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Original Research Article

Genotypic and Phenotypic Drug Resistance of Bacteria Associated with Diabetic Septic Foot Infections among Sudanese

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Abstract: Diabetes mellitus (DM) is one of the main problems in health systems and a global public health threat that has increased dramatically over the past two decades. According to epidemiological studies, the number of patients with DM increased from about 30 million cases in 1985, 177 million in 2000, 285 million in 2010, and estimated if the situation continues, to be more than 360 million people by 2030. Too many of the nearly 300 million people in the world with diabetes suffer from diabetes related foot complications. The understanding of the bacteriology of the diabetic septic foot is critical for guiding antibiotic selection and correlating culture results with appropriate therapy. This study is aimed to investigate the phenotypic and genotypic resistance among bacteria causing Diabetic Septic Foot patients. Bacterial isolates of diabetic patients with septic foot infection attending Jaber Abolez Diabetic Center from the 1st of February till the 31st of March, 2017 were recruited to the study. Total number of 204 wound swabs has been cultured during the study. Gram negative bacilli predominated as 183 isolates and Gram positive cocci as 30 isolates only. The commonest organism isolated was Proteus mirabilis as 96 isolates (45.07%). Regarding resistance; All Staphylococci identified are resistant to Oxacillin, resistance to Ciprofluxacin, Gentamicin and Amikacin is shown to be 25.7%, 50% and 27.9%, respectively. Bla_{CTX-M} gene was detected in 27 multi-drug resistant Gram negative isolates (64.29%). The preponderance of ESBLs among the isolates from diabetic foot infections as well as tremendous drug resistance is of concern. Further research is needed to support these findings**.**

Keywords: Diabetic Septic Foot, Diabetes, Drug resistance, ESBL.

INTRODUCTION

Diabetic patients with foot infection have several factors that might be associated with a high risk of multi-drug resistant microorganisms (MDR), such as inappropriate antibiotic treatment, chronic course of the wound, reduced antibiotic concentration in the tissue and frequent hospital admission. The most important cause of antimicrobial resistance is overuse or inappropriate use of antibiotics. The widespread incidence of MDR in both clinical and environmental settings is uprising and represents a potential threat to public health in most parts of the world, as some bacteria strains now show more than 50% resistance to several antibiotics belonging to different classes [1, 2].

Majority of the diabetic foot lesions are initially treated empirically based on the clinical knowledge and experience of the treating clinicians rather than scientific evidence and facts. Worldwide, several studies have been conducted with respect to the bacteriology and antibiotic sensitivity patterns of DSF concluded that aerobes are considered causing the most illnesses and pathogenicity. However, chronic wounds develop more complex colonizing flora [3, 4].

There are many studies of diabetic septic foot (DSF) infections along with their susceptibility patterns of antibiotic conducted from different parts of the world. However, - and to our knowledge only a few are available in literature from Sudan, none of them addressed a molecular characterization of multi- drug resistant isolates [5 -8]. Therefore, a prospective study is managed to be carried out to determine the relative frequency of *Enterobacteriaceae* bacterial isolates among Sudanese patients of diabetic foot infections and to assess their *in vitro* susceptibility to the commonly

used antibiotics, as well as to study their genetic resistance markers.

MATERIALS AND METHODS

The current descriptive cross-sectional study was conducted in Jaber Abolez Diabetic Center (JADC), which is specialized centre in Sudan for the treatment and follows up of diabetic patients with septic foot infections. All septic foot infection patient's samples attending JADC for wound dressing and culture inquiries from 1st February to 31st of March 2017 were recruited to the study after the approval of the laboratory administration.

Laboratory work

Collection and Cultivation of isolates on different types of media

For isolation and purification of bacterial isolates, culture from sterile cotton swabs was maintained on suitable sterile culture media (Blood agar and MacCONKEY media). The plates were incubated under aerobic condition at 37°C for 24 hours.

Identification of isolated bacteria

Identification of isolates was achieved according to cultural characteristics, gram staining properties and different biochemical tests (oxidase, Kligler Iron Agar, citrate, indole, motility, urease, DNAse, catalase and coagulase production tests).

Antibacterial sensitivity profile

The standard disc diffusion method was used (Kirby-Bauer). The antibiotic discs used were from Himedia (Himedia Laboratories Pvt. Ltd, Mumbai 400086, India). The following antibiotics were used: penicillin G (10mg) Oxacillin (1mg), gentamycin (10mg), erythromycin (10mg), cloramphenicol (30mg), Amoxillin-clavulinic acid (30mg), Ciprofluxacin (5mg), vancomycin (30mg), Fusidic acid (10mg), ceftazidime (30mg), amikacin (30mg), tetracycline (30mg), piperacillin (100mg), imipenem (10mg) and aztreonam (30mg) (Figure 4). Interpretation of results was conducted according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2010[9]. All gram negative multi-drug resistant isolates (resistant to three or more antibiotics belong to different classes) were stored as pure isolates for further molecular investigations.

Resistance genotyping by Multiplex PCR

DNA was extracted employing boiling, and PCR reactions were carried out using 5 µl DNA solutions, Qiagen HotStarTaq Master Mix (Qiagen). The amplification was done using TECHNE® Ltd peltier thermal cycler (Germany). Ten pmol of each gene-specific primer in a final volume of 25 µl. For ESBL genes detection, Primer-pair sequences (bla-SHV, TEM and CTX-M) were used in a single PCR assay per each, origin and expected PCR amplicon sizes are given in (Table 1). Initially, a PCR annealing temperature gradient was performed and PCR amplification conditions were as follows: initial denaturation step at 95 °C for 15 minutes; 30 cycles of denaturation at 94 °C for 30 seconds, (annealing at 60 °C for 30 seconds for bla_{SHV}, 54 °C for 30 seconds for bla_{TEM} and 57 °C for 30 seconds for bla_{CTX-M}), extension at 72 °C for 2 minutes, followed by a final extension step at 72 °C for 10 minutes as previously described by Monstein *et al*., [31] with modifications.

Table-1: Primer sequences and amplicon sizes used in the study.

Primer	Sequence (5'-3' direction)	Amplicon Size	Reference
bla-SHV.F	ATTTGTCGCTTCTTTACTCGC	1051	Monstein *et al*., [31]
bla-SHV-R	TTTATGGCGTTACCTTTGACC		
blaTEM-F	ATGAGTTTCAACATTTCCGTG	861	
blaTEM-R	TTACCAATGCTTAATCAGTGAG		
blaCTX-M-F	ATGTGCAGYACCAGTAA	500	
blaCTX-M-R	CCGCRATATGRTTGGTGGTG		

Agarose gel electrophoresis

In order to make 2% Agarose Gel; 1.0 gram of agarose was mixed with 50 ml 0.5x TBE buffer in an Erlenmeyer flask. Heat was applied for 2 minutes using microwave oven. Then it was left to cool to 50° C and 1 µl of 20 µg/ µl ethidium bromide solution was added, mixed well and poured in gel pouring chamber. Two combs were then placed in the chamber and left to cool for about 20 minutes. Electrophoresis chamber was then filled with the 1x TBE buffer. Samples and ladder were then loaded to the wells of the gel. Electrophoreses was conducted at 60Volt for 55 minutes.

RESULTS

Wound microbiology

Total number of 204 wound swabs has been cultured during the study. 153 samples (75%) revealed positive culture results, and 51(25%) cultures revealed no growth under aerobic condition. Among the 153 positive culture specimens monomicrobial isolates have been detected in 93 (60.8%) whereas 60 (39.2 %) showed two organisms per report (Data not shown). Microbiological culture isolated (213) bacterial isolates with an average of 1.39 organisms per positive culture. Gram negative bacilli predominated as 183 isolates and gram positive cocci as 30 isolates only. The commonest organism isolated was *Proteus mirabilis* as 96 isolates

(45.07%). Prevalence of different isolates is summarized in (Table 2).

Antimicrobial Susceptibility testing

All Staphylococci identified are resistant to Oxacillin. Vancomycin in another hand is showing high rates of resistance as 9 isolates (30%) are intermediately resistant and another 9 (30%) are resistant. All susceptibility results of gram positive isolates against different antibiotics are summarized in (Table 3&5).

The gram negative isolates response to Ceftazidime is showing higher rates of resistance rather than sensitivity, 90 isolates (57.4%) are resistant while 78 isolates (44.4%) are sensitive. For Imipenem, 41% of the isolates are found to be resistant while 55.7% are sensitive to the antibiotic. For the antibiotics Ciprofloxacin, Gentamicin and Amikacin the resistance is shown to be 25.7%, 50% and 27.9%, respectively. All susceptibility results of gram negative isolates against different antibiotics are summarized in (Tables 4&5).

Detection of ESBLs

A total of 42 multi-drug resistant gram negative isolates have been selected for further genetic detection of ESBL genes. Bla_{CTX-M} was found predominating as the gene was detected in 27 multi-drug resistant gram negative isolates (64.29%). Bla_{SHV} and bla_{TEM} genes follow as 35.71% and 28.75%, respectively. (84.5%) of all tested Gram negative organisms in the study are found to be ESBL producers representing (23.6%) of all Gram negative isolates in the study. 100% of ESBL producers detected in the study are either *Klebsiella* or *Proteus* species. All result is summarized in (Figure 1,2 & Table 7). The relationship between phenotypic and genotypic resistance as well as the cross positivity of different ESBL among isolates observed in the study is summarized in (Table 5).

Table-2: Frequency of isolated bacteria from patient with diabetic wound infections.

Isolate	Frequency	%	Cumulative %
Citrobacter species	6	2.8	2.8
CNS*	15	7.0	9.8
Enterococcus. faecalis	3	1.4	11.2
Escherichia.coli	18	8.4	19.6
Klebsiella species	12	5.6	25.2
Proteus.merabilis	96	44.9	70.1
Proteus.valgaris	18	8.4	78.5
Pseudomonas	15	7.0	85.5
Serratia marcescens	6	2.8	88.3
Staphylococcus. aureus	12	5.6	93.9
Unidentified**	13	6.1	100.0
Total	214	100.0	

*CNS: Coagulase Negative Staphylococci.

*Unidentified: Biochemical reactions gave no clear identification. Further advanced tests are required.

Table-3: Resistance of Gram positive isolates from patients with diabetic foot infections

	Isolate					
	Coagulase Negative Staphylococci		**Staphylococcus. aureus**		**Enterococcus. faecalis**	
	Count	%*	Count	%*	Count	%*
Erythromycin	9	60	3	25	3	100
Fusidic Acid	9	60	3	25	0	0
Cloramphenicol	9	60	0	0	3	100
Vancomycin	9	60	0	0	0	0
Gentamycin	15	100	0	0	0	0
Ciprofluxacin	9	60	0	0	3	100
Penicillin	15	100	9	75	0	0
Amoxicillin - clavulinic acid	15	100	12	100	0	0
Oxacillin	15	100	12	100	0	0

*The percentage of resistant isolates in the corresponding bacteria.

Table-4: Resistance of Gram negative isolates from patients with diabetic foot infections.

	Isolate													
	Citrobacter species		**E.coli**		**Klebsiella spp**		**P.merabilis**		**P.valgaris**		**Pseudomonas**		**S marcescens**	
	Count	%	Count	%	Count	%	Count	%	Count	%	Count	%	Count	%
Ciprofluxacin	0	0	3	16.5	3	16.5	24	25	0	0	3	20	3	50
Amoxicillin - clavulinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amikacin	0	0	6	33.3	3	16.5	33	34.3	0	0	0	0	6	100
Imipinem	0	0	0	0	3	16.5	51	53.1	3	16.7	6	40	0	0
Tetracycline	0	0	12	66.6	9	50	78	81.3	15	83.3	12	80	6	100
Ceftazidime	0	0	9	50	6	33.3	51	53.1	9	50	9	60	0	0
Piperacillin	3	100	15	80	6	33.3	54	56.2	6	33.4	3	20	0	0
Aztreonam	0	0	12	66.6	9	50	66	68.8	6	33.4	6	40	6	100

Table-5: Shows frequency and percentage of resistance of all antibiotics used in the study

Antibiotic	**Count**	**%***
Erythromycin	12	40
Fusidic Acid	12	40
Cloramphenicol	96	45.1
Vancomycin	9	30
Gentamycin	15	50
Ciprofluxacin	48	22.9
Penicillin	24	80
Amoxicillin - clavulinic acid	27	90
Oxacillin	27	90
Amikacin	48	26.2
Imipinem	75	41
Tetracycline	138	75.4
Ceftazidime	90	49.2
Piperacillin	93	50.8
Aztreonam	111	60.7

*i.e. 40% of times Erythromycin was tested against different isolates, bacteria resist its action.

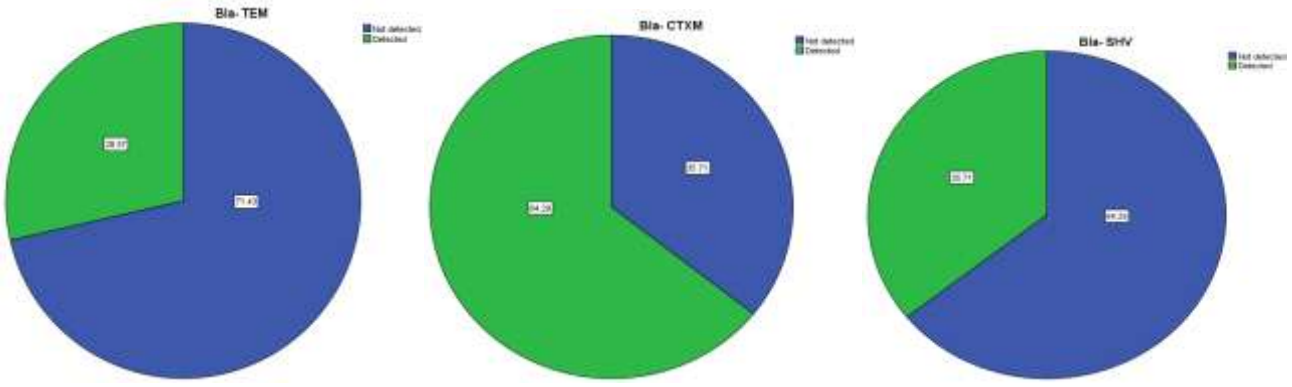

Fig-1: Percentage of different ESBL genes among multi-drug resistant gram negative isolated bacteria from diabetic food patients.

Table-6: Phenotypic and getotypic resistance among multi-drug resistant isolates.

Bacterial Isolates	Phenotype								Genotype		
	Clorampheniciol	Cipruflaxacin	Amikacin	Imipinem	Tetracycline	Ceftazidime	Piperacillin	Aztronam	TEM	SHV	CTXM
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	S	S	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	R	R	R	R	R	-	+	-
p.merabilis	S	S	R	R	R	R	S	R	+	+	+
p.merabilis	R	S	R	R	R	R	S	R	-	-	-
klebsiella	R	S	S	R	R	R	S	R	-	+	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	R	R	R	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	+	-	+
p.merabilis	S	S	S	R	S	R	R	R	+	+	+
p.merabilis	I	S	S	R	S	I	R	R	+	+	+
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	S	S	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	R	R	R	R	R	-	+	-
p.merabilis	S	S	R	R	R	R	S	R	+	+	+
p.merabilis	R	S	R	R	R	R	S	R	-	-	-
klebsiella	R	S	S	R	R	R	S	R	-	+	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	R	R	R	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	+	-	+
p.merabilis	S	S	S	R	S	R	R	R	+	+	+
p.merabilis	I	S	S	R	S	I	R	R	+	+	+
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	S	S	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	R	R	R	R	R	-	+	-
p.merabilis	S	S	R	R	R	R	S	R	+	+	+
p.merabilis	R	S	R	R	R	R	S	R	-	-	-
Klebsiella spp	R	S	S	R	R	R	S	R	-	+	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	R	R	R	R	R	R	R	R	-	-	+
p. valgaris	R	S	S	S	R	R	S	S	-	-	+
p.merabilis	S	S	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	-	-	-
p.merabilis	R	R	R	R	R	R	R	R	+	-	+
p.merabilis	S	S	S	R	S	R	R	R	+	+	+
p.merabilis	I	S	S	R	S	I	R	R	+	+	+

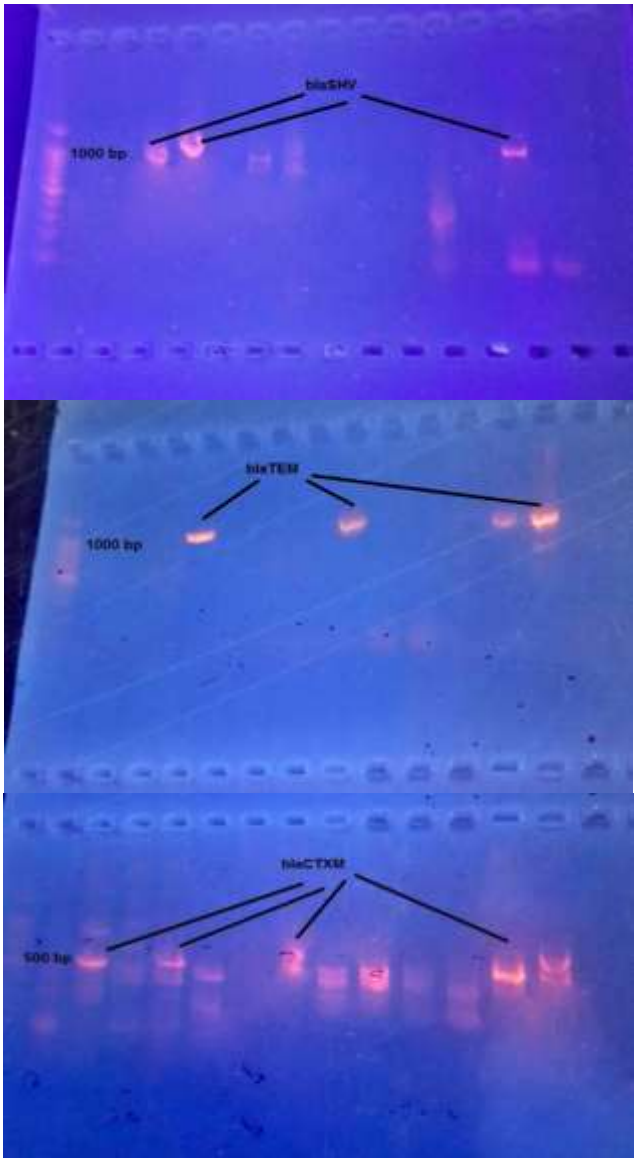

Fig-2: ESBL genes detected among multi-drug resistant gram negative isolated bacteria from diabetic food patients. On the Top: bla_{SHV}. In the Middle: bla_{TEM}. At bottom: bla_{CTXM}.

Table-7: Prevalence of ESBL genes among multi-drug resistant isolates

		Isolate								
		Klebsiella species			Proteus.merabilis			Proteus.valgaris		
		Count	%*	%**	Count	%*	%**	Count	%*	%**
Bla-TEM	Not detected	3	10.0	100.0	18	60.0	60.0	9	30.0	100.0
	Detected	0	0.0	0.0	12	100.0	40.0	0	0.0	0.0
Bla-SHV	Not detected	0	0.0	0.0	21	77.8	70.0	6	22.2	66.7
	Detected	3	20.0	100.0	9	60.0	30.0	3	20.0	33.3
Bla-CTXM	Not detected	0	0.0	0.0	12	80.0	40.0	3	20.0	33.3
	Detected	3	11.1	100.0	18	66.7	60.0	6	22.2	66.7

*Percentage among the corresponding gene.
**Percentage among the corresponding isolate.

DISCUSSION

Accompanying-to some extent with our results, DFU infection was reported to mostly be polymicrobial by [10]. In the year 1996, a study conducted earlier by Tan and colleagues concluded the same finding in Ohio, as single isolates were found in 21.1% of the study sample, and multiple organisms in 75.2 % [11].

Proteus mirabilis is the most predominant (45%) in our study. However, antagonizing our results; [12] several studies from different regions concluded that *S.*

aureus is the predominant. Sharma and colleagues indicated that *S. aureus* is the most predominant (38.4%) isolate followed by *P. aeruginosa* (17.5%) and *Proteus mirabilis* follows as low as (14%) [10-14].

Ako Nai and colleagues in their work characterized the bacteria isolated from DFU infection in Nigeria; (47.6%) were *S. aureus*, coagulase negative *staphylococci* (33%) and *Proteus species* comes third with frequency of 32.9%, *E. coli* 26.1%, *P. aeruginosa* 12.5%, *Klebsiella* species. 11.3%, *Enterobacter cloaca* 5.6%, *Citrobacter freundii* 6.8%. *Serratia* species contributed as 3.4% of all isolates in their study, which is nearly close to our finding as illustrated in (Figure 2) [15].

Citron and his team in a multicentric trial (the SIDESTEP trial) conducted in United States of America on 433 moderate to severely infected DFU patients isolated a total of 1,607 organisms from 454 specimens. 83.8% had polymicrobial infection and 16.2% had monomicrobial infection. Antagonizing our finding; Gram-positive comprised 80.3% of the aerobic organisms. The predominant aerobic species were *S. aureus* (76.6%). *Enterococci* were found in 35.7% of the patients. Among Gram-negative rods, 19.7% were aerobic organisms. *P. aeruginosa* was the predominant species followed by *Proteus mirabilis* [16].

(Alavi and colleagues agreed to the gram negative predominance in our study. They concluded that aerobic Gram-positive bacteria accounted for 42.9 % and 54.8% were Gram-negative rods. However, they indicate *S. aureus* to be the most frequent microorganism (26.2%), while *P. mirabilis* is (9.5%) frequent [17].

Raja in his research as well concluded the gram negative predominance as 52% were aerobic gram negative bacteria while 45% showed gram positive aerobic bacterial infection, and also indicated the organism *S. aureus* as the most predominant (17%), *Proteus* species follow with close proximity as (15%) [18]. Banashankari and colleagues in their work revealed- agreeing to our result that *Proteus species* is the predominantly isolated pathogens. DSF bacterial predominance is not conclusive and apparently different from region to region. There are several results of DSF bacterial predominance in the literature [3, 19-22].

The infection of DFU and the drug resistance in this group of patients is an important and major health issue which needs to be addressed. Different populations of DSF patients show wide variation in the level of antibiotic resistance encountered. High prevalence of antibiotic resistance, especially MRSA, affects treatment decisions concerning wounds and raises the question of whether the empirical regimens could cover these resistant organisms. Whilst the additional impact of antibiotic-resistant organisms on wound healing is not known, overall, the morbidity, mortality and cost associated with infections in hospitalized patients caused by antibiotic-resistant organisms has been shown to be 1.3- to 2-fold higher than those infections caused by antibiotic-sensitive organisms [23, 24].

In the present study, resistance against imipenim and ceftazidime are 41% and 49%, respectively as illustrated in (Table 5). Abdulrazak and his colleagues antagonizing our result showed that imipenem, meropenem, and cefepime sensitivity were between 80-100%. Ako-Nai in their work showed the resistance to erythromycin as 67.1% and tetracycline 61.4%, while in our study they were 40% and 75.4%, respectively as illustrated in (Table 5). Agreeing with our findings, they determined the resistance to chloramphenicol to be 45.7%. However, resistance to Augmentin was reported in their study as 38.6% of the isolates while it flares up to 90% in isolates of the current study [13, 15].

Alavi in their research revealed that *Pseudomonas aeruginosa* was resistant to all antibiotics used and have 100% sensitivity to Ciprofloxacin. However, contradicting our findings; more than 25% of *Pseudomonas aeruginosa* isolates in the current study are resistant to the antibiotic Ciprofloxacin, and 100% of *Pseudomonas aeruginosa* are sensitive to Amikacin [17].

Raja in his work reported the resistance to Methicillin in *S. aureus* isolates as 16%, while sensitivity to Vancomycin is reported in several studies to be 100% sensitive. Nevertheless, in the present study resistance to Oxacillin among *S. aureus* was found to be 100% and sensitivity of Vancomycin was found to be 75% [16, 18, 25, 26].

Lily and colleagues reported that 98% of Gram negative isolates as Imipenem sensitive, and 74% of *P. aeruginosa* as sensitive. However, only 55% of Gram negative isolates in the current study are found to be sensitive to the antibiotic Imipenem, and only 60% of *P. aeruginosa* are found to be sensitive [27].

Zubair and colleagues showed higher percentage of antibiotic resistance (67.1%) by coagulase-negative staphylococcal species. Accompanying to their result; coagulase-negative staphylococcal species are found to show high patterns of resistance as they found to be resistant to Erythromycin, Fusidic Acid, Cloramphenicol, Vancomycin, Gentamycin, Penicillin, Amoxicillin - clavulinic acid, Oxacillin as (60%, 60%, 60%, 60% 100%, 100%, 100% and 100%, respectively) [20].

Currently, there is paucity of data on the epidemiology of ESBL producing organisms isolated from DFU patients in the World. Furthermore, the extensive studies on infection with ESBL producing organisms in DFU patients in Sudan are scarce. In

India, Mathur and collaegues from India have reported 68.5% of their DSF isolates as ESBL producers [28]. Babypadmini &Appalaraju in their work have shown that 40% of *K. pneumoniae* isolates and 41% of *E. coli* isolates are ESBL producers in their study in India as well [29]. The prevalence of ESBL was found to be 6% in *E. coli* isolated from DSF patients among Brazilians [24]. Gadepalli and colleagues have reported that 54.5% of *E. coli* isolates as ESBL producers in diabetic foot infections in India [30]. Umadevi and his team determined that 56% of *Enterobacteriaceae* member were ESBL producers, in which 62.5% of *Proteus* species were ESBL positive followed by *Klebsiella pneumoniae* (60%) and *Escherichia coli* (56%). In our study more than 84% of tested Gram negative isolates are found to be ESBL producers. However, only multi-drug resistant organisms are tested for ESBL production in the current study [31].

CONCLUSION

This study showed a preponderance of gram negative bacilli among the isolates from diabetic foot infections as well as tremendous drug resistance of concern. Moreover, several indications are pointed toward possible contamination sources of microorganisms; further research is needed to support these findings.

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Competing Interest

The authors declare that they have no competing interests.

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