Antimicrobial profiling and molecular characterization of antibiotic resistant genes of *Proteus vulgaris* isolated from tertiary care hospital, Islamabad, Pakistan

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Abstract: Urinary tract infections (UTIs) are among the most common bacterial infections acquired from hospitals and community. *Pseudomonas* and *Proteus* species are the common cause of these UTIs. Generally, UTIs are self-limiting but have potential to re-occur. Extensive treatment therapy with antibiotics lead to the development of resistance in uropathogens. The development of antibiotic resistance is leading to the failure of currently available antibiotic based therapies thus making the situation worse. The objective of the present study was to access antimicrobial sensitivity and to characterize antibiotic resistant genes of *Proteus vulgaris* (*P. vulgaris*) isolated from patients suffering with UTIs. A total of 150 urine samples were collected and cultured on MacConkey agar medium followed by isolation and identification on blood agar medium. Biochemical characterization of all presumptive Proteus isolates was done using Remel Rap ID one kit. Antibiotic sensitivity for *P. vulgaris* isolates was performed by disc diffusion method. Presence of *bla*TEM and *qnr* antibiotic resistant genes was determined by PCR. The results showed that the overall prevalence of *P. vulgaris* in clinical samples was 11.3%. It showed maximum resistance (94%) to three antibiotics i.e. ampicillin, tigecycline and chloramphenicol, while least resistance was observed against imipenem (12%). Statistical analysis depicted that imipenem had a significantly larger zone of inhibition *(P=.01)*, while ampicillin had significantly smaller zone of inhibition *(P=.0004)* followed by chloramphenicol (p-value = 0.002). Imipenem should be considered as an effective antibiotic to treat urinary tract infections associated with *P. vulgaris.* Both *bla*TEM and *qnr* genes were found to be involved in conferring resistance to β-lactam and quinolones antibiotics.

Keywords: Antibiotic resistance, *Proteus vulgaris,* antimicrobial profiling.

INTRODUCTION

Among all the bacterial infections that are acquired outside hospitals, urinary tract infections (UTIs) account for 20% of these infections (Negut and Buiuc, 2008). Approximately 95% of UTIs are associated with uropathogens that emerge from the patient's own gastrointestinal tract (GIT) flora (Stickler *et al.*, 2003). Proteus species are responsible for ascending UTIs (Stickler and Feneley, 2010). *Proteus vulgaris (P. vulgaris)* is an opportunistic, Gram negative rod shaped bacterium and a member of *Enterobacteriaceae* family that inhabits GIT of humans and animals as normal flora. Under favorable conditions, it causes several infections including wound infections, meningitis in infants and neonates, urinary tract infections (UTIs) and rheumatoid arthritis. As compared to other uropathogens, UTIs caused by Proteus species are much complicated and mostly accompanied by kidney stones formation. Proteus has many virulence factors including flagella, fimbriae, enzymes, and toxins such as hemolysins, Proteus toxin agglutinin (Pta), and endotoxin lipopolysaccharide (Rozalski *et al.,* 2012).

Antibiotic resistant bacteria are re-emerging worldwide and posing serious threat to the public health although, this resistance pattern varies in different geographical regions (McGregor *et al.,* 2014; Melaku *et al.,* 2012). Occurrence of multidrug resistant bacterial infections is due to extensive use of antibiotics in treating bacterial infections. In general, this widespread misuse of antibiotics has led to the development of bacterial resistance to these antibiotics (Manikandan *et al.,* 2011). Over the period of many years, resistance pattern is varying in uropathogens responsible for UTIs. Emergence of resistance by Proteus species to β-lactam and quinolones has also been reported (Karlowsky *et al.,* 2002). The objective of the current study is to check the prevalence of *Proteus vulgaris* in patients suffering from UTIs and to perform the antibiotic sensitivity profiling. The presence of two antibiotic resistance genes; *bla*TEM and *qnr,* is also detected.

MATERIALS AND METHODS

The study was conducted in compliance with local Institutional Bioethics Committee (Approval No.CE 278),

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University of Agriculture, Faisalabad, Pakistan. A written consent was taken from each patient.

Sample collection and processing

A total of 150 urine samples from patients suffering from UTIs were collected. All the samples were transferred to the microbiology laboratory at Institute of Microbiology, University of Agriculture, Faisalabad in transport media under ambient temperature. MacConkey agar and Blood agar media were used as selective media to study the specific colony characteristics of Proteus isolates. All media were sterilized by autoclaving at a temperature of 121°C for 15-20min/15lbs. Gram staining was performed to differentiate between Gram positive and Gram negative bacteria according to standard protocol (Jones *et al.,* 1981).

Biochemical characterization of Proteus isolates

Following biochemical tests were performed for biochemical identification of *P. vulgaris* isolates; catalase test, IMVIC test, nitrate reduction, H_2S production, methyl red test, urease production and lactose fermentation by following the Manual of Methods for General Bacteriology (Wikler *et al.,* 2007). Biochemical confirmation of all presumptive Proteus isolates was done using RemelRapID one kit (Thermo Fisher Scientific, Catalog # A39900).

Antibiotic sensitivity profiling of P. vulgaris isolates

Antibiotic sensitivity of *P. vulgaris* was checked against different antibiotic discs (Oxoid, UK) such as amikacin, ciprofloxacin, imipenem, chloramphenicol, ampicillin, nitrofurantoin, tigecycline, and cefotaxime. Kirby-Bauer disc diffusion method was used for antibiotic sensitivity. Zones of bacterial growth inhibition were measured and results were interpreted according to the guidelines of Clinical Laboratory Standard Institute (CLSI) (Bergallo *et al.,* 2006).

Detection of antibiotic resistance genes (blaTEM & qnr)

In order to detect the presence of both *bla*TEM and *qnr* genes, genomic DNA from clinical isolates of *P. vulgaris* was extracted by illustra bacteria genomicPrep Mini Spin Kit according to the manufacturers guide (Thermo Fisher Scientific, US). The extracted DNA was amplified in thermal cycler (Thermo Fisher Scientific, USA) by using specific primers described in Table 1. PCR products of both genes were subjected to gel electrophoresis with 1% agarose gel and 0.5µg/ml ethidium bromide in order to detect the successful amplification (Lee *et al.,* 2012).

STATISTICAL ANALYSIS

Data was analyzed using R software. Using linear model function (lm), it was tested if the inhibitory zones of various antibiotics differed significantly from each other or not.

RESULTS

Prevalence of Proteus spp.

Out of total 150 urine samples, only 17 samples (11.33%) were found positive for *P. vulgaris,* while 6% (9/150) and 2.6% (4/150) were positive for *P. mirabilis* and *P. penneri,* respectively. Rest of the isolates were *Pseudomonas, E. coli,* and *S. aureus* (fig. 1).

Cultural characteristics

Proteus isolates gave pale color colonies on MacConkey agar medium due to the presence of bile salts (fig. 2a). Fig.2b represents Proteus isolates showing different zones of swarming growth due to peritrichous flagella. Under

Table 1: List of primers used to amplify *bla*TEM and *qnr* genes

Target genes	Primer sequence	Product size	References
*bla*TEM	F 5'-AGAGCAACTCGGTCGCCGCATA-3' R 5'-GCGCAACGTTGTTGCCATTGCT-3'	310 bp	Amador *et al*., 2011
qnr	F 5'-ACGCCAGGATTTGAGCGACAGC-3' R 5'-CGCTGAGGTTGGCATTGCTCCA-3'	410 bp	Chen *et al*., 2008.

Table 2: Comparison between zones of inhibitions of different antibiotics

Antibiotics	Estimate	Standard error	t value	p Value	Mean of zone of inhibition mm	S.D.
Amikacin	-0.311	1.424	-0.200	0.6120	12.1176	4.526068285
Ampicillin	-5.176	1.424	-3.634	0.0004 **	6.9411	2.74933147
Cefotaxime	-1.882	1.424	-1.321	0.1887	10.2352	6.220223185
Chloramphenicol	-4.352	1.424	-3.056	0.0027 **	7.7647	4.131122907
Ciprofloxacin	-0.411	1.424	-0.289	0.7730	11.7058	6.01835428
Imipenem	3.588	1.424	2.519	0.0130 *	15.7058	2.257340966
Nitrofurantoin	-1.000	1.424	-0.702	0.4839	11.1176	2.847857812
Tigecycline	-0.529	1.424	-0.372	0.7107	11.5882	2.1811357

*Significant **Highly significant

microscope, Proteus appeared as short Gram-negative rods, which were pink in color (Fig. 3). Results of different biochemical tests (RemelRapID one kit) confirmed the presence of *P. vulgaris* in 17 samples.

Antibiotic susceptibility profiling

Fig. 4 represents the antibiotic sensitivity pattern exhibited by *P. vulgaris* to various antibiotics used in the current study. It was found that *P. vulgaris* isolates showed maximum resistance (94%) to three antibiotics; ampicillin, chloramphenicol, and tigecycline. On the other hand, they showed maximum sensitivity to imipenem (88%) followed by amikacin (59%) (fig. 5). When it was tested whether these antibiotics differed significantly from each other regarding their zone of inhibitions against test bacteria, the results showed that imipenem had significantly larger zones of inhibition, while ampicillin had significantly smaller zones of inhibition (table 2).

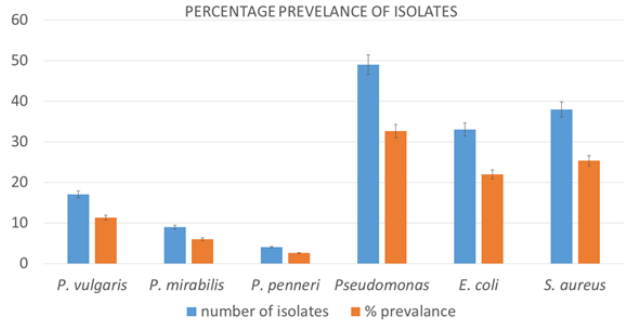

Fig. 1: Histogram representing the percentage prevalence of various bacterial isolates from clinical samples.

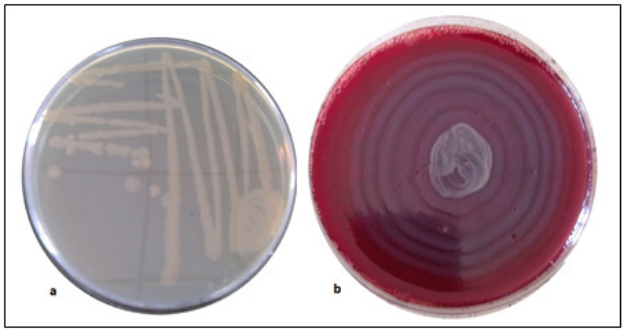

Fig. 2: a) Growth characteristics of Proteus isolates on MacConkey agar medium. b) Swarming growth of Proteus isolates on blood agar medium.

Detection of blaTEM and qnr genes

Out of 10 representative *P. vulgaris* isolates used for detection of antibiotic resistance genes, *bla*TEM gene was detected in all 10 isolates, while *qnr* gene was present in 5 (50%) of the isolates. Fig. 6 exhibits the amplified *bla*TEM and *qnr* genes with amplicon size of 310bp and 410 bps, respectively.

DISCUSSION

In the present study, out of 150 urine samples, 32.6% isolates were *Pseudomonas* followed by *Staphylococcus aureus* (25.3%), *E. coli* (22%) and *Proteus* spp. (19.9%). Among all Proteus isolates from the clinical samples, *P. vulgaris, P. mirabilis* and *P. penneri* account for 11.3%, 6% and 2.6%, respectively. Our results were in correspondence with other studies in which 7% prevalence for *Proteus* spp. in the urine samples was reported (Erum *et al.,* 2014). Our results were not in compliance with studies conducted by Lazm *et al.* (2018), in which a 33.3% prevalence rate of Proteus spp. in urine samples was reported. A similar higher prevalence (37.3%) of Proteus spp. was also observed by Laftaa (2001). The possible reason for such variation in prevalence rate of *P. vulgaris* could be attributed to different factors like duration of catheter, hospitalization, diabetes mellitus, and abnormalities in urinary tract in our study.

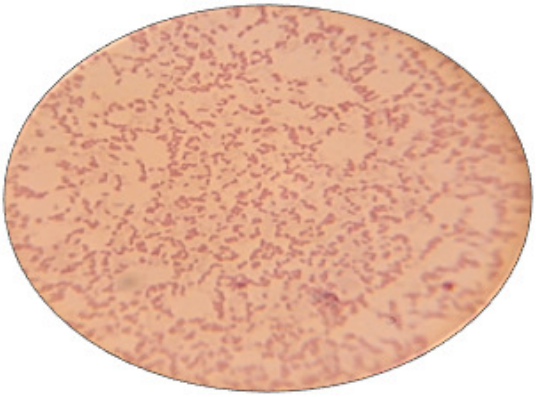

Fig. 3: Microscopic appearance of *P. vulgaris*. Under 100X (oil immersion lens), *P. vulgaris* appeared as Gram negative rods.

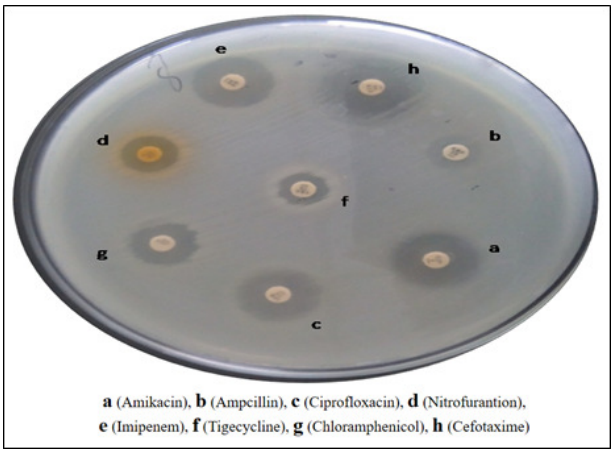

Fig. 4: Zone of inhibition of various antibiotics against *P. vulgaris* isolates. All the isolates showed similar pattern of inhibition zones for all the antibiotics used in this study. Small letters indicate the various antibiotic used in the current study; a (Amikacin), b (Ampicillin), c (Ciprofloxacin), d (Nitrofurantoin), e (Imipenem), f (Tigecycline), g (Chloramphenicol), h (Cefotaxime).

In present study, antimicrobial susceptibility of *P. vulgaris* showed that the percentage resistance of *P.*

vulgaris to different antibiotics was 94% in case of chloramphenicol, tigecycline, and ampicillin, 88% for cefotaxime, 76% for ciprofloxacin and nitrofurantoin and 50% in case of amikacin (59%). Similar findings were found in study conducted by Lazm *et al.* (2018) in which Proteus isolates were 93.3% and 80% resistant to amoxicillin and penicillin respectively, while 100% resistance was observed to cephalothin. Feglo *et al.* (2010) also found the similar resistance pattern for *P. vulgaris*. About 76% of *P. vulgaris* isolates were resistant to ciprofloxacin in present study which was not in compliance with previous study who reported a 53.4% isolates resistance to ciprofloxacin. The variation in resistance pattern of *P. vulgaris* isolates may be due to types of antibiotics and frequency in their use in different patients from whom the samples were taken. In another study, all *Proteus* isolates were found sensitive to ciprofloxacin (Fam *et al.,* 2013). However, studies of Daini *et al.* (2008) suggested the resistance of *P. vulgaris* to ciprofloxacin. Most of the Proteus isolates were resistant to amikacin. Okesola and Makanjuola (2009) reported contrasting results as their findings showed that *P. vulgaris* isolates were sensitive to amikacin. Lazm *et al.* (2018) also observed amikacin sensitivity to *P. vulgaris* isolates. *P. vulgaris* showed 70% resistance to nitrofurantoin. Some studies suggested contradictory findings as they found that *P. vulgaris* isolates were sensitive to nitrofurantoin (Schaeffer, 2003; Kippax, 1957). In the current study, only imipenem was found to be sensitive for 88% of *P. vulgaris* isolates, while sensitivity to all other tested antibiotics was less than or equal to 12%. Htoutou *et al.* (2011) also reported sensitivity of *P. vulgaris* to imipenem.

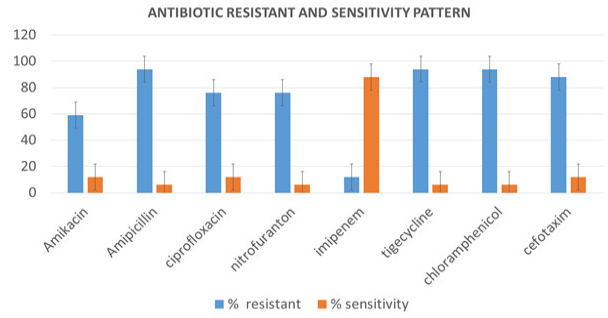

Fig. 5: Percentage resistance and sensitivity showed by *P. vulgaris* isolates against different antibiotics.

In our study we detected the occurrence of two important genes; *bla*TEM and *qnr*, which are thought to be involved in conferring resistant to β-lactam and quinolone antibiotics. β-lactam antibiotics are utilized on large scale to treat bacterial infections. Presence of *bla*TEM gene allows *P. vulgaris* to resist β-lactam antibiotics by producing wide range of β-lactamases (Bonnet *et al.,* 1999). PCR results of present study showed a 100% detection of the *bla*TEM gene in representative *P. vulgaris* isolates that were subjected to PCR mediated amplification. These results were in accordance with the results of others in which a higher rate of occurrence for *bla*TEM gene in Proteus isolates was reported (Dallenne *et al*., 2010). Moreover, Tissera and Mae Lee (2013) also reported similar kind of findings for *bla*TEM gene. The increased resistance of *Proteus* to β-lactam antibiotics is mediated by the presence of extended spectrum β-lactamase. Such an increased antibiotic resistance could due be due horizontal gene transfer, transposons and integrons (Fam *et al.,* 2013). On the other hand, 50% (5/10) occurrence of *qnr* gene in selected isolates of *P. vulgaris* was found in the present study. These results were in accordance to findings of EO and NO (2006) in which an average resistance of 42.7% to 66.7% was exhibited by Gram negative bacteria to certain quinolones with Proteus showing the least mean resistance of 42.7%. Daini *et al*. (2008) also highlighted resistance of Gram negative bacteria to ciprofloxacin. Initially a low level of resistance to Quinolones is due to acquisition of resistance genes followed by high level of resistance that consents bacteria to widen their resistance spectrum up to second generation quinolones such as ciprofloxacin (Morgan-Linnell and Zechiedrich, 2007).

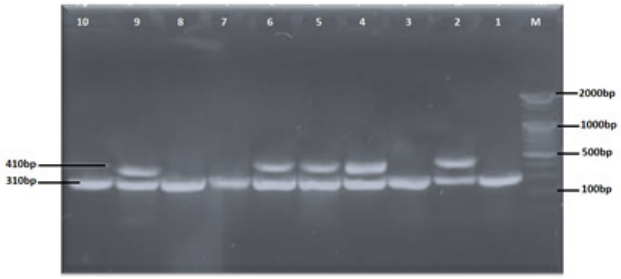

Fig. 6: depicts the amplified *bla*TEM and *qnr* genes with 410bps and 310bps, respectively. The right lane indicates DNA ladder (ThermoFisher Scientific, catalog # 15628019), while other lanes (from 1 to 10) represent the presence of *bla*TEM and *qnr* antibiotic resistant genes in clinical isolates of *P. vulgaris.*

CONCLUSION

Overall prevalence of *P. vulgaris* in clinical samples was 11.3%. *P. vulgaris* showed maximum resistance (94%) to three antibiotics i.e. ampicillin, tigecycline and chloramphenicol, while least to (12%) to imipenem. Statistical findings indicated that imipenem had a significantly larger zone of inhibition while ampicillin had significantly smaller zone of inhibition followed by chloramphenicol. Gene *bla*TEM was detected in all the representative *P. vulgaris* isolates (100%), while *qnr* gene was found in 50% of the isolates.

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