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Molecular Detection of Beta-lactamase Genes in *Proteus* Species Isolated From Urinary Tract Infections in a Pakistani Hospital

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ABSTRACT

Antimicrobial resistance in *Proteus* species (spp)is increasing, such as the resistance to cephalosporins due to the production of extended-spectrum β-lactamases (ESBL) genes in hospitalized patients, which is a matter of great concern.For this purpose, 150 *Proteus* spp.isolates were collected from urinary tract infection (UTI) patientsby standard microbiological procedures.Phenotypic detection of ESBLs, MBLs, and pAmpC was conducted by combined disk diffusion and Double Disk Synergy Test (DDST). Conventional PCR was done for*ureC*and NDM-1, while ESBLs, MBLs, and pAmpC genes were screened through multiplex PCR.Of the total, 50 were confirmed as *Proteus* spp. (*Proteus mirabilis* (46) and *Proteus vulgaris*(4)).All isolates displayedβ-lactam resistance. The highest resistance was examined against Erythromycin (80%) and Rifampicin (72%), while resistance towards Levofloxacin and Imipenem was noticed as 60% and 58% respectively. Detection of ESBLs, MBLs, *pAmpC,* and NDM-1 were recorded as 48%, 28%, 36%,and 56% respectively.This is probably the first report from Pakistan reporting the high prevalence of β-lactamase genes in *Proteus* spp. of hospital origin. The current status of multidrug resistance(MDR) and increase in β-lactamase production in *Proteus* spp.in a Pakistani hospital is very alarming for healthcare workers.

Key Words: ESBLs, MBLs, Antibiotic resistance, NDM-1, *Proteus* spp.

Introduction

Urinary tract infections (UTI) are applied to various clinical conditions; asymptomatic to symptomatic and cause severe infections of the kidney with resulting sepsis (1). The most common organisms isolated from UTI infections are the members of the *Enterobacteriaceae*, such as *Escherichia coli* causes 65-90% of UTIs, while *Proteus*spp. are involved in 46% of cases (2).*Proteus mirabilis* (*P. mirabilis*) involved in empyema, meningitis, gastroenteritis, and osteomyelitis, causes nosocomial infections of the lower respiratory tract (30%),surgical wounds (24%), and bacteremia(17%), in elderly patients (3).

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In Pakistan,one report observed a 6.5% prevalence of *Proteus* spp. in pregnant UTI patients (4). Another study recently reported the prevalence of *Proteus mirabilis* with 13.8% (5).*Proteus* spp.shows resistance to various classes of antibiotics(6) such as Penicillin, Imipenem, Ureido-penicillin, and Cephalosporin which are mainly plasmid-mediated.Multidrug-resistant(MDR) *Proteus mirabilis* withpAmpC-typechromosomallyencodedβ-lactamase was reported in Europe (7,8).

Intrinsic resistancewasfound against tetracyclineandnitrofurantoinin *Proteus* spp.(9),while susceptibility to aminoglycosides, trimethoprim-sulfamethoxazole, and fluoroquinolones,co-resistance to these drugs was recurrently examined among ESBLs producers in *Proteus* species(10).

Transmission can occur by direct contact with a colonized carrier, extensively dispersed in the soil, manure, natural environment, and contaminated water. *P. mirabilis* is usually found in the gastrointestinal tract and periurethral area of humans. *Proteus* spp. spread easily from species to species, so its transmission between animals and human beings can occur (11).Like other *Enterobacteriaceae*members, MDR *Proteus* spp. emergesdue to the inappropriate and misuse of frequently used antibiotics in clinical practices, causing health complications(12). This study was aimed to isolate and *Proteus* spp.from UTI patientsscreenfor different antibiotics and their respective antibiotic resistance genes, through conventional and multiplex PCR.

Materials and Methods

A total of 150 urine samples were collected from UTIpatientsfromthe Urology ward at Hayatabad Medical Complex (HMC), Peshawar, and brought to the microbiology laboratory within 2 hours at 4°C. The isolates were thentransported to the Department of Biotechnology and Genetic Engineering, Kohat University of Science and Technology (KUST) Kohat, Pakistan.

Microbiology and Molecular Procedures

Isolates were cultured on Nutrient, MacConkey, and on Blood agar, (Oxoid Cambridge, UK)and incubated at 37ºC for 24 hours.The isolates were identified by using biochemical tests. The genomic DNA of all isolates was isolated, using the boiling method (13). Molecular identification was done by using species-specific primers for the *P. mirabilisureC* gene, as shown in Table 1, using conventionalPCR (Thermocycler- Applied Biosystem, Singapore) (14). PCR product was run on gel electrophoresis (Fischer Scientific, UK) and analyzed ongel documentation system (Boimetra, Germany) using DNA marker of 100bp (Thermo Scientific, UK) as standard.

Table 1: Primers for detection of *Proteus* species

Organism	Gene	Primers	Amplicon size
Proteus spp	ureC1 ureC2	5'-CCGGAACAGAAGTTGTCGCTG- GA-3' 5'-GGGCTCTCCTACC GACTTGATC-3'	533-bp

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was done on Mueller-Hinton agar (MHA) media (Oxoid, England) for all the isolates,using a panel of antibiotics like,Ceftazidime(30µg),Azithromycin(15µg),Levofloxacin(5µg),Ceftriaxone(30µg),Sulfamethox

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azole/trimethoprim(24/1.25µg),Imipenem(10µg),rifampicin(5µg),Amoxicillin(25µg),Ciprofloxacin(5µg),Erythromycin(15µg),Doxycycline(30µg),Chloramphenicol(30µg),Gentamicin(10µg),Streptomycin(10µg),Cephalothin (30µg) and Amikacin (30µg),using Kirby-Bauer disc diffusion method (15). *E.coli* (ATCC 25922) was used as a reference strain. Interpretation of the results was done by following Clinical and Laboratory Standards Institute (CLSI) guidelines, 2017.

Phenotypic detection of Beta-Lactamases

Double Disk Synergy Test (DDST)was done forexamining phenotypic detection of ESBLs.EDTA-inhibition testwas performed for MBLs, whilecombined disk diffusion method was followedfor pAmpC in table 2.1, 2.2, 2.3, as described previously(16,17).

Table 2.1: Antimicrobials agent with their codes and potencies for MBL

Sr.No	Antibiotics Used	Class of Antibiotics	Code	Concentration
1	Imipenem	Carbepenams	IMP	10µg
2	Imipenem-EDTA	Carbepenams/EDTA	IMP-EDTA	10/10µg
3	EDTA	EDTA	EDTA	10µg

Table2.2: Antimicrobials agent with their codes and potencies for ESBLs

Sr.No	Antibiotics Used	Class of Antibiotics	Code	Concentration
1	Ceftriaxone	Cephalosporins	CRO	30µg
2	Ceftazidime	Cephalosporins	CAZ	30µg
3	Cefotaxime	Cephalosporins	CTX	30µg
4	Aztreonam	Monobactam	ATM	30µg
5	Amoxicillin/clavulanic acid	Penicillin/beta-lactamase inhibitors	AMC	20/10µg

Table 2.3: Antimicrobials agent with their codes and potencies for AmpC

Sr.No	Antibiotics Used	Class of Antibiotics	Code	Concentration
1	cefoxitin	Cephalosporins	FOX	30µg
2	cefotaxime	Cephalosporins	CTX	30µg

Genotypic detection of beta-lactamase genes

Phenotypically confirmed MBL, ESBLs, and *pAmpC*were further processed through multiplex PCR for ^{bla}*SHV*, ^{bla}*TEM*, and ^{bla}*CTX-M-U1/U2*genes, in Table 3 from already published data (16). Phenotypically MBL producing isolates were screened for the presence of*imp*, *vim*, *gim*, *spm*, and *sim* genes in Table 4(17), and plasmid-encoded pAmpC, isolates were screened for the^{bla}MOXM, ^{bla}CITM, ^{bla}DHAM, ^{bla}ACCM, ^{bla}EBCM, and ^{bla}FOXM genes using primers mentioned in Table 5(18).All the *Proteus* isolates were screened for NDM-1 in Table 6(19).

Table 3: Primers for detection of ESBLs

Gene	Primers	Amplicon size

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Bla-SHV.SE-F BlaSHV.AS-R	5' - ATGCGTTATATTCGCCTGTG -3' 5' - TGCTTTGTTATTCGGGCCAA -3'	747bp
TEM-164.SE-F TEM-165.AS-R	5'- TCGCCGCATACACTATTCTCAGAATGA -3' 5' - ACGCTCACCGGCTCCAGATTTAT -3'	445bp
CTX-M-U1 CTX-M-U2	5' - ATGTGCAGYACCAGTAARGTKATGGC -3' 5'TGGGTRAARTARGTSACCAGAAYCAGCGG -3'	593bp

Table 4: Primers for detection of MBL

Gene	Primers	Amplicon size
Imp-F Imp-R	5' -GGA ATA GAG TGG CTT AAY TCT C -3' 5' - CCA AAC YAC TAS GTT ATC T -3'	188 bp
Vim-F Vim-R	5' - GAT GGT GTT TGG TCG CAT A -3' 5' - CGA ATG CGC AGC ACC AG -3'	390 bp
GIM-F GIM-R	5' - TCG ACA CAC CTT GGT CTG AA -3' 5' - AAC TTC CAA CTT TGC CAT GC -3'	477 bp
Spm-F Spm-R	5' - AAA ATC TGG GTA CGC AAA CG -3' 5' - ACA TTA TCC GCT GGA ACA GG -3'	271 bp
Sim-F Sim-R	5' - TAC AAG GGA TTC GGC ATC G -3' 5' - TAA TGG CCT GTT CCC ATG TG -3'	570 bp

Table 5: Primers for detection of AmpC

Gene	Primers	Amplicon size
MOXMF MOXMR	5' - GCT GCT CAA GGA GCA CAG GAT -3' 5' - CAC ATT GAC ATA GGT GTG GTG C -3'	520bp
CITMF CITMR	5' - TGG CCA GAA CTG ACA GGC AAA -3' 5' - TTT CTC CTG AAC GTG GCT GGC -3'	462bp
DHAMF DHAMR	5' - AAC TTT CAC AGG TGT GCT GGG T -3' 5' - CCG TAC GCA TAC TGG CTT TGC -3'	504bp
ACCMF ACCMR	5' - AAC AGC CTC AGC AGC CGG TTA -3' 5' - TTC GCC GCA ATC ATC CCT AGC -3'	346pb
EBCMF EBCMR	5' - TCG GTA AAG CCG ATG TTG CGG -3' 5' - CTT CCA CTG CGG CTG CCA GTT -3'	302bp
FOXMF FOXMR	5' - AAC ATG GGG TAT CAG GGA GAT G -3' 5' - CAA AGC GCG TAA CCG GAT TGG -3'	190bp

Table 6: Primers for detection of NDM-1

Gene	Primers	Amplicon size
NDM-1-F NDM-1-R	5' - ACC GCC TGG ACC GAT GAC CA -3' 5' - GCC AAA GTT GGG CGC GGT TG -3'	264bp

Results

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Out of 150 isolates, 50 isolates were confirmed as *Proteus* spp. Forty-six (46)were *P. mirabilis* while 4 were *P.vulgaris*. All the *P. mirabilis*isolates were positive for*ureC*gene, while in the case of *P.vulgaris*no *ureC* was detected.

Age-Wise Distribution of *Proteus* Infection

Age-wise distribution showed that *Proteus* spp.was more common in females(30%), as compared to males(20%), in Table 7.

Table 7: Samples Distribution of Patients (age)

Age group	Sample size	*Proteus* spp
10-20	15(30%)	*Proteus mirabillis*
21-30	24(48%)	*Proteus mirabillis, Proteus vulageris*
31-40	7(14%)	*Proteus mirabillis, Proteus vulageris*
41-50	4(8%)	*Proteus mirabillis, Proteus vulageris*

Antibiotic Susceptibility Test

Antibiotic sensitivity profile of *Proteus* spp. against16 different antimicrobial agents showed no 100% effectivenessagainst *Proteus* spp.Maximum resistance was examined against erythromycin (80%) in *Proteus* spp. followed by rifampicin and streptomycin, with resistance patterns as 72% and 70%, respectively. Amoxicillin and ceftriaxone displayed 68%resistance. The highest sensitivity was observed in 60%isolates against levofloxacin and imipenem (58%), while 50%and 48% sensitivity was observed in the case of ciprofloxacinand ceftazidime respectively, inFigure 1.

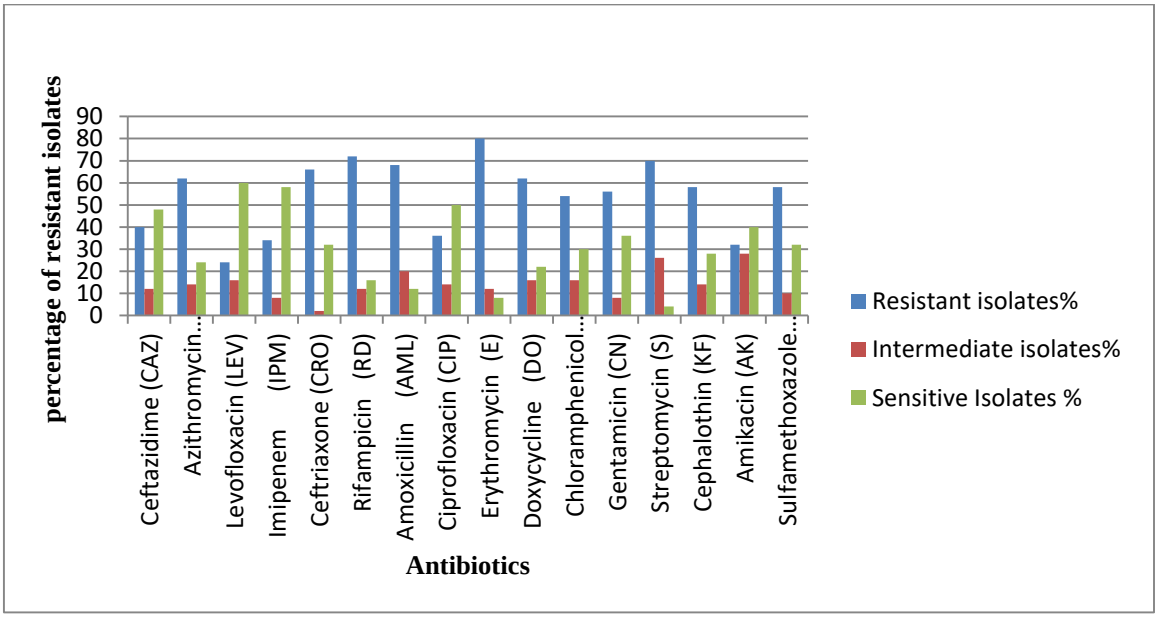

Figure 1: Pattern of Antibiotic susceptibility profile in *Proteus* spp.

Molecular detection of ESBLs

Out of 50 isolates, twenty-four (48%) isolates were ESBLs positive in which *bla*TEM was detected in 16.6% in*P. mirabilis*, while 7% in *P. vulgaris*.*bla*CTXwas displayed by 45% in *P. mirabilis* and 4% in*P. vulgaris*. *bla*TEM+*bla*CTX with25% noticed in *P. mirabilis*while

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absentin *P. vulgaris. bla*SHV was absent both in *P. mirabilis* and *P. vulgaris* as shown in Table 8.

Table 8: ESBLsgenes detected

Genes Detected	Species	Number of Isolates	% of Genes	Source
TEM	*P mirabilis*	4	16.6	
	P vulageris	2	7	Urine
CTX	*P mirabilis*	11	45	
	P vulageris	1	4	Urine
TEM+CTX	*P mirabilis*	6	25	
	P vulageris	0	0	Urine
SHV	*P mirabilis*	0	0	Urine
	P vulgaris	0	0	

Molecular Detection of MBL

bla$_{IMP}$ was detected in 14% *P. mirabilis* and 7% in *P.vulgaris.bla*$_{VIM}$ was observed in 21% *P. mirabilis* and 0% in *P.vulgaris.bla*$_{GIM}$ gene was observed in 14% *P. mirabilis* and 0% in *P.vulgaris.bla*$_{SPM}$ with 21% in *P. mirabilis* and 0% in *P. vulgaris.bla*$_{SIM+IMP}$was share by 14% isolates in the case of *P. mirabilis* while 7% in *P. vulgaris*, by multiplex PCR. Fourteen (28%) isolates were positive for MBL genes, in Table 9.

Table 9: MBL genes detected

Genes Detected	Species	Number of Isolates	% of Genes	Source
VIM	*P. mirabilis*	3	21	Urine
	P. vulageris	0	0	
IMP	*P. mirabilis*	2	14	Urine
	P. vulageris	1	7	
GIM	*P. mirabilis*	2	14	Urine
	P. vulageris	0	0	
SPM	*P. mirabilis*	3	21	Urine
	P. vulageris	0	0	
SIM+IMP	*P. mirabilis*	2	14	Urine
	P. vulageris	1	7	

Molecular detection of *pAmpC*

bla*$_{MOXM}$*,*bla$_{FOXM}$*,*bla*$_{EBCM}$, was absent in both*P. mirabilis* and *P. vulgaris*. *bla*$_{CITM}$,gene was detected in 27.7% in *P. mirabilis* and 0% in *P. vulgaris. bla*$_{DHAM}$ gene was displayed by 33.33%of *P. mirabilis* and 0% in *P.valgaris.bla*$_{ACCM}$,11.11% in *P. mirabilis*, and 0% in *P. vulgaris* through multiplex PCR, 18 (36%) isolates were pAmpCpositive. Both *bla*$_{ACCM}$+*bla*$_{DHAM}$ were displayed by 22.22% in *P. mirabilis* and 0% in *P. vulgaris*. *bla*$_{CITM}$+*bla*$_{DHAM}$wererecorded

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as 5.5% in *P. mirabilis* and 0% in *P. vulgaris*. Other genes such as *bla*$_{FOXM}$, *bla*$_{MOXM}$, *bla*$_{EBCM}$ genes were absent in both species (*P. mirabilis, P. vulgaris*)in Table 10.

Table 10: *pAmpC*genes detected

Genes Detected	Species	Number of Isolates	% of Genes	Source
CITM	*P. mirabilis*	5	27.7	Urine
	P. vulageris	0	0	
ACCM	*P. mirabilis*	2	11.11	Urine
	P. vulageris	0	0	
DHAM	*P. mirabilis*	6	33.33	Urine
	P. vulageris	0	0	
ACCM+DHAM	*P. mirabilis*	4	22.22	Urine
	P. vulageris	0	0	
CITM+DHAM	*P. mirabilis*	1	5.56	Urine
	P. vulageris	0	0	
MOXM	*P. mirabilis*	0	0	Urine
	P. vulageris	0	0	
FOXM	*P. mirabilis*	0	0	Urine
	P. vulageris	0	0	
EBCM	*P. mirabilis*	0	0	Urine
	P. vulageris	0	0	

Molecular detection of NDM-1

NDM-1 was examined in 28 isolates (56%) as 94.42% in *P. mirabilis* and 5.58% in *P. vulgaris* as shown in Table 11.

Table 11: NDM-1 gene detected

Gene Detected	Species	Number of Isolates	% of Gene	Source
NDM-1	*P. mirabilis*	27	94.42	Urine
	P. vulageris	1	5.58	

Discussion

To the best of my knowledge, this is thefirst report ofthe β-lactamases prevalence in *Proteus*spp. from Pakistan. In the current study,*Proteus* spp. showed that 46% isolates were multi-drug, the previous study from Pakistan reported approximately the same MDR *Proteus* spp. prevalenceof 52% from Peshawar (20).In Pakistan, most of the studies were limited only to the prevalence of *Proteus* spp. no comprehensive study was available on the carriage of β-lactamases (ESBLs, MBLs, *pAmpC*) in *Proteus* isolates.The emergence ofMDR *Proteus* strains in hospital patients presents a major health problem to clinicians with limited options, as these strains show resistance against commonly used antibiotics.The high rate of β-lactamase-producing *Proteus* pathogens could be due to poor hygiene, irrational and uncontrolled use of

broad-spectrum antibiotics, which left only limited options to be effective. The data about the mortality rate in these health care settings are not available, so we cannot comment on whether the mortality rate has been affected by these resistance pathogens or not. In*Proteus* spp. the prevalence of β-lactamases was found to be as high as 56% which is an alarming situation for clinicians which requires immediate considerations. In the present study, 50% of isolates were susceptible to ciprofloxacin, showing close resemblance with another study at Peshawar(20).Inthe current study, the highest resistance was observed against erythromycin 80%, rifampicin 72%, streptomycin 70%, amoxicillin 68%, and ceftriaxone 66% highest sensitivity was observed against levofloxacin, followed by imipenem 58%, ciprofloxacin 50%, and ceftazidime48%.Antibiotic susceptibility profile of *Proteus* spp. from different cities of Pakistan was different from each other. This may be due to the geographical location, sample size, and hospital policies.The MDR *Proteus*spp.presents major health problems, as these strains show resistance to various classes of antibiotics. The prevalence of *Proteus* spp. in different cities in Pakistan increased.In Karachi,*Proteus* spp. was found to be11% in 2004(21), 8% from Peshawar (20),and 1% in Lahore during 2008 and 2015(22),2.6% fromIslamabad (23),and 13.8% from Kohat(4).Since the early studies from different countries of the world have widely recorded the presence of ESBLs, MBLs, and *pAmpC* in *Proteus* spp. (24-30).In the current study, the prevalence of *Proteus* spp. was noticed as 33.33%, which is alarming for clinicians. The variation in the prevalence of *Proteus* spp. in different cities of Pakistan may be due to the geographical variation, sample size, hospital policies,misuse, and uncontrolled use of antibiotics.

Conclusion

The emergence of MDR *Proteus* spp.in hospital setup is threatening for public health. The high prevalence of β-lactamases is also alarming, as they regulate the expression of β-lactamasegenes, which is a challenge for clinicians.

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Conflict of interest: Nothing to declare.

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