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Incidence and Antibiogram of β Lactamases-Producing *Citrobacter freundii* Recovered from Clinical Isolates in Peshawar, Pakistan

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SK performed the lab work. NR, Asadullah and IK collected and processed the samples. RT supervised and conceptualized the work. SUR analysed results and finalized the manuscript.

Key words
***Citrobacter freundii*, Metallo-β lactamase, Extended spectrum *β*-lactamases, Antimicrobial susceptibility.**

ABSTRACT

Citrobacter freundii, a common cause of nosocomial infections leading to diarrhea, urinary tract infection and meningitis, is increasingly becoming multidrug resistant. The current study was aimed to determine the prevalence and antibiotic susceptibility pattern of extended spectrum *β*-lactamases producing *C. freundii* clinical isolates. A total of 2950 clinical samples were collected from both hospitalized and non-hospitalized patients of Khyber Teaching Hospital (KTH), Peshawar, and processed by for isolation of *C. freundii* followed by phenotypic detection of *β*-lactamases and antimicrobial susceptibility profile. Our results indicated that a total of 130(4.40%;130/2950) samples were positive for *C. freundii* comprised of urine 58.46%, pus 33.85% and wounds 7.69%, respectively. More samples from females (n=70) than males (n=60) with majority (43.08%) from age group 21-40 years were found positive for *C. freundii*. Of the 130 isolates, 28 (21.53%) were ESBL producers comprised of 10 (35.71%) from males and 18(64.29%) from females with majority recovered from age group of 21–40 years (57.14%). Furthermore, 10(7.69%) isolates were metallo-β lactamase (MBL) producers containing 4(40%) from males and 6(60%) from females with majority from age group of 41–60 years (60%). Of the ESBL producers, 78.57%, 71.42%, 64.29%, and 42.86% isolates displayed resistance against trimethoprim-sulphamethoxazole, levofloxacin, ciprofloxacin and piperacillin-tazobactam, respectively, while, tygacil and colistin showed 100% susceptibility. On the other hands, 60%, 80%, 40% and 20% of metallo-β lactamase producers were found resistant to trimethoprim-sulphamethoxazole, levofloxacin, ciprofloxacin and piperacillin-tazobactam, respectively. Overall, current results show a high number of isolates resistant to commonly used drugs, and hence appropriate usage of antibiotics must be ensured to avoid further dissemination.

INTRODUCTION

Antimicrobial resistance (AMR) is a global threat and it is more challenging for developing countries like Pakistan (Khattak *et al.,* 2018; Rahman *et al.,* 2018). AMR is one of the serious problems of health care systems of Pakistan. Antibiotics are available without prescription in Pakistan and hence are excessively used. On top of this, due to lack of nationwide surviellance of AMR, its extent and pattern of emergence and evolution is not known. Random reports show that resistance microbes have been reported from different origins such as environment, food-producing animals, patients and community depicting the widespread dissemination of resistant microbes (Adnan *et al.,* 2017; Ali *et al.,* 2016, 2017; Hussain *et al.,* 2014a, b; ur Rahman *et al.,* 2018). Particularly, resistance to *β*-lactams and carbapenems is quite alarming as these drugs are proven safe and efficient, but are losing its value and efficacy due to emerging AMR. Bacteria achieve resistance to *β*-lactams by acquiring the ability to produce extended spectrum β-lactamases (ESBL) enzymes that inactivate many antimicrobials including third-, fourth-generation cephalosporins and monobactams, however, could not inactivate cephamycins and carbapenems. Similarly, resistance to carbapenems is mainly achieved by expression carbapenemase enzymes such as metallo-β-lactamase enzymes (El Salabi *et al.,* 2013; ur Rahman *et al.,* 2018a; Ullah *et al.,* 2009). Metallo-β-lactamase enzymes are ambler class B carbapenemase enzyme of mainly five types (IMP, VIM, SPM, GIM, and SIM), offering resistance against all β-lactam drugs except aztreonam thereby reducing the value of carbapenem drugs (Nordmann and Poirel, 2002).

Citrobacter freundii is a member of the genus

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Citrobacter within the family of Enterobacteriaceae and is known to reside as commensal in the digestive tract of human and animals. However, in certain situation, by expressing various virulent factors, it can lead to severe infections of digestive and urinary tract leading to diarrhea, food poisoning and urinary tract infection *etc*. (Bai *et al.,* 2012). In certain cases, *C. freundii* infections can be fatal, with over all death rates of 33-48% in adults while around 30% among neonates (Begum *et al.,* 2013). Antibiotic resistance and mainly resistance against cephalosporins and carbapenem drugs among *C. freundii* has been increased worldwide mainly due to expression ESBL and MBL enzymes (Choi *et al.,* 2007). However, unfortunately, incidence of ESBL- and MBL- producing-*C. freundii* and its resistance profile has not been reported from Pakistan. We have isolated *C. freundii* from different sites of indoor and outdoor patients who visited Khyber Teaching Hospital and assayed the ability of these species for the production of ESBL and MBL and its antimicrobial resistance profile. The information would be useful for clinicians, policy makers and global scientific community.

MATERIALS AND METHODS

Ethical approval

The study was approved from departmental ethical review committee of the Institute of Chemical Sciences, University of Peshawar and samples were collected and processed following local and national guidelines of ethics.

Study design

This is a cross sectional study performed during July, 2016 to July, 2017. Samples were clinical specimens (pus, urine and fluids) of both genders of all age groups, and patients on treatment or with parasitic and/ or fungal infections were excluded from the current study.

Sample collection

A total 2950 clinical samples (pus, urine and fluids) were collected in sterile conditions from both hospitalized (indoor patients-IDP n=1906) and non-hospitalized patients (outdoor patients-ODP n=1044) of Khyber Teaching Hospital (KTH), Peshawar according to the guide lines of Clinical Laboratory and Standard Institute (2014). The collected samples were processed in Microbiology Section, Department of Pathology, KTH.

Isolation of C. freundii

The collected clinical samples were inoculated on various culture media like Blood Agar, MacConkey Agar and Cysteine Lactose Electrolytes Deficient Agar and were incubated for 24 h at 37°C for the bacterial growth. Gram negative bacteria were purified by sub culturing. *Citrobacter* were identified by colonial characteristics, gram staining and biochemical profile as described earlier (Liu *et al.,* 2018).

Determination of phenotypic expressions

Extended spectrum-β-lactamase

Phenotypic expression of ESBL was confirmed by double disc synergy test on Mueller Hinton agar using following guidelines of CLSI (2014). *K. pneumoniae* ATCC 700603 was used as positive and quality control.

Metallo-β-lactamase (MBL)

MBL production was detected by double disc potentiation method using Ethylene Diamine Tetra Acetic acid (EDTA) was used as inhibitor. Two discs of imipenem or meropenem were placed on inoculated MHA plate while 7ul of 0.5M EDTA solution was added to one imipenem or meropenem and incubated at 37°C for 24 h. Increase in zone size (>7 mm) in EDTA containing disc represented the MBL production as reported earlier (Pitout *et al.,* 2005).

Statistical analysis

For analysis of susceptibility and resistance to antibiotics, descriptive statistic was used. For this SPSS version 23 was used by implying Chi-Square test. P value ≤ 0.05 was considered as significant.

RESULTS

Prevalence of C. freundii *isolates*

A total of 2950 samples (urine pus and swabs of infected wounds) were collected from male and female patients. Out of 2950 samples 130 (4.40%) samples yielded the growth of *C. freundii*. Among the positive, 60 patients were males while 70 samples were females. Among 1044 OPD patients, 46 (4.4%) were positive for *C. freundii* while in 1906 IPD patients, 84 (4.4%) cases were positive. The highest prevalence was observed among the age group of 21-40 years (43.08%), followed by age groups 41-60 years (32.31%) and 11-20 years (12.31%), while the lowest prevalence was recorded in the age group of 00-10 and 61 and above years *i.e.* 6.15%. The high frequency of 76 (58.46%) was recorded in patients suffering from UTIs followed by systemic infections (SSIs) 44 (33.85%) and pulmonary infections 10 (7.69%) (Table I).

Frequency of extended spectrum-β-lactamase

The current study detected 28 (21.53%) clinical isolates of *C. freundii* as ESBL producers among all 130 clinical isolates. Amongst 28 ESBLs positive isolates, 10 (35.71%) were from males and 18 (64.29%) isolates were from females, while 18 were from hospitalized

patients and 10 cases were from non-hospitalized patients. The highest prevalence of ESBL producing *C. freundii* was observed in the age group of 21-40 years (57.14%), followed by in the age group 41-60 years (28.57%), while the lowest prevalence was observed in age group of 11-20 years (14.28%), 00-10 years and 61 and above years (0%) as shown in Table II.

Table I.- Frequency distribution of *C. freundii* in different types of clinical samples (n=130).

Features	Frequency	Percentage	P value
Gender			
Male	60	46.15%	0.182
Female	70	53.85%	
Age group			
00–10	8	6.15%	0.000
11–20	16	12.31%	
21–40	56	43.08%	
41–60	42	32.31%	
> 60	8	6.15%	
Specimen type			
Pus	44	33.85%	0.000
Urine	76	58.46%	
Wound	10	7.69%	
Clinical features			
UTI	76	58.46%	0.000
SSIs	44	33.85%	
PIs	10	7.69%	

Table II.- Frequency distribution of ESBLs producing *C. freundii* in clinical isolates (n=130).

Features	ESBLs positive n=28	ESBLs negative n=102	P value
Gender			
Male	10 (35.71%)	50 (49.02%)	0.211
Female	18 (64.29%)	52 (50.98%)	
Age group			
00 – 10	0	8 (7.84%)	0.178
11 – 20	4 (14.28%)	12 (11.76%)	
21 – 40	16 (57.14%)	40 (39.22%)	
41 – 60	8 (28.57%)	34 (33.33%)	
> 60	0	8 (7.84%)	
Specimen type			
Pus	10 (35.71%)	32 (31.37%)	0.094
Urine	14 (50%)	66 (64.71%)	
Wound	4 (14.29%)	4 (3.92%)	
Clinical features			
UTI	14 (50%)	64 (62.75%)	0.107
SSIs	10 (35.71%)	34 (33.33%)	
PIs	4 (14.29%)	4 (3.92%)	

Table III.- Frequency distribution of MBLs producing *C. freundii* in clinical isolates (n=130).

Features	MBL positive n=10	MBL negative n=120	P value
Gender			
Male	4 (40%)	56(46.66%)	0.685
Female	6(60%)	64(53.33%)	
Age Group			
00 – 10	0	8 (6.66%)	0.261
11 – 20	0	16 (13.33%)	
21 – 40	4 (40%)	52 (43.33%)	
41 – 60	6 (60%)	36 (30%)	
> 60	0	8 (6.66%)	
Specimen type			
Pus	4 (40%)	38 (31.67%)	0.597
Urine	6 (60%)	72 (60%)	
Wound	0	10 (8.33%)	
Clinical features			
UTI	6 (60%)	70 (58.33%)	0.619
SSIs	4 (40%)	40 (33.33%)	
PIs	0	10 (8.33%)	

Frequency of metallo-β-lactamase

A total of 10 (7.69%) MBL-producers were detected among all 130 isolates. Amongst these, 4 (40%) were recovered from males population and 6 (60%) were from females, while 8 were from admitted patients and 2 were from OPD patients. The highest prevalence of MBLs producers was detected in the age group of 41-60 years (60%) while the lowest prevalence was recorded in the age group of 21-40 years which is 40% as shown in Table III.

Antimicrobial susceptibility profile

A total of 12 various classes of antibiotics were tested against all these isolates. Results showed that tygacil (TGC) and colistin (CO) showed 100% susceptibility against the ESBL positive isolates followed by amikacin (AK), meropenum (MEM) and piperacillin-tazobactam (TZP) 57.14%), while the highest resistivity pattern was observed in trimethoprim-sulphamethoxazole (SXT) (78.57%) followed by levofloxacin (LEV) (71.42%), CH (64.29%) and TZP (42.86%) as shown in Table IV. Statistical analysis showed that there was significant difference among the ESBL positive and negative isolates against MEM, TZP and doxycycline (DO). A significant number of ESBL-producing *C. freundii* isolates were resistant to the above three antibiotics as compared to the non-ESBL producers.

Table IV.- Antibiotic resistance among ESBLs producing *C. freundii* in clinical isolates (n=130).

Antibiotics		ESBLs positive n=28	ESBLs negative n=102	P value
SCF	Sensitive	20 (71.42%)	88 (86.27%)	0.063
	Resistant	8 (28.57%)	14 (13.72%)	
TZP	Sensitive	16 (57.14%)	84(82.35%)	0.005
	Resistant	12 (42.86%)	18(17.65%)	
MEM	Sensitive	18 (64.29%)	92(90.20%)	0.001
	Resistant	10 (35.71%)	10(9.80%)	
AK	Sensitive	18 (64.29%)	80(78.43%)	0.124
	Resistant	10 (35.71%)	22(21.57%)	
DO	Sensitive	18 (64.29%)	30(29.41%)	0.001
	Resistant	10 (35.71%)	72(70.59%)	
CIP	Sensitive	10 (35.71%)	30(29.41%)	0.522
	Resistant	18 (64.29%)	72(70.59%)	
LEV	Sensitive	8 (28.57%)	38(37.25%)	0.395
	Resistant	20 (71.42%)	64(62.75%)	
SXT	Sensitive	6 (21.42%)	18(17.65%)	0.648
	Resistant	22 (78.57%)	84(82.35%)	
CH	Sensitive	10 (35.71%)	64(62.75%)	0.011
	Resistant	18 (64.29%)	38(37.25%)	
FOS	Sensitive	22 (78.57%)	72(70.59%)	0.403
	Resistant	6 (21.42%)	30(29.41%)	
TGC	Sensitive	28 (100%)	102(100%)	-
	Resistant	0	0	
CO	Sensitive	28 (100%)	102(100%)	-

SCF, cefoperazone + sulbactam; TZP, piperacillin-tazobactam; MEM, meropenum; AK, amikacin; DO, doxycycline; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim-sulphamethoxazole; FOS, fosfomycin; TGC, tygacil; CO, colistin.

Similarly, antibiotic sensitivity of MBL-producers was also performed and compared with that of non-MBL producers. Results suggest all MBL-producers were found resistant to meropenum (Table V), while, TGC and CO showed 100% susceptibility. Other effective antibiotics were FOS, TZP, Cefoperazone + Sulbactam (SCF) with 80% susceptibility rate. Overall, results show that MEM, CO, FOS, TZP and SCF were found to be most effective antibacterials against under study *C. freundii* isolates.

DISCUSSION

Citrobacter is increasingly showing resistance against a large number of antimicrobial agents, particularly against *β*-lactam antibiotics by producing ESBLs and AmpC *β*-lactamases resulting major health threats (Liu *et al.,* 2018). Carbapenems were used as a first option for many infections during the last decades of 19th century, but now developing resistance due to bacterial ability to produce MBL enzymes (Kim and Lim, 2005). The current study showed 4.4% isolation rate of *C. freundii* 4.4% from different clinical samples (n=2950) obtained from different outdoor and indoor patients. Similar reports of 3.33% ESBL producers was reported in a study conducted in Germany (Wiegand *et al.,* 2007), and 5.72% in Aligarh India (Rizvi *et al.,* 2009). Contrary to our findings, few reports have shown increased frequency of ESBL producers of *C. freundii* like 13.81% and 8.79% from Korea and India, respectively (Kim and Lim, 2005; Oberoi *et al.,* 2013). This increased resistance show the ability of *C. freundii* to acquire resistance elements from the surroundings microbial population. These resistance elements are acquired by bacteria in response to excessive use of antibacterials creating passive pressure in analogy to the toxin producing phenomenon of gram negative bacteria (ur Rahman *et al.,* 2014; ur Rahman and van Ulsen 2013; van Ulsen *et al.,* 2014). Gram negative bacteria mostly produce various kinds of toxins in response to specific signals or molecules in the surrounding.

Table V.- Antibiotic resistance among MBLs producing *C. freundii* in clinical isolates (n=130).

Antibiotics		MBL positive n=10	MBL negative n=120	P value
SCF	Sensitive	8 (80%)	102 (85%)	0.674
	Resistant	2 (20%)	18 (15%)	
TZP	Sensitive	8 (80%)	92 (76.66%)	0.810
	Resistant	2 (20%)	28 (23.33%)	
MEM	Sensitive	00	110 (91.66%)	-
	Resistant	10 (10%)	10 (8.33%)	
AK	Sensitive	8 (80%)	90 (75%)	0.724
	Resistant	2 (20%)	30 (25%)	
DO	Sensitive	4 (40%)	44 (36.66%)	0.834
	Resistant	6 (60%)	76 (63.33%)	
CIP	Sensitive	6 (60%)	34 (28.33%)	0.37
	Resistant	4 (40%)	86 (71.66%)	
LEV	Sensitive	2 (20%)	44 (36.66%)	0.290
	Resistant	8 (80%)	76 (63.33%)	
SXT	Sensitive	4 (40%)	20 (16.66%)	0.068
	Resistant	6 (60%)	100 (83.33%)	
CH	Sensitive	4 (40%)	70 (58.33%)	0.261
	Resistant	6 (60%)	50 (41.66%)	
FOS	Sensitive	8 (80%)	86 (71.66%)	0.572
	Resistant	2 (20%)	34 (28.33%)	
TGC	Sensitive	10 (100%)	120 (100%)	-
	Resistant	00	-	
CO	Sensitive	10 (100%)	120 (100%)	-
	Resistant	00	-	

For abbreviatios, see Table IV.

In the current study, we analyzed samples from both indoor and outdoor patients to assess diversity in patterns of ESBL and MBL production as well as antimicrobial susceptibility. Results showed that ESBL producers from both outdoor and indoor patients were similar (4.4%), while interestingly, almost all MBL producers were isolated from indoor patients suggesting a possible hospital acquired features of the isolates. This goes along with 28 ESBL producers comprising a total of 18 isolates recovered from indoor patients strongly suggesting that ESBL and MBL expressing genetic elements are widespread in hospitals and nosocomial infections with these organisms may lead to severe morbidity and mortality. Finally, the co-occurrence of high level of ESBL or MBL producers among the hospitalized patients particularly among age group of 21-40 years is alarming.

We observed that 21.53% of *C. freundii* isolates were ESBL producers in agreement with earlier reports from Islamabad, Pakistan (Begum *et al.,* 2013). In contrast, high prevalence of 35.4% ESBL producers has been reported in India (Rizvi *et al.,* 2009). Generally speaking, high prevalence of ESBL producing microorganisms are observed in developing countries mainly where the infections control systems are not fully developed and most of the peoples get their treatment through self-medication. Thus excessive usage of antimicrobials lead to emergence of AMR. ESBL producers generally exhibit MDR phenotype by showing resistance to more than two classes of antibiotics. Our results mulls this general notion as most of the ESBL or MBL producers were found to be MDR showing resistance against more than two classes of antimicrobials. Other reports from Pakistan suggest a widespread prevalence of ESBL producers (Khan *et al.,* 2010a, b). All MBL producers were found resistant to meropenum, while they were sensitive to colistin. Surprisingly, ESBL expression did not actually increase the spectrum of resistance among majority of the isolates. Only four ESBL-producing isolates showed high level of resistance to a number of antibiotics as compared to non-ESBL producers (Table IV).

CONCLUSIONS

A total of 130 isolates (4.40%) were recovered out of which 28(21.53%) were ESBL producers, while 10 (7.69%) were MBL producers. Majority of the ESBL and MBL producers were recovered from indoor patients. All these isolates showed a varied degree of resistance against commonly used drugs with tygacil and colistin found to be the most effective antibiotics. Overall, these results suggest prudent use of antimicrobials and initiation of an overall structural surveillance program to monitor the usage of antibiotics and emergence of AMR.

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Statement of conflict of interest

The authors declare no conflict of interest.

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