 1996-0808

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Chemical analysis of the biomass of a native strain of *Spirulina subsalsa* Oersted ex Gomont 1892 (Spirulinaceae) cultivated in low-cost saline medium

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Received 23 May, 2019; Accepted 19 July, 2019.

Spirulina subsalsa, **a filamentous cyanobacterium, was first described by Gomont in 1892. This microorganism has been subject to biotechnological evaluations, due to their high content of proteins and pigments. The objective of this study was to analyze the biochemical composition of the biomass of a native strain of *S. subsalsa* cultivated in low-cost saline medium and harvested in the exponential and stationary phases of growth. The highest protein contents (58.5%) were obtained in the exponential phase; while the highest amounts of carbohydrates (20%), lipids (19.7%), chlorophyll (51.6 µg/ml), total carotenoids (218,215 µg/ml), exopolysaccharides (7.30 ± 0.7 mg/ml) and phycocyanin (25.8 µg/ml) were accumulated in the stationary phase. Additionally, in the biomass of *S. subsalsa*, the presence of saponins and polyphenols was detected in both growth phases, whereas basic alkaloids and flavonoids were detected only in the stationary phase. This article concludes information on the potential future biotechnological applications of the cyanobacterium strain, *S. subsalsa.***

Key words: Cyanobacterium, biotechnology, *Spirulina subsalsa*.

INTRODUCTION

Spirulina subsalsa Oersted ex Gomont is a filamentous cyanobacteria originally described by Gomont (1892, 1893). This microorganism inhabits saline and fresh waters all over the world (Szulbert et al., 2018). In Venezuela, Spirulina has been reported by Rodriguez (2001), Bernal (2002), González et al. (2003) and Petrash et al. (2012).

This cyanobacterium forms mantles on the substrate, usually blue-green in color and has sometimes been observed to be part of the cyanobacteria blooms that cause poisoning in flamingos (Ballot et al., 2004) and shrimp (Lightner, 1978); however, there is no evidence that this cyanobacterium produces any cyanotoxin.

The biotechnological potential of *S. subsalsa* has been little studied, being used as a bioremediator agent of residual contaminants (Jiang et al., 2015), biosensor for

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the evaluation of the toxicity of estuarine waters (Campanella et al., 2001), and producing bioactive metabolites (Mazur-Marzec et al., 2015). In addition, *S. subsalsa* is a source of polyhydroxyalkanoates (PHA), which are biopolymers for construction of implants and artificial tissues (Shrivastav et al., 2010).

Spirulina cultures are usually carried out in fresh water and need expensive culture media, due to the inclusion of a large number of analytical grade salts. Between these means, Zarrouk medium was emphasized (Zarrouk, 1966), *Spirulina* (Aiba and Ogawa, 1977), BG-11 (Rippka, 1988), and some modified media (Amala and Ramanathan, 2013; Kumari et al., 2014a, b). This situation has led to the search for alternative sources of culture media that allow obtaining high yields of biomass at low cost. Furthermore, it is necessary to evaluate new strains of this cyanobacterium, since it has been demonstrated that the responses of microalgae to changes in abiotic factors vary considerably from one species to another, between strains of the same species and even between clones originating from the same unialgal culture, which would be due to morphological and physiological differences, attributable to intraspecific genetic variations (Gómez and González, 2005; Guevara et al., 2016).

The objective of this investigation was to analyze the biomass of a native strain of *S. subsalsa* cultivated in low-cost saline medium and harvested in exponential and stationary phases of growth.

MATERIALS AND METHODS

A native strain of S. subsalsa, isolated from the Clavellino Reservoir, Sucre State, Venezuela (coordinates: between 10° 19 'to 10° 23' Lat. N and between 63° 35 'to 63° 40' Long. O) and deposited in the Algae Germplasm Bank of the Oceanographic Institute of Venezuela, Universidad de Oriente, with the code BGAUDO 161, was cultivated in seawater (9‰) previously treated, according to the methodology of Faucher et al. (1979).

The cultures were carried out in quadruplicate, discontinuously, for 30 days, in 45 cm diameter plastic bags, placed in cylindrical metal frames (Figure 1), containing 100 L of culture medium each with a nitrate concentration of 14 mM, 0.036 mM phosphate, 95.23 mM sodium bicarbonate, 0.0013 mM Fe and 0.0009 mM Mn. The bags were located in a controlled laboratory environment (T: 32 ± 1°C, continuous irradiance of 39 µmol/m^2/s provided by 3 white light lamps of 40 W and photoperiod 12:12) and aerated with plastic hoses and diffuser stones. The salinity and nitrate concentration used were selected according to results in previous experiments (Romero et al., 2018).

The cultures were started with inocula previously acclimated to the mentioned environmental conditions. From the beginning of the test and every 48 h, samples were taken from each of the replicas to determine the pH and population growth according to the criteria of Pelizer and Oliveira (2014).

When the culture reached the exponential phase (2 replicas) and stationary (2 replicas), the entire culture was harvested, filtering it in permaline sleeve. The filtrate was used to quantify the exopolysaccharide content according to the methodology of Vicente et al. (2004). The harvested biomass, after several washes with acidulated water (pH 4), was maintained at low temperatures (-20°C) until the moment of realization, in triplicate, the protein analysis, according to Lowry et al. (1951); total lipids, according to Bligh and Dyer (1959) and Pande et al. (1963); carbohydrates, according to Dubois et al. (1956); secondary metabolites according to Domínguez (1973) and Marcano and Hasegawa (2002), and pigments according to Sharma et al. (2014) and Murugan and Rajesh (2014).

Analysis of the results

The data of the values of exopolysaccharides, proteins, lipids, carbohydrates, and *Spirulina* pigments obtained in the exponential and stationary growth phases were contrasted by a one-way analysis of variance (phases), following recommendations of Sokal and Rolhf (1995).

RESULTS

Growth and pH

Figure 2 shows the population growth of the microalgae *S. subsalsa* in the low-cost culture medium during the 30 days of the trial. It is observed that during the first 6 days, this microalga was in adaptation phase; after which, the culture entered the exponential growth phase till day 12. Followed by and until the end of the trial, the culture remained in the stationary phase, and no signs of a descent phase were observed. The pH of the cultures was between 9 and 10.2.

Expolisaccharides

The exopolysaccharide content obtained in the *S. subsalsa* cultures presented significant differences ($p < 0.05$) between the growth phases (Figure 3). The concentration of these exocompounds was 7.30 ± 0.7 and 5.4 ± 0.4 mg/ml on stationary and exponential phase.

Proteins, carbohydrates, lipids and pigments

The contents of proteins, carbohydrates, lipids and pigments of *S. subsalsa* cultivated in a low-cost saline medium are shown in Table 1. Total proteins showed significant differences ($p < 0.05$) between the phases, reaching their highest contents in the exponential phase (58.5 ± 0.58%). The rest of the analyzed compounds, like the proteins, showed significant differences between the phases ($p < 0.05$), but with the difference that their highest values were obtained in the stationary phase. In this way, carbohydrates, lipids, chlorophyll, phycocyanin and total carotenoids had percentages of 20.0 ± 2.71%, 19.7 ± 1.41%, 51.6 ± 0.64 µg/ml, 25.8 ± 0.40 µg/ml and 218.215 ± 2.27 µg/ml, respectively.

Table 1. Content of proteins, carbohydrates, lipids and pigments of *S. subsalsa*.

Content	Exponential phase	Stationary phase
Proteins (%)	58.5 ± 0.58[a]	41.37 ± 0.90[b]
Carbohydrates (%)	17.1 ± 0.95[a]	20.0 ± 2.71[b]
Lipids (%)	14.1 ± 0.20[a]	19.7 ± 1.41[b]
Pigments (µg/ml)		
Chlorophyll *a*	42.7 ± 0.40[a]	51.6 ± 0.64[b]
Total carotenoids	157.9 ± 2.36[a]	218.215 ± 2.27[b]
Phycocyanin	20.2 ± 1.05[a]	25.8 ± 0.40[b]

[a, b]Different letters between rows denote significant differences ($p < 0.05$).

Table 2. Secondary metabolites in *Spirulina subsalsa* grown in low-cost saline medium and harvested during the phases of exponential and stationary growth.

Secondary metabolite	Crop phase	
	Exponential	Stationary
Saponins	+	+
Alkaloids (basic)	-	+
Anthraquinones	-	-
Flavonoids	-	+
Phenolic compounds (polyphenols)	+	+
Phenolic compounds (tannins)	-	-
Cyanogenic glycosides	-	-
Cardiotonic glycosides	-	-
Pentacyclic triterpenes	-	-
Unsaturated sterols	-	-

Secondary metabolites

As shown in Table 2, the presence of saponins and polyphenols in the fresh biomass of *S. subsalsa* was positive in both phases of growth; however, basic alkaloids and flavonoids were only evidenced in the stationary phase (Table 2).

DISCUSSION

The population growth observed in *S. subsalsa* in this study is related to the results presented by Rodríguez and Triana (2006), who indicated that in the *Spirulina* species, the adaptation phase usually lasts between zero and four days, because the microalga is coupled to the culture conditions and has a low specific growth rate. From there, the growth of the microalga gradually increases, entering the phase of exponential growth, where cell multiplication is at its maximum. This phase continues until it reaches its maximum value (days 12-16), where depletion of nutrients has been observed, hence a decrease in growth. The stationary phase begins, due to the decrease in the rate of growth, increased cellular respiration and accumulation of enhancement of toxic wastes. At this point, it is important to take care of the cultivation conditions to extend the phase and avoid unfavorable conditions that might cause the death of the cells (death phase). In the present study, death of the cells did not occur during the present test.

The amount of maximum biomass obtained in this study was 3.1 mg/ml. This biomass value is higher than those reported by Oliveira et al. (1999), where they determined an amount of 2.4 mg/ml at 30°C, in *Spirulina platensis* and *Spirulina maxima*. This difference may be due to the temperatures used for the culture, in this work the maximum temperature recorded was 32 ± 1°C.

Volkmann et al. (2008) and Licet et al. (2014) obtained higher biomass than those achieved in this research when cultivating *Arthrospira platensis* viz. 4.95 and 3.5 mg/ml, respectively. This difference may be due to the fact that the previous authors used different culture conditions, among these are the irradiance (140 and 390 µmol/m^2/s, respectively), which were greater than those

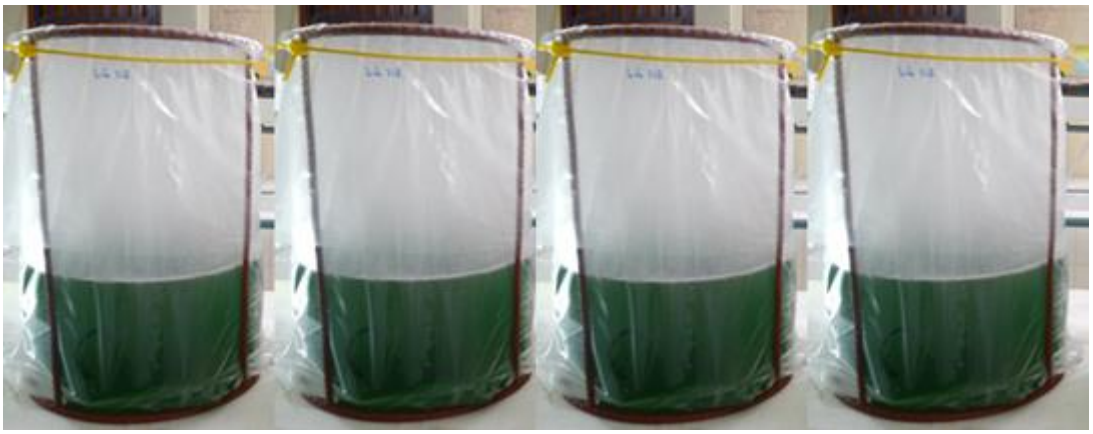

Figure 1. Cultivation of *S. subsalsa* in 45 cm diameter plastic bags, placed in cylindrical metal frames, containing 100 L each of culture medium.

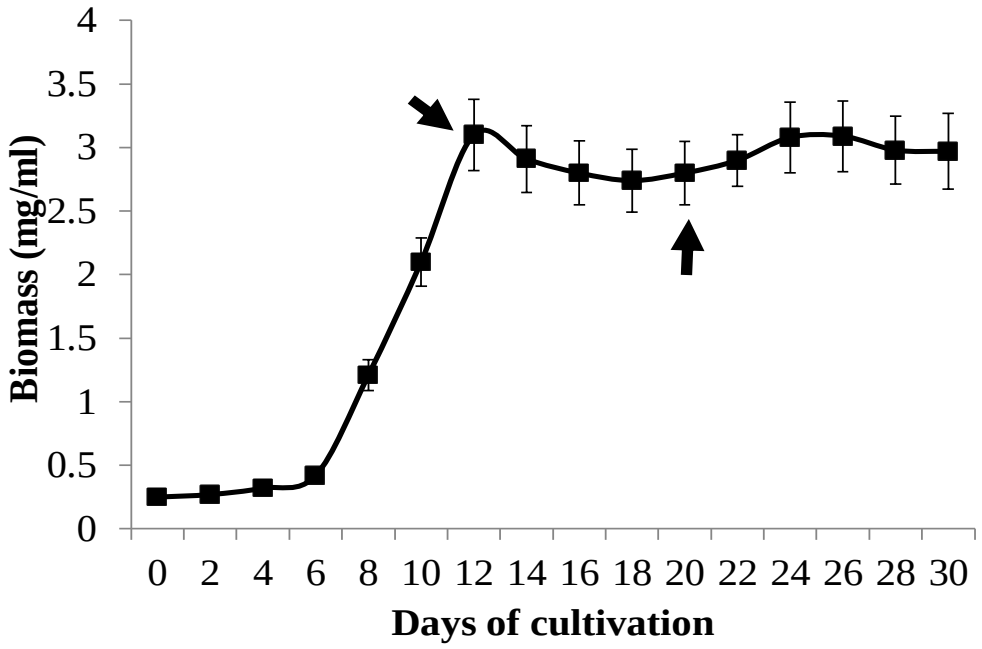

Figure 2. Population growth of *S. subsalsa*. The arrows indicate the days of harvest (exponential at 12 days and stationary at 20 days).

implemented in this research (39 µmol/m^2/s).

The pH of the cultures remained between 9 and 10.2, which is within the values reported for this cyanobacterium, according to the criteria of Rincón et al. (2013).

Several studies have reviewed the ability of cyanobacteria to adapt to variations in salinity (Thajuddin and Subramanian, 2005; Nagle et al., 2010; Joset et al., 1996), but not all cyanobacteria are halotolerant (Blumwald and Tel-Or, 1982). The ability of cyanobacteria to grow at high concentrations of Na^+ may be related to their ability to regulate respiration (Gabbay-Azaria et al., 1992), the flow of Na^+ (Molitor et al., 1986) and the production of osmolytic compounds (Reed et al., 1986), which help the cells to withstand the pressure caused by the large amount of sodium ions present in the medium. One of these compounds are the exopolysaccharides, which are exuded into the environment, as an osmoprotective effect.

The higher content of exopolysaccharides in the

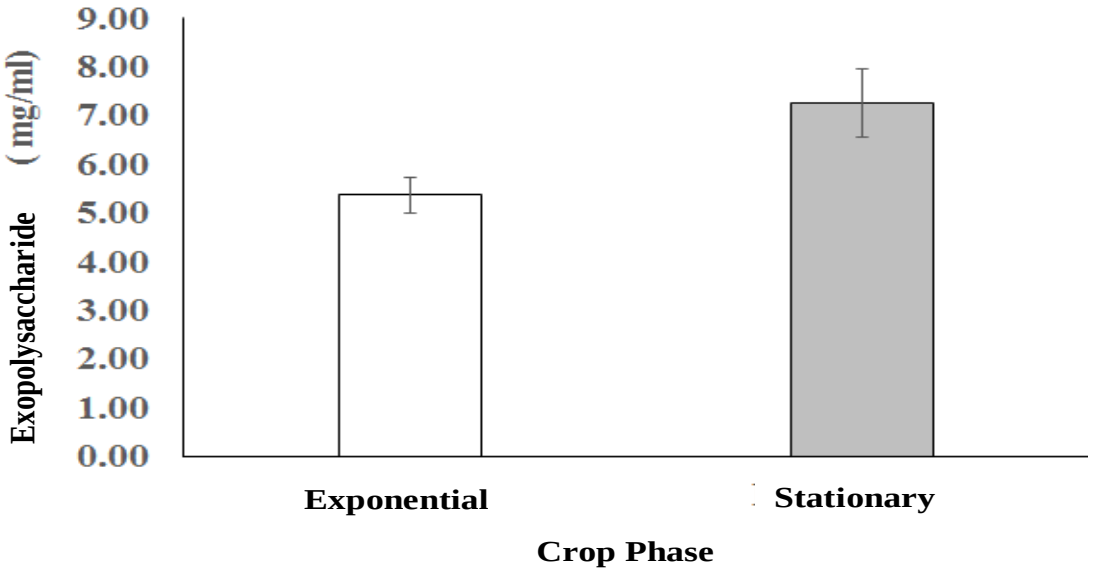

Figure 3. Exopolysaccharide content (mg/ml) of a native strain of *S. subsalsa*, cultured in a low-cost saline medium and harvested in the phases of exponential and stationary growth.

stationary phase may be due to the deficiency of nitrogen that occurs in this phase, as indicated by De Philippis et al. (1993) and Otero and Vincenzini (2003). This situation probably contributes to the increase in the C: N ratio, which promotes the incorporation of carbon in polymers (Otero and Vin-cenzini, 2003; Kumar et al., 2007).

The higher contents of exopolysaccharides together with the growth of the cyanobacterium and increase in the pH of the medium, limit the availability of light, which leads to an increase in the content of accessory pigments and phycobiliproteins, thus reducing the phosphorus and nitrogen content, and subsequently the redirection of the cellular metabolism towards the synthesis of carbohydrates (Laloknam et al., 2010; Magro et al., 2018).

Although the characterization of the obtained exopolysaccharides was not satisfied in the development of this work, some authors have managed to isolate and identify some sulphated type of *Spirulina* polysaccharides, called spirulan calcium Ca-SP, in which antiviral (*in vitro* and *ex vitro*) microbiological tests has inhibited the replication of HIV, Herpes simplex, human cytomegalovirus, influenza A virus, mumps and measles (Chamorro et al., 2002). *In vitro* studies suggest that the polysaccharides, unique to *Spirulina*, improve the enzymatic activity of the cell nucleus and the synthesis and repair of DNA (Premkumar et al., 2004).

The highest total protein contents of *S. subsalsa*, cultivated in low-cost saline medium, were obtained in the exponential phase (58.5%). These results may be due to the fact that in this phase, the culture medium did not present nutrient limitations, which favors protein synthesis. In addition, the salinity used in crops does not represent extreme stress levels that can interfere with protein accumulation.

Andrade et al. (2018) have observed protein content in *Spirulina* between 50 and 70%. These differences in biochemical composition, including proteins, are attributed to the variation between genera and species, and in the culture conditions (availability of nutrients, pH, light, temperature) of a particular species (Colla et al., 2007).

The highest contents of lipids (19%) and carbohydrates (20%) were observed in the stationary phase; this could be due to the fact that in this phase, the supply of nutrients usually decreases and the irradiance received by the culture becomes less, motivated by the overshadowing caused by the massive growth of this cyanobacterium, which have been referred to as stimulants of the accumulation of lipids and carbohydrates (Möllers et al., 2014).

Similar to carbohydrates and lipids, the pigment contents showed their highest values in the stationary phase. Chlorophyll *a* reached contents of 51.6 µg/ml and total carotenoids of 218.215 µg/ml. These results differ from that reported by Marrez et al. (2013), who obtained values of chlorophyll *a* and total carotenoids of 147.43 and 139.88 µg/ml, respectively for *S. platensis*. The discrepancies may be due to the dissimilarity of the salinities, since 9‰ was used in the present investigation and the mentioned authors cultivated salinities of 4.83‰.

Senthilkumar and Jeyachandran (2006) reported that the cultivation of cyanobacteria with high salt concentrations significantly affects the chlorophyll content. The results of Ayachi et al. (2007) supports this, who

observed that the inhibition of chlorophyll synthesis under salt stress is due to a decrease in the energy level caused by the pumping of sodium ions entering the cell, and that also causes a significant inhibition of the chain of electron transport and transport of electrons in the photosystem (PS-II), due to damage in the PS-II reaction center and alterations in the water oxidation complex (Pulz and Gross, 2004).

The highest values of phycocyanin were 25.8 µg/ml, which is lower than those reported (55.37 µg ml^{-1}) by Marrez et al. (2013) obtained in *S. platensis* and cultivated in SHU medium. It is evident here that the composition of the culture medium exerts influence on the chemical composition of cyanobacteria (Marrez et al., 2014). The optimization of the culture conditions to maximize the accumulation of phycocyanin is due to the fact that this compound is indicated as being responsible for the antioxidant activity of this cyanobacterium (Ahmed et al., 2014).

The presence of saponins and flavonoids in both phases of cultivation, and basic alkaloids and flavonoids in the stationary phase, coincides with that reported by Borowitzka (1995), who proposes that almost all biologically active compounds of interest are secondary metabolites, thereby tending to be more abundant in the stationary phase or in slow-growing crops.

Some reports show that microalgae and cyanobacteria can contain many kinds of phenolic compounds, such as flavonoids (Klejdus et al., 2010). Hamouda and Doumandji (2017) performed the phytochemical analysis of *S. platensis*, testing with some solvents: acetone, methanol, ether, dichloromethane and hexane, and found the presence of flavonoids, phenolic compounds, alkaloids and cardiac glycosides.

Although no calculations were made to estimate the production costs of *S. subsalsa* with the culture medium used in this research, it can be inferred that this medium is less expensive, since it only has 5 commercial grade salts, while the zarrouk medium, the most widely used in the cultivation of *Spirulina*, has 21 analytical grade salts, with which 1000 L of medium can be prepared at a price of US$ 79.5 (Raoof et al., 2006).

The results obtained on the growth, as well as the contents of proteins, lipids, carbohydrates and pigments in the native strain of *S. subsalsa* when cultivated in low-cost saline medium, permit us to suggest the use of this cyanobacterium in the biotechnological industries with a view to their use as food in aquaculture and in humans, making it necessary to specify the degree of toxicity, since some strains can be toxic in certain culture conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 13(26), pp. 446-456, August, 2019
DOI: 10.5897/AJMR2019.9175
Article Number: 20B31B761776
ISSN: 1996-0808

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Antibiotic resistant phenotypes of *Staphylococcus aureus* isolated from fresh and fermented milk in parts of Nasarawa State, Nigeria

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Received 22 July, 2019; Accepted 20 August, 2019.

This work was aimed at determining the occurrence and antibiogram of *Staphylococcus aureus* isolated from fresh and fermented milk samples in parts of Nasarawa State, Nigeria. A total of 180 samples comprising of fresh raw milk, bulk milk, *nono*, and *kindirmo* were collected over a period of 6 months (May to October, 2017). Standard microbiological procedures were employed in the isolation, identification, characterisation, and determination of the antibiogram of *S. aureus* from the milk samples. Characterisation of the *S. aureus* isolates was by morphological, biochemical characteristics using conventional methods, Microgen® STAPH-ID kits. Confirmed isolates were tested for susceptibility or resistance to a panel of 11 commonly used antibiotics using the agar disc diffusion technique. Out of the 180 milk samples examined, 9 *S. aureus* were isolated giving a prevalence of 5.0%. The occurrence of *S. aureus* was higher in *nono* (12.1%) and *kindirmo* (10.6%) than in fresh raw milk (5.9%). The high occurrence of *S. aureus* in *nono* disproved the assertion that fermented foods are not good media for the survival and growth of *S. aureus*. The antibiotic susceptibility profile of the *S. aureus* isolates indicated all of the nine isolates were completely resistant to cefoxitin, ampicillin, and amoxicillin/clavulanic acid. The isolates were moderately resistant to erythromycin (22.2%), sulphamethoxazole/trimethoprim (22.2%), and tetracycline (44.4%). Five antibiotic resistance patterns were recorded among the isolates. All of the isolates had a multiple antibiotics resistance (MAR) index of 0.3 and above, an indication of possible antibiotic misuse in the areas studied.

Key words: Milk, *Staphylococcus aureus*, antibiotic resistant phenotypes, Nasarawa State, Nigeria.

INTRODUCTION

Raw milk and fermented milk of various types are produced and consumed as supplement to normal meals in homes and even for sale (Maduka et al., 2013). Traditionally produced dairy products especially those

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produced from raw milk under neglected hygienic conditions, are potential vehicles for the transmission of different foodborne pathogens especially toxigenic *Staphylococcus aureus* (Kadariya et al., 2014). *S. aureus*, including those associated with animals have been frequently recovered from raw milk and milk products worldwide (Peton and Le Loir, 2014). Despite the fundamental roles that milk and milk products play in human nutrition, they serve as vehicles for the transmission of many bacterial pathogens to man. For example, in Europe, milk and other dairy products are found to be responsible for 5% of staphylococcal outbreaks (Bianchi et al., 2014). Health risk to consumers can be associated with milk due to the presence of zoonotic pathogens and antimicrobial drugs residues (Vyletělová et al., 2011). Milk quality can be lowered by a number of factors such as contamination during and after milking, and the presence of udder infections. Pathogenic microorganisms in milk can be derived from the cow itself, the human hand, or the environment (Esron et al., 2005).

Antibiotic usage has become common place in human medicine and animal production. The extensive use of antibiotics in both human medicine and agriculture particularly in disease prevention and growth enhancement in animal production is a considerable cause of the selection and prevalence of antibiotic resistant microorganisms (Jamali et al., 2015). The use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacterial pathogens and transmission of resistance bacteria to humans via the food chain (Jamali et al., 2015).

Food contamination with antibiotic resistant pathogens poses a major public health threat as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance (Jamali et al., 2014). Contamination of food products with microorganisms influence considerably, the safety of the products, endanger the health of consumers, lower their shelf life resulting in foodborne infections, intoxications, and economic losses due to food spoilage (Shiferaw and Ahmad, 2016). Milk is considered as a good substrate on which *S. aureus* grow and produce enterotoxins (Korpysa-Dzirba and Osek, 2011). *S. aureus* has been reported as one of the most common causative agents of food poisoning associated with the consumption of raw milk and milk products (Spanu et al., 2012). Contamination of food stuff occurs directly from infected food-producing animals or may result from poor hygiene during production processes or the storage and retail of food since humans also harbours microorganisms (Vázqnez-Sánchez et al., 2012).

The hygienic standard of milk may be assessed based on the level of contamination with *S. aureus* and studying the antibiotic resistant phenotypes of this bacterial pathogen will provide valuable information on antibiotic usage and infection control strategy in animal production. Considering the aforementioned points, this study was conducted with the aim of isolating, characterizing and determining the antibiotic resistant patterns of *S. aureus* from fresh and fermented milk samples in parts of Nasarawa State, Nigeria.

MATERIALS AND METHODS

Study area

This study was carried out in Nasarawa State, Nigeria. Two Local Government Areas were selected in the three Senatorial Zones in the State viz: Akwanga and Wamba (Nasarawa North); Lafia and Doma (Nasarawa South); Keffi and Nasarawa (Nasarawa West) (Plate 2). Nasarawa State is situated between latitude 70^0 40 and 90^0 40, and longitude 70^0 0 and 90^0 30. The State has thirteen Local Government Areas and is bounded to the north by Kaduna State, Plateau State to the northeast, Taraba State to the southeast, Benue State to the south, Kogi State to the southwest, and the Federal Capital Territory (FCT), Abuja, to the west (Plate 1). These positions were taken using Taiwan-made Etrex® high sensitive Geographic Positioning System (GPS) receiver.

Study design

Sample size

The sample size was determined using the 12.6% prevalence of *S. aureus* as reported by Umaru et al. (2013). The sample size was determined by using the equation described by Naing et al. (2006):

$$n = \frac{Z^2 P (1-P)}{d^2}$$

Where n is the sample size;
P is the prevalence from a previous study = 12.6% = 0.126;
Z is the standard normal distribution at 95% confidence interval = 1.96;
d is the absolute desired precision at 5% = 0.05.

Therefore,

$$n = \frac{(1.96)^2 \times 0.126 \times (1-0.126)}{(0.05)^2} = \frac{0.4231}{0.0025} = 169 \text{ samples}$$

Sample collection

For the purpose of this study, the sample size was rounded up to 180. Thirty cow milk and milk products samples were randomly collected from each of the Local Government Areas selected for this study viz: Akwanga and Wamba (Nasarawa North); Lafia and Doma (Nasarawa South); Keffi and Nasarawa (Nasarawa West) respectively from May to October, 2017.

Proportionate sampling method was used in collecting fresh raw milk samples from lactating cows at the accessible Fulani settlements. This was done by taking 50% of the number of lactating cows in a herd at the settlements. On the whole, 34 fresh raw milk samples were collected. Herds were visited during milking time, where 30 ml of composite fresh cow milk samples were

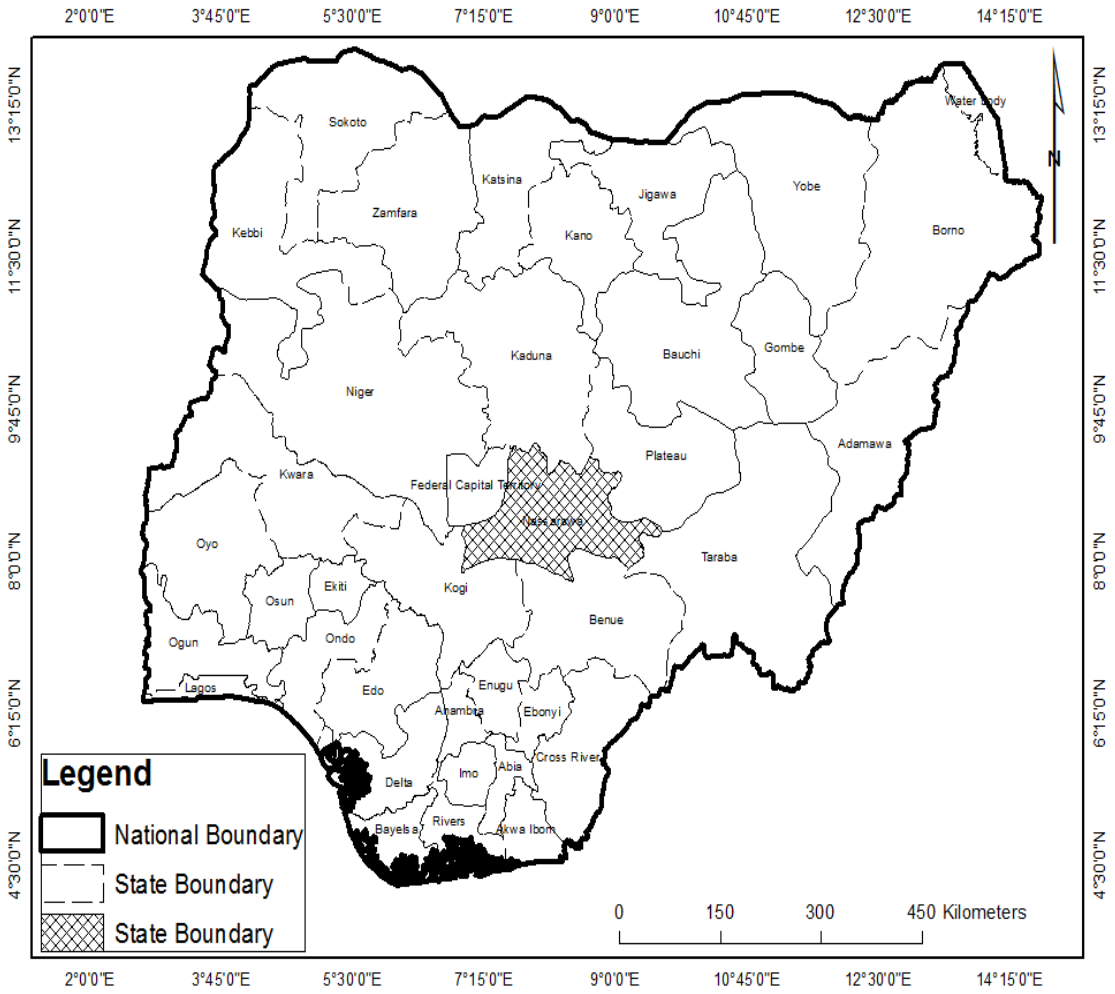

Plate 1. A Map of Nigeria showing the Position of Nasarawa State.

collected directly from the milking cows and placed into sterile bottles. Each sample (30 ml) was collected into sterile screw-capped plastic bottles and labelled appropriately.

Fourteen bulk milk samples were collected from the accessible Fulani settlements in each town selected for this study. The bulk fresh milk samples were collected after the milk have been collected and pooled. Twenty two *nono* and locally-pasteurised milk (*kindirmo*) samples were randomly purchased from vendors in the towns selected for this study. All samples were placed in separate sterile plastic bags to prevent spillage and cross contamination. Samples were the stored in a cooler with ice blocks and then transported to the Biochemical Laboratory of the Institute for Agricultural Reearch (IAR), Samaru, Zaria and the Postgraduate Students' Laboratory of the Department of Microbiology, Ahmadu Bello University, Zaria, for proximate and microbiological analyses respectively.

Isolation and identification of *S. aureus*

All samples were inoculated with the aid of a sterile wire loop onto the surface of prepared Baird-Parker agar plates (Oxoid, Basingstoke, England) supplemented with 5% egg yolk tellurite emulsion (Baird-Parker, 1962). Representative colonies were selected after incubation at 37°C for 24 h based on the appearance of presence of black colonies on the medium which occur as a result of the ability coagulase-positive staphylococci to reduce tellurite, and clear zones of lypolysis which is due to the lecithinase of staphylococci that break down the egg yolk. Discrete colonies were further sub-cultured on to freshly prepared plates of the selective media and nutrient agar plates for biochemical tests and identification (Patrick et al., 2013). *S. aureus* ATCC 25923 strain was used as a positive control.

Observation of colony morphology and characteristics

Presumptive morphological identification of the colonies was done by observing their individual appearance on the selective media that was used for the isolation and Gram reaction.

Biochemical tests

The conventional biochemical tests carried out to identify the suspected *S. aureus* colonies were Catalase test, slide coagulase

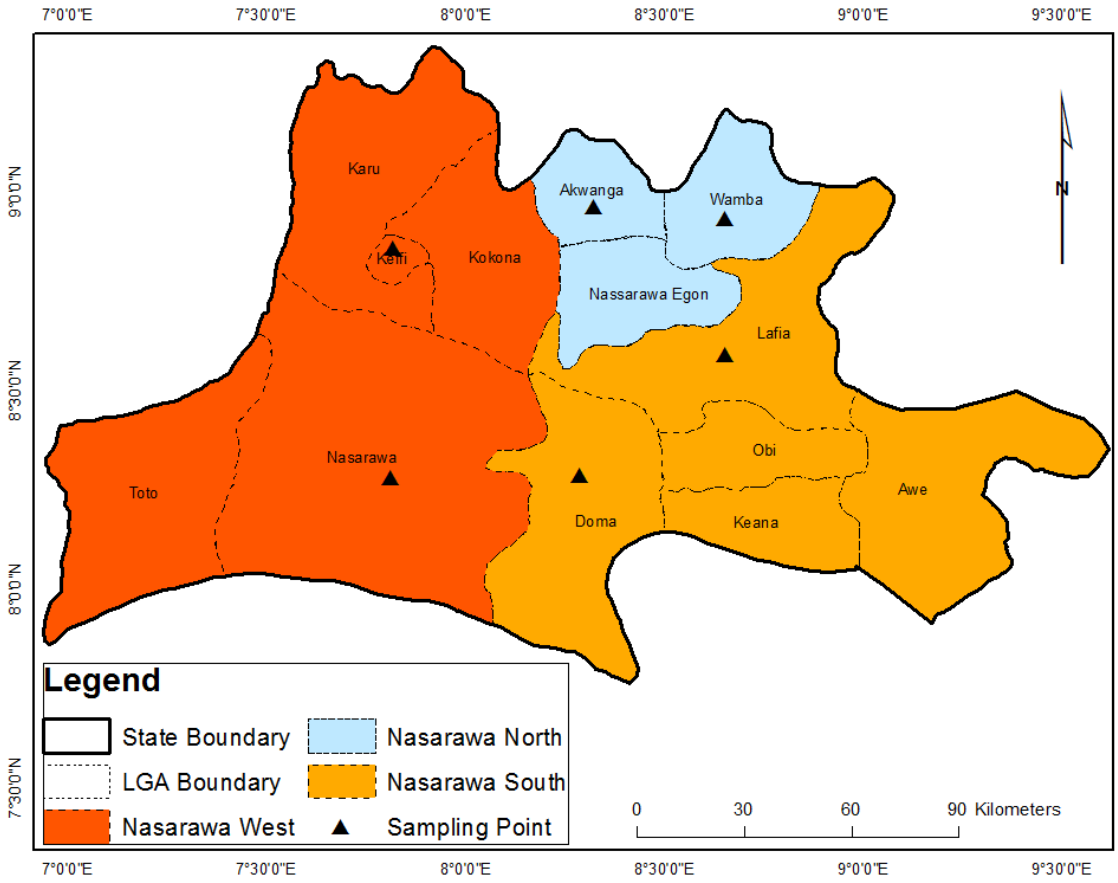

Plate 2. Map of Nasarawa State showing the sampling areas.

test, haemolysis on blood agar, and DNase test (Japoni et al., 2004).

Microgen® staphylococci identification (STAPH identification) kits

The Microgen Staph-ID system comprises of a single microwell test strip containing 12 standardised biochemical substrates which have been selected on the basis of extensive computer analysis that is, each well contains dehydrated substrates, namely: nitrate, sucrose, tetrahalose, mannitol, n-acetyl glucosamine, mannose, turanose, N-acetyl glucosamine, β-glucosidase, β-glucuronidase, urease, arginine, and1-pyrrolidonyl-α-naphthylamide (www.microgenproducts.com UK). A colour change occurs if the individual substrates are metabolised by the organism during incubation, or after addition of specific reagents.

Determination of the antibiogram of the *S. aureus* isolates

The antibiogram of the isolates was determined using the Kirby-Bauer agar disc diffusion method as described by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2016). The isolates were tested against a panel of eleven antibiotics with the following concentrations: ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), chloramphenicol (30 µg), imipenem (10 µg), erythromycin (15 µg), gentamicin (30 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), vancomycin (30 µg), sulphamethoxazole/trimethoprim (25 µg), and tetracycline (30 µg) (Oxoid, England). The determination of sensitive, intermediate, or resistant isolates depend on the diameter zone of growth inhibition of Clinical Laboratory Standards Institute's (CLSI) breakpoint (CLSI, 2016). *S. aureus* ATCC 25932 obtained from the National Veterinary Research Institute (NVRI), VOM, Plateau State, Nigeria was included in each batch analysis as the quality control standard strain.

Determination of minimum inhibitory concentration (MIC) of vancomycin

The MIC of vancomycin against the isolates was determined by broth microdilution method as recommended by the Clinical Standards Institute (CLSI, 2016). Standard powders of the antibiotic (vancomycin) were obtained from GlaxoSmithKline Pharmaceutical Companies. The MIC of vancomycin against the isolates was determined using the procedure described by Wayne (2002). Readings were taken and recorded according to the guidelines of CLSI (2016).

Determination of multiple antibiotics resistance (MAR) index of the *S. aureus* isolates

The multiple antibiotics resistance (MAR) index was determined for each of the isolate using the formula: MARI = x/y, where 'x' is the

Table 1. The occurrence of *S. aureus* in relation to the type of milk samples collected from parts of Nasarawa State, Nigeria.

Type of Milk Sample	No. examined	No. positive (%)	X^2	p-value
Nono	66	4(6.06)		
Bulk Milk	14	1(7.14)		
Fresh Milk	34	1(2.94)	0.646	0.886
Kindirmo	66	3(4.55)		
Total	180	9(5.0)		

Table 2. The prevalence of *S. aureus* in fresh and fermented milk in relation to the local government areas sampled.

LGAs	No. Examined	No. Positive (%)	X^2- value	p-value
Nasarawa	35	1(2.86)		
Keffi	29	2(6.89)		
Akwanga	28	2(7.14)		
Wamba	28	1(3.57)	1.233	0.942
Lafia	31	2(6.45)		
Doma	29	1(3.45)		
Total	180	9(5.0)		

Key: LGAs – Local Government Areas.

number of antibiotics to which the isolate display resistance, and 'y' is the total number of antibiotics to which the test organism had been evaluated for sensitivity (Olayinka et al., 2004; Tula et al., 2013).

Statistical analyses

The chi-square test was used to determine the statistically significant difference between the occurrence of *S. aureus* and the different sample types, and also the occurrence of *S. aureus* in fresh and fermented milk in the different sampling areas. $P \leq 0.05$ was considered statistically significant for all comparisons.

RESULTS AND DISCUSSION

Table 1 shows the occurrence of *S. aureus* in relation to the type of milk samples collected from parts of Nasarawa State, Nigeria. Out of the 66 *nono* samples collected and analysed in the course of this study, four were found to be contaminated with *S. aureus* giving a prevalence of 6.06%; of the 14 bulk milk samples analysed, one was found to be positive for *S. aureus* giving a prevalence of 7.14%; of the 66 *kindirmo* samples examined, three were found to be positive for *S. aureus* giving a prevalence of 4.55%; and of the 34 fresh milk samples examined, one was found to be contaminated with *S. aureus* giving a prevalence of 2.94% (Table 1). The results obtained from this study revealed that, *S. aureus* were present in fresh and fermented milk in parts of Nasarawa State, Nigeria. This is of public health significance since it is a commonly recovered pathogen in outbreaks of food poisoning attributed to dairy products (Junaidu et al., 2011). The occurrence of *S. aureus* (5.0%) in the study area is an indication of defective or absence of public health measures and poor sanitary habits among the people that are concerned with milking, milk handling, and transportation as these have been documented to be factors that predisposes milk to contamination with pathogens (Akram et al., 2013). The use of traditional method of *nono* and *kindirmo* production also exposes the products to bacteria found on the hands and clothes of the people that are concerned with the production and also in the containers used. The unsanitary conditions of the places where the products (*nono* and *kindirmo*) are marketed might have also contributed to their contamination.

Table 2 shows the prevalence (%) of *S. aureus* obtained from fresh and fermented milk in the six sampling areas selected for this study. Out of the 180 samples collected in the course of the study, nine (5.0%) were found to be contaminated with *S. aureus*. Out of the 35 samples collected from Nasarawa Local Government Area, one yielded positive result for *S. aureus* with a prevalence of 2.86%; of the 29 samples collected from Keffi Local Government Area, two yielded positive results for *S. aureus* giving a prevalence of 6.89%; of the 28 samples each collected from Akwanga, two yielded positive results for *S. aureus* with a prevalence of 7.14%; of the 28 samples each collected from Wamba, one yielded a positive result for *S. aureus* with a prevalence

of 3.57%; of the 31 samples collected from Lafia Local Government Area, two were found to be contaminated with *S. aureus* with a prevalence of 6.45%; and out of the 29 samples collected from Doma Local Government Area, one was found to be contaminated with *S. aureus* giving a prevalence of 3.45% (Table 2). The percentage occurrence of *S. aureus* in fresh and fermented milk in parts of Nasarawa State, recorded in this study was 5.0%, which was lower than the 12.6 and 12.14% recorded by Umaru et al. (2013) and Usman and Mustapha (2016), in studies conducted to determine the occurrence of *S. aureus* in fresh and fermented milk in Kaduna and Zaria, respectively. It was also lower than the 8.7% prevalence recorded by Okpo et al. (2016) in fresh and fermented milk in parts of Kaduna State, Nigeria. Higher occurrence of *S. aureus* of 32, 56, 25.53 and 55.26% were reported by Patrick et al. (2013), Gundogan and Avci (2014), Jahan et al. (2015), and Chaalal et al. (2016). Higher occurrence of the organism in dairy products in Kenya, Turkey, Bangladesh, and Algeria respectively. The paucity of information on *S. aureus* in milk and foods in general in the study area made it difficult to make any comparison and to assess the level of *S. aureus* in dairy products in the areas studied.

The isolation of *S. aureus* from fresh and fermented milk is a cause for public health concern because many people in the area consume the products. The findings of this work lend credence to the assertion that, dairy products is one of the major vehicle for the transmission of *S. aureus* to man. No statistically significant difference ($p>0.05$) was found using chi-square in the occurrence of *S. aureus* in fresh and fermented milk with respect to the different sample types collected in the course of this study, indicating that, the milk samples might have been exposed to the same levels of contamination. This may be due to similar handling procedures employed during milking, milk collection, and processing and production of fermented milk (*nono* and *kindirmo*). This trend of occurrence of *S. aureus* is in contrast with was documented by Umoh (1989) that fermented foods are not good media for the survival and growth of *S. aureus*. The occurrence of the organism in these processed foods implies recontamination during and/or after processing. Proper heat treatment and refrigeration can minimise the chances of contamination with *S. aureus*. It has been observed that, during the heat treatment of milk to make *kindirmo*, the temperature does not rise up enough to achieve effective pasteurisation. The occurrence of *S. aureus* in fresh and bulk milk in this study may be attributed to the presence of sub-clinical mastitis in the milked cows, poor sanitary practices during milking, and unclean milking utensils. The main source of *S. aureus* in milk is the udder of infected cows which could be transferred via the milkers hands, milking utensils, towels, and the environment (Radostitis et al., 1994). *S. aureus* can adapt to and survive in the udder of cow and establish chronic and sub-clinical infections. From the udder, it is shed into the milk which serves as a primary source of infection to individuals who drink unpasteurised milk.

The results antibiotics susceptibility profile of the nine *S. aureus* isolated from fresh and fermented milk in parts of Nasarawa State using 11 antibiotics are as presented in Table 3. The results shows that, six (66.7%) of the isolates were susceptible to sulphamethoxazole/ trimethoprim, 8(88.9%) of the isolates were susceptible to vancomycin, seven (77.8%) were susceptible to chloramphenicol, four (44.4%) were susceptible to erythromycin, nine (100%) were susceptible to gentamycin and ciprofloxacin, eight (88.9%) were susceptible to imipenem, while only three (33.3%) were susceptible to tetracycline (Table 3). However, nine (100%) of the *S. aureus* isolates were resistant to cefoxitin, ampicillin, and amoxicillin/clavulanic acid, four (44.4%) were resistant to tetracycline, two (22.2%) were resistant to both erythromycin and sulphamethoxazole/ trimethoprim. The results shows that, gentamycin, ciprofloxacin, imipenem, vancomycin, chloramphenicol, and sulphamethoxazole/trimethoprim were the antibiotics to which the isolates were most susceptible; whereas, erythromycin, and tetracycline were the antibiotics to which the isolates were less susceptible. All the nine *S. aureus* isolates obtained in this study were resistant to cefoxitin, an indication that they are all methicillin-resistant *S. aureus* (MRSA) strains. Cefoxitin is used as a surrogate test for oxacillin to check whether *S. aureus* isolates are MRSA or not (CLSI, 2016). The antibiotic susceptibility profile of the *S. aureus* isolates showed that, they were highly susceptible to gentamycin (100%), ciprofloxacin (100%), imipenem (88.9%), vancomycin (88.9%), and chloramphenicol (77.8%).

The high susceptibility of the isolates to gentamycin, ciprofloxacin, and chloramphenicol was in consonance with the findings of Okpo et al. (2016) and Rodrigues et al. (2017) who recorded high susceptibility of *S. aureus* to isolated from dairy products to the three antibiotics in parts of Kaduna State, Nigeria in Brazil, respectively. The high performance of these antibiotics to could be attributed to their small molecular sizes - a factor that enhances the their solubility in diluents thus enhancing their penetration power through the cell wall into the cytoplasm of the target organism where they exert their effects (Okpo et al., 2016). This agrees with the assertion of Mailard (2002) who opined that, the high efficacy of antibiotics may be attributed to their molecular sizes.

High level of susceptibility (88.9%) of the *S. aureus* obtained in this study to vancomycin was observed. None of the isolates was found to be resistant to the antibiotic (vancomycin). This finding is not surprising because vancomycin is rarely used in the treatment of diseases in livestock and in routine chemoprophylaxis in the study area which could lead to resistance among bacteria as a result of selective pressure. This result is in consonance

Table 3. The antibiotic susceptibility profile of *S. aureus* Isolated from fresh and fermented milk in parts of Nasarawa State.

Antibiotics	Disc Conc. (µg)	N= 9		
		S (%)	I (%)	R (%)
Ampicillin	10	0(0.0)	0(0.0)	9(100.0)
Amoxicillin/ clavulanic cid	30	0(0.0)	0(0.0)	9(100.0)
Cefoxitin	30	0(0.0)	0(0.0)	9(100.0)
Gentamycin	30	9(100.0)	0(0.0)	0(0.0)
Chloramphenicol	30	7(77.8)	2(22.2)	0(0.0)
Vancomycin	30	8(88.9)	1(11.1)	0(0.0)
Ciprofloxacin	5	9(100.0)	0(0.0)	0(0.0)
Erythromycin	15	4(44.4)	3(33.3)	2(22.2)
Imipenem	10	8(88.9)	1(11.1)	0(0.0)
Tetracycline	30	3(33.3)	2(22.2)	4(44.4)
Sulphamethoxazole/Trimethoprim	25	6(66.7)	1(11.1)	2(22.2)

with the findings of Suleiman et al. (2012) and Rodrigues et al. (2017) who opined that, the non-use of vancomycin for routine chemoprophylaxis and therapy in an area can result in *S. aureus* exhibiting high susceptibility to it. This finding also agrees with the results of Alian et al. (2012) in Iran who recorded 0% resistance among *S. aureus* isolated from dairy products. However, this finding contrasted starkly with that of Umaru et al. (2013) and Usman and Mustapha (2016), who reported 42.6 and 66.7% resistance of *S. aureus* isolated from fresh and fermented milk in to vancomycin in Kaduna and Zaria, respectively. The disparity between the findings of the present study and the aforementioned could be as a result of contamination of the milk with vancomycin-resistant *S. aureus* derived from human sources. The present study observed that all the nine *S. aureus* isolates were completely resistant to ampicillin and amoxicillin/clavulanic acid. This could be attributed to the use and misuse of these antibiotics in the study area. This finding is not surprising because, outside the hospital environment, people have easy access to various antibiotics at any drug store without any prescription from qualified personnel. This agrees with the findings of Anueyiagu and Isiyaku (2015) who reported 100% resistance of *S. aureus* isolated from dairy products in Jos, Plateau State, Nigeria, and Jahan et al. (2015) in Bangladesh. β- lactam antibiotics are commonly used as dry-cow treatment. This might have contributed to the increasing resistance among *S. aureus* strains to the drugs due to selective pressure. *S. aureus* resistant to one β-lactam drug can develop resistance to β-lactams because they have the same mechanism of activity.

Relatively high levels of resistance of *S. aureus* to tetracycline (44.4%), erythromycin (22.2%), and sulphamexazole/trimethoprim (22.2%) were recorded in this study. The relatively high level to tetracycline as observed in this study could be attributed to tetracycline being the most commonly available antibiotic that is used as a growth promoter and routine prophylaxis in livestock management in Nigeria (Olatoye, 2010). This finding is a cause for concern considering the fact that, tetracycline is a first-line drug in Nigeria. This is one drug that people with cases of gastro-intestinal infections in most developing countries readily purchase over-the-counter for self-medication (Chigor et al., 2010). This was in consonance with the findings of Usman and Mustapha (2016), and Tessema (2016), who reported 55.5 and 40% resistance of *S. aureus* isolated from dairy products in Kaduna State, Nigeria, and Ethiopia respectively. This trend is a cause for concern in human medicine and livestock disease management and production generally due to the existing emergence of bacterial strains that are resistant to major antibiotics. The use of antibiotics in in food animals have been established to promote the spread of antibiotic-resistant bacteria via the food chain to humans resulting in human infections (Phillips et al., 2004). The relatively high level of resistance to erythromycin could be a reflection of the frequent use and misuse of the antibiotic in the study area. Higher levels of resistance of 76 and 85.7% among *S. aureus* isolated from dairy products have been reported Mirzaei et al. (2012) and Anueyiagu and Isiyaku (2015) in Iran and Kaduna State, Nigeria, respectively.

The relatively high level of resistance to sulphamethoxazole/trimethoprim is this study is baffling considering the fact that, the drug is not routine used in veterinary practice in Nigeria. This suggests cross contamination of the dairy products by handlers with the drug-resistant strains of the pathogen. Mixed fermentation is known to occur in dairy products like *nono* and *kindirmo* and as the fermentation process is uncontrolled and that different organisms can occur at different times, transfer of determinants of antibiotic resistance can occur between organisms. Food is an important medium through which the transfer of determinants of antibiotic resistance among bacteria occurs. Such transfer can occur by means of residues of antibiotics in foods, through the transfer of antibiotic-

Table 4. The antibiotic resistance patterns of *S. aureus* isolated from fresh and fermented milk in parts of Nasarawa State, Nigeria.

No. of Antibiotics	Resistance Pattern	No. (%) of Isolates	LGA
3	Amp, Amo, Fox	4(44.4)	NS, KF, AK, AK
4	Amp, Amo, Fox, Tet	2(22.2)	LF, WM
5	Amp, Amo, Fox, Tet, Sul,	1(11.1)	KF
5	Amp, Amo, Fox, Ery, Tet	1(11.1)	LF
5	Amp, Amo, Fox, Ery, Sul	1(11.1)	DM

AMP = Ampicillin, Amo = Amoxillin/clavulanic acid, Fox = cefoxitin, Tet = Tetracycline, Ery= Erythromycin, Sul = Sulphamethoxazole/trimethoprim, NS = Nasarawa, KF = Keffi, AK = Akwanga, WM = Wamba, LF = Lafia, DM = Doma.

resistant foodborne pathogens, or through the ingestion of drug-resistant strains of the original food microflora, and transfer of transfer of antibiotic-resistance determinants in bacteria (Pereira et al., 2009).

The results of the antibiotic resistance patterns of the nine *S. aureus* isolates obtained from the fresh and fermented milk samples examined are as presented in Table 4 and the multiple antibiotics resistance (MAR) index of the nine isolates is as shown in Figure 1. Five antibiotic resistance phenotypes were obtained, all from the multiple resistance types with varying combinations of three, four and five antibiotics. No antibiotics resistance phenotype was found with a single or two antibiotics as all of the isolates were found to be resistant to three antibiotics and above. The highest frequency three (isolates showing resistance to a combination of antibiotics) was a found in a combination of five antibiotics. Multidrug resistance is defined as resistance of an isolate to two or more antibiotics (Olayinka et al., 2004). This finding is in consonance with the findings of Umaru et al. (2013), Anueyiagu and Isiyaku (2015), Tessema (2016), and Chaalal et al. (2016) who reported cases of multidrug resistance among *S. aureus* isolated from dairy products in Zaria, Jos, Ethiopia, and Algeria respectively. The isolates were resistant to a combination of three, four, five, and six of the antibiotics tested. Isolates obtained from Keffi, Akwanga, and Lafia, showed higher frequencies of multi-drug resistance. Multi-drug resistance in *S. aureus* may be attributed in part, to the spread of mobile genetic elements like plasmids, transposons, and integrons that may confer resistance to numerous antimicrobial agents (Zhao et al., 2001). According to Aarestrup (1995) and Levin et al. (1997), determinants of multi-drug resistance are capable of being disseminated in a region or between regions as a result of antibiotic selective pressure in either livestock or humans. Empirical evidence abounds which indicate that drug-resistant strains of bacteria can be transmitted to humans via food (Khachatourians, 1998).

The five antibiotic resistance patterns among the *S. aureus* isolates recorded in this study varied with the nine and 25 antibiotic resistance patterns recorded among *S. aureus* isolated from dairy products by Usman and Mustapha (2016), and Shiferaw and Ahmad (2016) in Kaduna State, Nigeria and Bahir Dar, Ethiopia, respectively. The disparity in the antibiotic resistance patterns of *S. aureus* isolates recorded in the present study and the one recorded in Ethiopia could be as a result of the different levels of use and misuse of antibiotics in the two areas. 100% of the *S. aureus* isolates obtained in this study had a MAR index of 0.3 and above. MAR index was calculated as the ratio of the number of antibiotics to which an organism is resistant, to the total number of antibiotics to which an organism is exposed (Furtula et al., 2013). MAR index gives an indirect suggestion of the probable source of an organism. MAR index greater than 0.2 is an indication that, an organism originates from an environment where there are no strict regulations regarding the use of antibiotics (Furtula et al., 2013). Residues of antibiotics have also been found in the tissues of food animals and their products (Kabir et al., 2004; Adesokan et al., 2013).

The public health significance of the findings of this study is that, antibiotic resistant strains of *S. aureus* from fresh and fermented milk (or dairy animals) may be transmitted to humans via the food chain, contact through occupational exposure, or wastewater run-off from *nono* and *kindirmo* production sites to the neighbourhood. Indiscriminate use of antibiotics in livestock production in the country could also have been responsible for the antibiotic resistance patterns recorded in this study.

Conclusion

The detection of *S. aureus* in fresh and fermented milk in parts of Nasarawa State, Nigeria, suggests that consumption of dairy products especially those that are produced using traditional methods constitute a hazard to consumers as the transmission of pathogens via foods has been well documented. The antibiotic susceptibility profile of the *S. aureus* isolates revealed high performance of gentamicin, ciprofloxacin, imipenem, vancomycin, and chloramphenicol, while relatively high

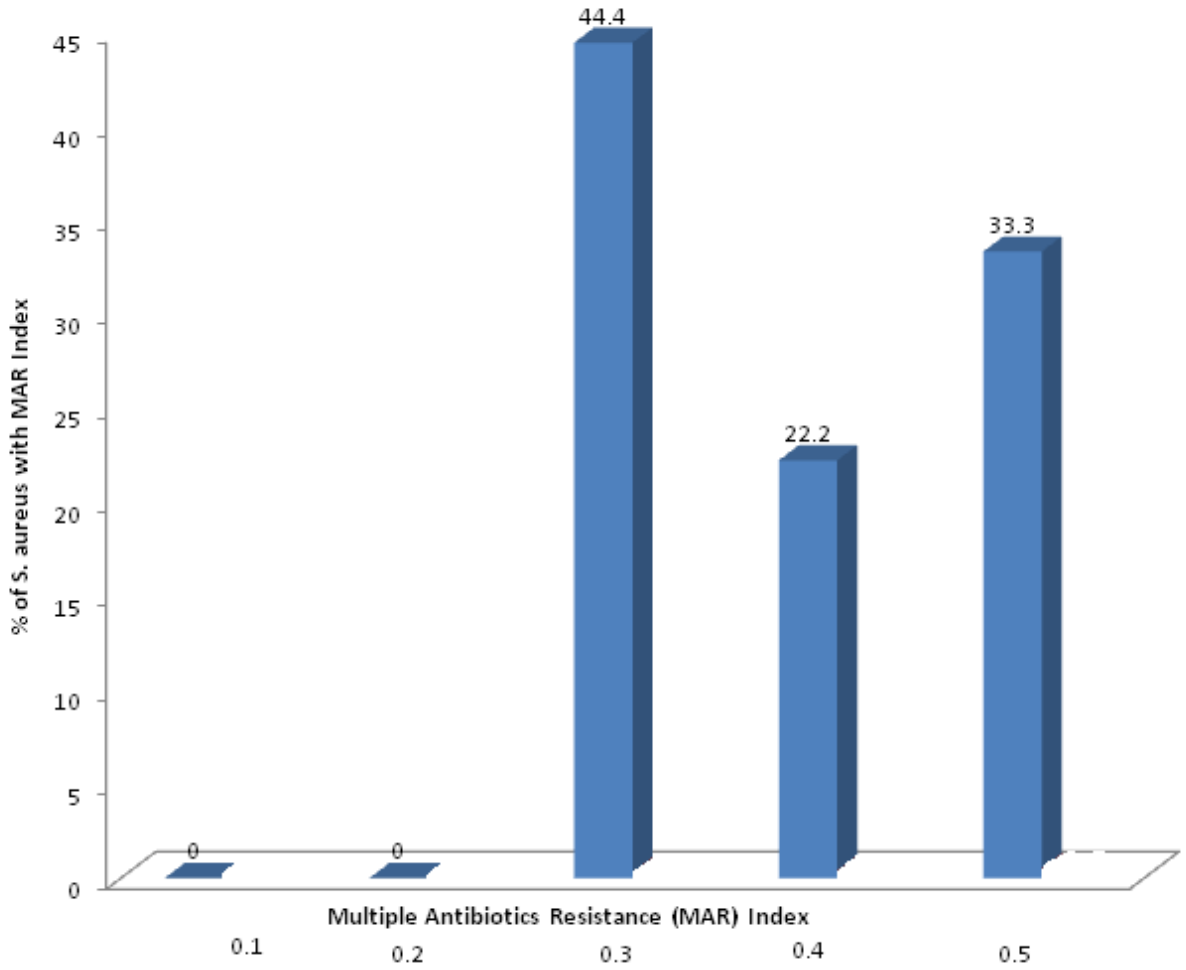

Figure 1. Multiple antibiotic resistance (MAR) Index of *S. aureus* isolated from fresh and fermented millk in parts of Nasarawa state, Nigeria.

levels of resistance to tetracycline was recorded. This is of public health concern because tetracycline is a commonly used antibiotic. The data obtained in this study suggests that, selection pressure imposed by the use of antibiotics whether therapeutically in human and veterinary medicine, or in routine chemoprophylaxis in livestock production is a key driving force in the promotion of antibiotic resistance in *S. aureus.*

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Vol. 13(26), pp. 457-463, August, 2019
DOI: 10.5897/AJMR2018.9030
Article Number: CE1EF4961778
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial resistance profile of *Escherichia coli* isolates recovered from diarrheic patients at Selam Health Center, Addis Ababa, Ethiopia

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Received 28 November, 2018; Accepted August 2, 2019

Antimicrobials have been playing an important role in preventing illness and death associated with infections due to bacteria. However, the emergence and spread of resistance by pathogens have decreased the effectiveness of the commonly prescribed antimicrobials. Intestinal *Escherichia coli* are among bacterial pathogens that are endowed with such resistance traits because they are important source and reservoir of genes that encode antimicrobial resistance. To determine the antimicrobial resistance profile of fecal isolates of *E. coli* from diarrheic patients. Stool samples were collected consecutively from 100 individuals who visited Selam Health Center during the study period, April to June 2018. Samples were collected and transported under sterile condition to the National Clinical Bacteriology and Mycology reference Laboratory, Ethiopian Public Health Institute. The samples were streaked on MacConkey agar and incubated overnight at 37°C. *E. coli* isolates were further confirmed using conventional biochemical tests. Antimicrobial susceptibility status was determined using the disk diffusion method on Mueller Hinton agar as recommended by the Clinical Laboratory Standard Institute. The raw data was compiled and entered to spreadsheet and analysis was done using SPSS Version 20 with p-value ≤0.05 considered statistically significant. Out of the 100 patients, 43 were female and the rest were male. Confirmed *E. coli* were isolated from 73 individuals. Antimicrobial susceptibility testing showed that *E. coli* isolated in this study were highly resistant to trimethoprim-sulfamethoxazole 49 (67.1%) and amoxicillin-clavulanic acid 47(64.4 %). No isolates showed resistance to gentamicin and tobramicin. Of all the isolates, 11(15.1%) were multidrug resistant. No association was observed between antimicrobial resistance status and sex of individuals included in this study. However, there was an association between age and resistance patterns. Resistance to commonly prescribed antibiotics among *E. coli* isolated in this study was high and a considerable proportions of the strains were multidrug resistant. This is an indication for an alarming rate of resistance of intestinal *E. coli* to first line antimicrobials. To reduce the problem, regular monitoring and education for the community are very important.

Key words: *Escherichia coli*, antibiotic susceptibility, multidrug resistant, Ethiopia, biochemical tests, disk diffusion.

INTRODUCTION

Antimicrobials have been playing an important role in preventing illness and death associated with bacteria infections. However, the emergence and spread of resistant pathogenic and commensal bacteria is

increasing all over the world (Aarestrup et al., 2008). Increased resistance to antibiotics has made it difficult to treat infections due to bacteria and even impossible in the extreme cases which in turn results in morbidity and mortality. The problem is particularly serious in developing countries where the availability of alternative antimicrobials is very low and too expensive (Eliopoulos et al., 2003). The World Health Organization (WHO) has raised this issue as a global challenge and a major threat of healthcare in the society today (WHO, 2014).

The emergence of antimicrobial resistance is believed to have a positive association with the way antimicrobials have been used. This is possibly the most important factor that increases the emergence of antimicrobial-resistant microorganisms. These are the result of misuse of antimicrobials by physicians, unskilled practitioners, weak integration between private and governmental health facilities and pharmacy outlets (Bailey et al., 2010; Vila and Pal., 2010).

E. coli is Gram-negative facultative anaerobe bacteria and they are the component of the human gastrointestinal tract. Most of them are usually commensal bacteria and seldom cause disease in healthy individuals. However, in immuno-suppressed patients and when they breached the gastrointestinal barriers, commensal *E. coli* can cause infection. Other group of *E. coli* are pathogenic when they gain virulence factors which enable them to cause intestinal and extraintestinal infections including, diarrhea, septicemia, urinary tract infections, and meningitis not only in immunecompromised patients but also in healthy individuals. Lippolysaccharide (O) and flagellar antigens are the features of pathogenic *E. coli* which can define serotypes or serogroup of these bacteria (Kaper et al., 2004; Skjøt-Rasmussen et al., 2012).

The gastrointestinal area provides favorable environmental conditions for the transmission of resistance genes within and between bacterial species through horizontal gene transfer and other mechanisms. The most abundant organisms in the fecal flora of warm blooded animals including humans are *E. coli* (WHO 2014). *E. coli* are used for monitoring antimicrobial drug resistance in fecal bacteria because they are found more frequently in a wide range of hosts, acquire resistance easily, and are reliable indicator of resistance in salmonellae (Tadesse et al., 2012). Apart from their pathogenecity, fecal *E. coli* have been used as sensitive indicators in surveillance and spread of antimicrobial resistance (WHO, 2014; Tadesse et al., 2012).

In Ethiopia, a number of hospital based studies have been conducted on the profile of antimicrobial resistant *E. coli* isolated from different clinical specimens (Tuem et al., 2018). However, studies from primary healthcare settings are limited. Therefore, the aim of this study was to determine the antimicrobial profile of fecal *E. coli* isolated from patients presenting with gastrointestinal problem at Selam Health Center (SHC) in Addis Ababa from April to June 2018. The study provided important information regarding the pattern of antimicrobial resistance in a primary healthcare setting where over 75% of all healthcare antibiotics are prescribed as reported data from other countries (Hopkins, 2016).

MATERIALS AND METHODS

Study subjects and sample collection

Consecutive non-duplicate 100 diarrheic patients who visited SCH for stool examinations, from April to June 2018 were included in this study. The study participants were informed about the purpose of the study and written consent was obtained from each participant.

All information related to personal identity was kept with strict confidentiality and samples were identifiable only via a generic code. Information collected include age and sex of the patients. Stool samples were collected in sterile cup and then using sterile cotton swabs, the samples were immediately transferred to Cary-Blair transport media and taken to the National Clinical Bacteriology and Mycology Reference Laboratory of the Ethiopian Public Health Institute.

Plating and identification of *E. coli*

At National Clinical Bacteriology and Mycology Reference laboratory, stool specimens were inoculated on MacConkey agar (Oxoid) to select lactose fermenting *E. coli* using the cotton swabs on the first quadrant of the plate and then streaked using sterile plastic inoculation loop. The plates were incubated overnight at 37°C. After overnight incubation, a pink colony was randomly picked and sub-cultured on the same plate media using a sterile inoculation loop to get well isolated colonies. Colonies suspected as *E. coli* were further confirmed using conventional biochemical tests. Briefly, motile, positive indole test, citrate negative, urea test negative and lysine decarboxylase test positive isolates were characterized as *E. coli* after overnight incubation at the same temperature. Isolates confirmed as *E.* coli were selected for antimicrobial susceptibility testing.

Antibiotic susceptibility testing

Susceptibility testing was determined using the disk diffusion method on Muller Hinton agar (MHA) as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2017). Susceptibility test was performed against amoxicillin/clavunate (20/10 µg), gentamicin (10 µg), tobramycin (10 µg), trimethoprime-sulfamethoxazole (Co-trimoxazole)) SxT (1.25/23.75 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), cefepime (30 µg) and nalidixic acid (30 µg). Following 16 to 18 h incubation,

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the plates were examined, and the inhibitory zone diameters for individual antimicrobial agents were measured and recorded as susceptible and resistant based on the breakpoints for respective antimicrobial susceptibility of CLSI 2017. A standard culture of *E. coli (*ATCC 25922) was used as a control with each batch of antimicrobial susceptibility test. The isolates showed resistance to three or more different groups of antibiotics were designated as multi drug resistant (MDR) *E. coli.*

Phenotypic characterization of extended spectrum beta-lactamases (ESBLs)

E. coli resistance to third generation cephalosporins (cefotaxim) and fourth generation cephalosporin (cefepim) were classified as ESBL producer and were further confirmed for the production of the enzymes using combination disk tests. Briefly, each isolate was sub-cultured on blood agar plate and incubated overnight at 37°C. After overnight incubation, a 0.5 McFarland standard was prepared by the direct colony suspension method in normal saline. Using a sterile cotton swab, the suspension was inoculated on the surface of MHA plate by streaking the entire surface in three different directions as well as the outer rim of the plate. Once the plate was dried, antimicrobial disks were applied. cefotaxime (30 µg) alone and cefotaxime-clavulanic acid (30/10 µg) and ceftazidime (30 µg) alone and ceftazidime-clavulanic acid (30/10 µg) were used. Plates were then incubated at 35±2°C for 16 to 18 h and the zones of inhibition surrounding each disk were measured.

The interpretation of positive results was based on an increase in zone diameter by ≥ 5 mm for the agents tested (cefotaxime and ceftazidime) in combination with clavulanic acid as compared to that agent alone. Parallel to all tests, *E. coli* ATCC 25922 (ESBL negative and *K pneumonia* ATCC 700603 (ESBL-positive) were run for quality control.

Statistical analysis

The data was captured and computed using Microsoft Excel. Percentage of MDR strains were analyzed using SPSS Version 20. Tables and graphs were used to summarize the results.

Ethical considerations

Ethical clearance for this study was obtained from the Ethiopian Public Health institute scientific and ethical review committee.

RESULTS

E. coli isolates and study participant's characteristics

A total of 100 stool samples were collected and processed for this study. Of all the study participants, 62% (n= 62) were females (Table 1). Since the selection of colonies from the primary media for further analysis was random, 73 *E. coli* isolates from the collected stool samples could be obtained, of which 43 isolates were derived from female participants and 30 *E. coli* isolates were from male patients. The age distribution of the patients with regards to *E. coli* obtained is shown in Table 2. It was found that the 21-30 year age group was the highest in prevalence (30.2%) among female patients. While 31-40 year age group was the patients with highest frequency (33.3%) among male participants. Less study participants were from patients with age > 50 both in female and male (Tables 1 and 2). Median age of the study participants was 25.64 year and standard deviation=16.40 year.

Antimicrobial resistance profile of *E. coli*

All biochemically confirmed *E. coli* isolates were tested by agar disk diffusion to determine their susceptibility profile to a panel of eight antimicrobial agents. The antimicrobials tested were from five classes of antibiotics used commonly in clinical practices. These include: Aminoglycosides (Gentamycin and Tobramycin), Cephalosporin (third generation Cefotaxime and fourth generation Cefepime), Penicillin combinations (Amoxicillin/clavulanate), Quinolones/Fluoroquinolones (Ciprofloxacin) and nalidixic acid and Sulfonamides (Trimethoprim-Sulfamethaxazole) (Co-trimoxazole). The result showed that a greater percentage of *E. coli* isolates were resistance to Co-trimoxazole (67.1 %) and Amoxaicillin/clavulanate (64.4%).

Among the Quinolones/Fluoroquinolone, resistance to nalidixic acid and Ciprofloxacin was 27.4 and 2.7%, respectively. Resistance to cephalosporins, cefotaxime and cefepime was 6.9% and all of them were phenotypically ESBL producers. Apart from ESBL productions which hydrolyze extended spectrum cephalosporins, the isolates were co-resistance with other antimicrobial agents. However, no isolates showed resistance to Aminoglycosides, Gentamicin and tobramycin (Figure 1).

The pattern of *E. coli* resistance to antibiotics in terms of sex was further analyzed and the finding indicated that antimicrobial resistance in *E. coli* was not significantly associated with the sex of the participant patients (P-value=0.93) (Table 3).

Another question was whether there were any associations between the age of individuals and antibiotic resistance. Interestingly, highest resistant strains (82.4%) to Co-trimoxazole were obtained from 0-10 age groups. As age increases, the resistance pattern of *E. coli* to co-trimoxazole was relatively decreasing. However, in the case of amoxicilli/clavulanate, *E. coli* strains isolated from patients who are younger (0-10 year) and the elderly above 40 year age group, exhibited increased incidence of resistance (Table 4).

Multi-drug resistance patterns (MDR)

MDR bacteria are defined as bacterial strains that are non-susceptible to at least one antimicrobial agent in three or more antimicrobial classes (Sweeney et al., 2018). Accordingly, 17.8 % (n=13), of 73 studied *E. coli* strains were susceptible to all antimicrobial classes tested. With regards to resistant *E. coli* strains, 27.4%

Table 1. Age and sex distribution of the study participants.

Age group	Number =100		
	Male	Female	Total
0-10	10	11	21
11-20	3	12	15
21-30	8	22	30
31-40	11	10	21
41-50	3	3	6
51-60	2	3	5
>60	1	1	2
Total	38	62	100

Table 2. Prevalence of *E. coli* in diarrheic patients based on age and sex distribution.

Age group	Male (n=30)	Female (n=43)	Total (N=73)
	n (%)	n (%)	n (%)
0-10	7(23.3)	10(23.3)	17(23.3)
11- 20	3(10)	6(14)	9(12.3)
21-30	6(20)	13(30.2)	19(26)
31-40	10(33.3)	9(20.9)	19(26)
41-50	2(6.7)	3(7)	5(6.8%)
51-60	1(3.3)	1(2.3)	2(2.7)
≥61	1(3.3)	1(2.3)	2(2.7)

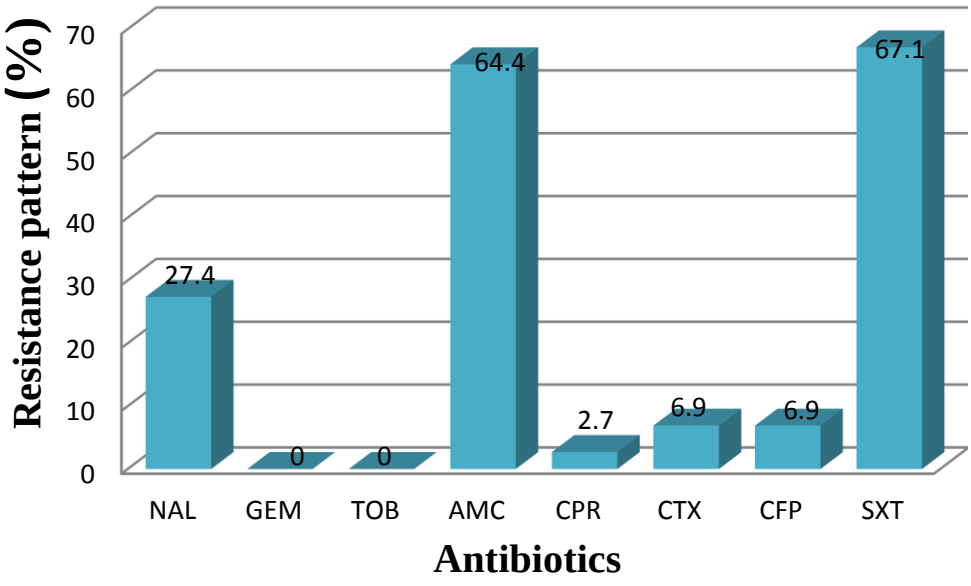

Figure 1. Over all antibiotic resistance profile of *E. coli*: NAL= nalidixic acid, GEM = gentamicin, TOB = tobramycin, AMC = amoxicillin–clavulanic acid, CPR = ciprofloxacin, CTX = cefotaxime, CFP = cefepime, SXT = trimethoprim–sulfametoxazole.

(n=20) were resistant to a single antimicrobials, 39.7% (n=29) strains were resistant to two antimicrobials and 15.1% (n=11) *E. coli* strains were resistant to three and more than three antimicrobials of different classes which

Table 3. Comparison of sex-based antibiotic resistance patterns of the *E. coli* strains isolated from patients with diarrhea.

Antimicrobial agents	Male (n=30)			Female (n=43)			T-test (P-value)
	R	S	% R	R	S	%R	
AMC	19	11	63.3	28	15	65	
GEN	0	30	0	0	43	0	
TOB	0	30	0	0	43	0	
CTX	3	27	10	2	41	4.7	0.92
CFP	3	27	10	2	41	4.7	
CPR	1	29	3.3	1	42	2.3	
SXT	20	10	67	29	14	67.4	
NAL	8	22	27	11	32	25.6	

Table 4. Association between age group and antimicrobial resistance of *E. coli* isolates recovered from diarrheic patients.

Age group	Resistance percentage	
	AMC (R%)	SXT (R%)
0-10	70.6	82.4
11-20	66.7	77.8
21-30	57.9	47.4
31-40	31.6	52.6
>40	77.8	40

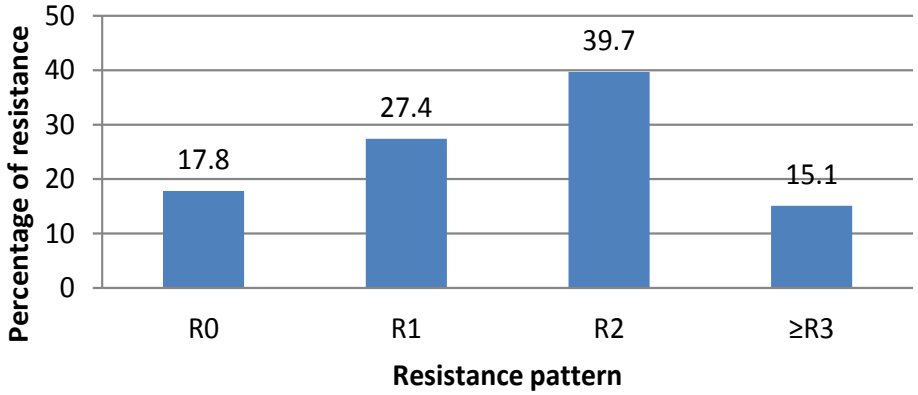

Figure 2. MDR *E. coli* isolated in this study (≥R3). R0: No resistance, R1: Resistance to one antibiotic, R2: Resistance two antibiotic, ≥R3: Resistance to three or more antibiotics (MDR).

are considered as MDR strains (Figure 2).

DISCUSSION

This study was conducted on stool specimen collected from SHC. Even though, there is no compiled Ethiopian study data on antibiotic prescription dosage, studies in other part of the world indicate that the greatest proportion of antibiotics for human use is prescribed at primary healthcare sector (Fernando et al., 2017), where use is strongly correlated to antibiotic resistance rates highlighting this sector as an important area for research and intervention (Bell et al., 2014). Therefore, antimicrobial resistance of *E. coli* isolates from patients visited the center due to diarrhea were examined. To do so, after overnight incubation as indicated in materials and methods section, a single isolate representing each sample

was randomly picked and sub-cultured for biochemical analysis and susceptibility test. This is because it was reported that most of the *E. coli* strains isolated from one stool samples are identical (Bok et al., 2018).

The pattern of antibiotic resistance of *E. coli* strains was tested against trimethoprim-sulfamethoxazole, β-lactams (cephalosporins and β-lactamases inhibitor combinations, AMC), fluoroquinolones and aminoglycosides. These antibiotics are used to treat community and hospital infections due to *E. coli (*Pitout, 2012*)*.

Resistance to Trimethoprim-sulfamethoxazole was one of the most common antibiotics resistance patterns identified among *E. coli* isolates. Trimethoprim-sulfamethoxazole resistance results from alterations of different substrate enzymes or their overproduction, loss of bacterial drug-binding capacity, and decreased cell permeability and this is often associated with acquisition of the resistance genes *sul1* and *sul2* (Kozak et al., 2009). Sulfonamide resistance genes are commonly associated with mobile genetic elements, and these elements play a major role in dissemination of multiple antimicrobial drug resistance genes in *E. coli* isolates (Bean et al., 2009). 67.4% of *E. coli* strains examined for this study were resistant to trimethoprim–sulfametoxazole. This result is almost similar with 66% of compiled study finding from community settings in South Asia and Sub-Saharan Africa (Ingle et al., 2018) and higher than 57.47% of resistance rate reported from Ethiopia (Tuem et al., 2018).

Amoxicillin (AMX), broad-spectrum β-lactam penicillin in combination with the β-lactamase inhibitor clavulanic acid is used for treating lower respiratory tract infections and abdominal infections caused by Enterobacteriaceae and other group of bacteria. The resistant Enterobacteriaceae including *E. coli* isolated from patients with abdominal infections have been typically associated with administration of AMC (Lund et al., 2001). Frequent use of these antibiotics increases the concern for emerging development and spread of antibiotic resistance genes (Duytschaever et al., 2013). The highest incidence of resistant *E. coli* strains to SXT and AMC in this study may be the indication of frequently prescription of these antibiotics by physicians and misuse of the antibiotics in the community.

Cephalosporins are β-lactam antibiotics and are the major drug classes used to treat community-onset or hospital-acquired infections caused by *E. coli* (Pitout, 2010). The production of β-lactamase by *E. coli* is the most important contributing factor to β-lactam resistance. The enzymes β-lactamases inactivate β-lactam antibiotics by hydrolysis, which results in ineffective the compounds (Pitout, 2010). In this study, Cefotaxime and Cefepim third generation and fourth generation cephalosporins respectively, were tested. These antibiotics are called expanded-spectrum cephalosporins because they are developed to treat infection due to Enterobacteriaceae including *E. coli* producing narrow-spectrum β-lactamases such as TEM-1, TEM-2 and SHV-1 enzyme (Bush and Jacoby, 2010). However, the bacteria developed resistance to the expanded-spectrum cephalosporins by producing plasmid-mediated extended-spectrum β-lactamases such as TEM derivatives, SHV derivatives and CTX-M types (Pitout, 2012). Among *E. coli* isolated in this study, 6.9% were resistant to expanded-spectrum cephalosporins. These groups of *E. coli* are assumed to produce extended-spectrum-beta-lactamases and were phenotypically confirmed as all of them were ESBL producer in this study. Extended spectrum beta lactamase producing enterobacteriaceae including *E. coli* are the major public health concern because few antibiotics remain active against these bacteria and can be disseminated easily into the community as the genes encoding these enzymes are found on plasmids (Ruppé et al., 2013)**.**

Reducing the susceptibilities of *E. coli* to fluoroquinolone is due to the up regulation of efflux pumps and plasmid-mediated resistance mechanisms such *qnr* determinants. In addition, 1-2 point mutations within the quinolone resistance determining regions of *gyrA* and *parC,* are required for high level resistance to the fluoroquinolones in *E. coli* (Johnson et al., 1013). The results indicate that 27.4% fecal isolated *E. coli* were resistant to nalidixic acid and these isolates may produce these genes and might have mutation as a result of selective antibiotic pressure.

The patterns of antibiotic resistance in *E. coli* strains were further analyzed in terms of sex of the participants. The results of this study indicate that no direct relation exists between the sex of the patients and the resistance patterns of the isolates. This was explained by studies done elsewhere in that, the susceptibility patterns of bacteria depending on exposure of the individuals to antimicrobial agents which may result in acquiring mutation that confers resistance to these drugs regardless of the sex of the patients (Cho et al., 2011; Sahuquillo-Arce et al., 2011

The associations between the age of individuals and antibiotic resistance among the most frequent *E. coli* strains resistant to Trimethoprim-Sulfamethoxazole and Amoxicillin/clavulanate documented in this study, can be resulted from intensively prescribed and over abused antimicrobials for mild infection particularly in young individuals. These have been well explained by many studies that, the selective pressure produced by antibiotic prescribing in community contribute to such problem (Cho et al., 2011). The higher resistance of AMC in the greater than 40-year-old group of patients may be explained by the longer exposure of these individuals to these antibiotics which has been reported as the age of the patients are one of the factors for antimicrobial resistance (Garcia et al., 2017).

Multidrug resistance among *E. coli* isolates observed in the present study sends alarming message as these

group of organisms have significant clinical implication.

Conclusion

Resistance to commonly used primary care antibiotics particularly Trimethoprim-Sulfamethoxazole (co-trimoxazole) and Amoxicillin/clavulanate in faecal *E. coli* isolates from this study was very high. Over prescribing of these antibiotics at primary healthcare for mild or self-limiting infections, may be responsible for the major problem (Costelloe et al., 2010). To reduce the problem, education for prescribers and patients at facility and community level is essential. Moreover, extensive research that can show on the relation between antibiotic prescriptions and resistance burden is needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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