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A Study on Antibiogram and Beta-lactam Resistance of *Proteus mirabilis* Isolated from Animals and Humans in Andhra Pradesh, India

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ABSTRACT

Background: *Proteus mirabilis* is one of the organisms which is often associated with urinary tract infections of humans as well as animals. As a member of *Enterobacteriaceae* family, the level of antimicrobial resistance tend to pose a significant public health risk. Hence, the present study was undertaken to study antibiogram profiles, multidrug resistance *P. mirabilis* isolates and detection of β-lactamase activity in them.

Methods: A total of 175 *P. mirabilis* isolates from different sources were subjected to antibiotic sensitivity/ resistant test by Kirby Bauer disc diffusion method. Detection of ESBL production was done phenotypically by Phenotypic Screening Test and Phenotypic Confirmation Test as recommended by CLSI guidelines and genotypically using multiplex PCR assay to detect different classes of β-lactamase genes. This study was carried out from March 2017 to August 2018 in and around areas of Krishna District Andhra Pradesh.

Result: Out of 175 *P. mirabilis* isolates screened, antibiogram revealed highest sensitivity towards gentamicin (76.57%), followed by ampicillin (64.57%), kanamycin (61.14%), amikacin (60.57%) and streptomycin (43.42%). Higher resistance was observed for erythromycin (71.42%), nalidixic acid (62.85%), ciprofloxacin (62.85%), tetracycline (60%), polymyxin-B (60%), cefoxitin (49.14%) and amikacin (36%). β-lactamase genes were detected in a total of 23 isolates (13.14%). Prevalence rates of β-lactamase genes among different samples was 23.6%, 11.1%, 10.8% and 42.8% from chicken, pork, poultry cloacal swabs and human urine samples, respectively with *bla*TEM being the predominant gene detected (69.56%) followed by *bla*OXA (26.08%), *bla*AmpC gene FOX (13.04%), *bla*CTX-M group I (4.34%), *bla*SHV (4.34%) and *bla*AmpC gene CIT (4.34%) among all the tested *P. mirabilis* isolates.

Key words: Antibiogram, β-lactamase genes, *Proteus mirabilis*.

INTRODUCTION

Increased antibiotic resistance in human bacterial pathogens continues to be a major public health concern. Animals and their products act as potential source of MDR bacteria, hence the emergence of MDR among food-borne pathogens is a cause of grave concern to public health (Tiwari *et al.,* 2013). Recent studies suggest that the inappropriate treatment and misuse of antibiotics leads to the emergence of antibacterial multidrug resistance in *Proteus* spp. (Alabi *et al*., 2016; Mandal *et al*., 2015). The emergence of antimicrobial resistance in *P. mirabilis* isolates from companion animals has been documented (Authier *et al*., 2006; Pedersen *et al*., 2007; Grobbel *et al*., 2007) and drug resistant strains of *P. mirabilis* were also detected in clinical and food samples (Nahar *et al*., 2014; Wong *et al*., 2013; Kim *et al*., 2005). This highlighted the importance of checking antimicrobial resistance in *Proteus mirabilis*, so as to safeguard the health of humans and animals. As the information regarding prevalence of MDR and ESBL producing *Proteus mirabilis* in Andhra Pradesh, India is scarcely available, this study was carried out.

MATERIALS AND METHODS

Bacterial strains and isolates

A total of 175 *Proteus mirabilis* isolates which were selected for the present study were isolated from different sources like Chicken, mutton, pork, poultry cloacal swabs, pig rectal swabs, human urine and diarrhoeic samples and water samples in and around areas of Krishna District Andhra Pradesh. The present study was carried out during the period of March 2017 to August 2018 at Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram Andhra Pradesh.

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Antimicrobial susceptibility and phenotypic detection of β-lactamase production

All the 175 *P. mirabilis* isolates from different sources were subjected to antibiotic sensitivity/ resistance test using 12 most commonly used antibiotics in veterinary practice by Kirby Bauer disc diffusion method (Bauer *et al*., 1966). The antibiotics used were gentamicin, ampicillin, kanamycin, amikacin, streptomycin, erythromycin, nalidixic acid, ciprofloxacin, tetracycline, polymyxin-B, cefoxitin and colistin.

Resistance to at least 3 antibiotic classes was taken as MDR (minimum one in each class) (Ziech *et al*., 2016). Direct colony suspension of each isolate was made in PBS (pH 7.4) and the turbidity was adjusted to 0.5 McFarland units (equivalent to an approximate cell density of 1.5×10^8CFU/ml). About 200 µl of each inoculum was seeded on the MH agar using sterile cotton-tipped swab. The plates were allowed to dry and antibiotic discs were placed aseptically with sterile forceps. The plates were incubated at 37°C for 24 h. The diameter of inhibition zones was measured and susceptibility/resistant patterns of *P. mirabilis* were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018).

Detection of ESBL production was done phenotypically by phenotypic screening test (PST) and phenotypic confirmation test (PCT) as recommended by CLSI (2018) guidelines. PST was carried out using four indicator β-lactam antibiotics: Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Ceftriaxone (CTR, 30 µg) and Aztreonam (AT, 30 µg). Resistance to at least one of the four antibiotics was considered to be positive PST for ESBL production. The positive PST isolates were then subjected to PCT by combination disc method using three pairs of antibiotic discs: Ceftazidime (CAZ, 30 µg), Ceftazidime plus Clavulanic acid (CAC, 30/10 µg), Cefotaxime (CTX, 30 µg), Cefotaxime plus Clavulanic acid (CEC, 30/10 µg) and Ceftriaxone (CTR, 30 µg), Ceftriaxone plus Tazobactum (CIT, 30/10 µg). ESBL production was confirmed when zone diameter around the combination discs was more than or equal to 5 mm when compared to discs containing respective cephalosporin alone (Drieux *et al*., 2008).

Genotypic detection of β-lactamase genes

Multiplex PCR assay can detect different classes of β-lactamase genes. In the present study, bla_{TEM}, bla_{SHV} and bla_{OXA} gene primers have been used for the detection of broad spectrum as well as extended spectrum TEM, SHV and OXA β-lactamases, $bla_{CTX\text{-}M}$ group 1, 2 and 9 gene primers for CTX-M ESBLs (Dallenne *et al*., 2010) and bla_{AmpC} primers for detection of AmpC β-lactamases *viz*., CIT, DHA and FOX (Manoharan *et al.,* 2012).

DNA was extracted by boiling and snap chilling method (Suresh *et al*., 2018) from all the PCT positive *P. mirabilis* isolates and subjected to PCR assays for detection of ESBL genes (Table 1 and 2). PCR assays were optimized in 25 µl reaction mixture containing 2 µl of DNA template, 12.5 µl of 2x master mix (Go Taq Green Master Mix, Promega), 0.5 µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease free water, under standardized cycling conditions: initial denaturation

Table 1: Oligonucleotide primers used for the detection of ESBL genes.

Primer	Target	Nucleotide sequence	Amplicon size (bp)
m-PCR I			
MultiTSO-T	*bla*TEM gene	CATTTCCGTGTCGCCCTTATTC	800
		CGTTCATCCATAGTTGCCTGAC	
MultiTSO-S	*bla*SHV gene	AGCCGCTTGAGCAAATTAAAC	713
		ATCCCGCAGATAAATCACCAC	
MultiTSO-O	*bla*OXA gene	GGCACCAGATTCAACTTTCAAG	564
		GACCCCAAGTTTCCTGTAAGTG	
m-PCR II			
MultiCTXM-Gp1	*bla*CTX-M group 1 gene	TTAGGAAATGTGCCGCTGTA	688
		CGATATCGTTGGTGGTACCAT	
MultiCTXM-Gp2	*bla*CTX-M group 2 gene	CGTTAACGGCACGATGAC	404
		CGATATCGTTGGTGGTACCAT	
MultiCTXM-Gp9	*bla*CTX-M group 9 gene	TCAAGCCTGCCGATCTGGT	561
		TGATTCTCGCCGCTGAAG	

Table 2: Oligonucleotide primers used for the detection of AmpC β-lactamase genes

Primer	Target gene	Sequences (5′-3′)	Amplicon size (bp)
CITMF	*LAT-1* to *LAT-4,CMY-2* to *CMY-7, BIL-1*	TGG CCA GAA CTG ACA GGC AAA	462
CITMR		TTT CTC CTG AAC GTG GCT GGC	
DHAMF	DHA-1, DHA-2	AAC TTT CAC AGG TGT GCT GGG T	405
DHAMR		CCG TAC GCA TAC TGG CTT TGC	
FOXMF	FOX-1 to FOX-5b	AAC ATG GGG TAT CAG GGA GAT G	190
FOXMR		CAA AGC GCG TAA CCG GAT TGG	

at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min and a final elongation step at 72°C for 7 min for TEM, SHV, OXA and CTX-M ESBLs and initial denaturation at 95°C for 2 min; 30 cycles of 94°C for 45 s, 62°C for 45 s and 72°C for 1 min and a final elongation step at 72°C for 5 min for bla_{AmpC} gene amplification.

RESULTS AND DISCUSSION

The emergence and spread of bacterial pathogens that have evolved mechanisms of drug resistance to several antibiotics continues to be a major public health concern. As *P. mirabilis* is one of the most common species identified in several infections, this study was undertaken to observe the antimicrobial susceptibility/ resistance and prevalence of antibiotic resistant genes.

In this context, an *in vitro* antibiotic sensitivity test was conducted for all 175 *P. mirabilis* isolates to detect the sensitivity/ resistance pattern by disc diffusion method using twelve most commonly used antibiotics in veterinary practice that are commercially available as discs (Table 3). It was found that most of the *P. mirabilis* isolates showed sensitivity to gentamicin (76.57%), followed by ampicillin (64.57%), kanamycin (61.14%), amikacin (60.57%), colistin (45.7%) and streptomycin (43.42%). Higher resistance was observed for erythromycin (71.42%), nalidixic acid (62.85%), ciprofloxacin (62.85%), tetracycline (60%), polymyxin-B (60%), cefoxitin (49.14%) and amikacin (36%). Notable percentages of isolates were intermediately resistant against streptomycin (33.14%), erythromycin (20.57%) and cefoxitin (18.28%). The results in the present study were more or less similar to the earlier findings, where along with multiple resistance of *P. mirabilis* to several antibiotics, a varying degree of sensitivity was reported for amikacin, ampicillin, kanamycin and gentamicin (Rana *et al*., 2016, Nachammai *et al*., 2015, Senthamarai *et al*., 2015; Mordi and Momoh, 2009). Among human clinical isolates, 28% resistance was reported to ampicillin which was in contrast to the findings of Nachammai *et al*. (2015) who reported a higher resistance (100%) to ampicillin. Higher sensitivity to gentamicin (100%) from human clinical samples in the present study was in correlation with the findings of Nachammai *et al*. (2015) and Pandey *et al*. (2013) who reported 100% sensitivity to gentamicin among *P. mirabilis* isolated from human clinical samples. Ahmed *et al*. (2014) reported sensitivity to kanamycin and streptomycin among *P. mirabilis* isolates isolated from water samples in Bangladesh. Those findings were in agreement with the present study with a sensitivity of 50% for both the antimicrobial agents. Jabur *et al*. (2013) reported 100% resistance pattern of *P. mirabilis* isolated from human clinical samples towards erythromycin and tetracycline which were in contrast with the present study which showed 71% resistance to erythromycin and 42% to tetracycline. Sensitivity to ciprofloxacin (43%) and gentamicin (100%) among human urine samples observed in the present study was in correlation with the studies conducted by Latif *et al*. (2017) where 97.3% sensitivity was observed for ciprofloxacin and 81.5% for gentamicin, whereas Jabur *et al*. (2013) reported 53.3% and 46.6% sensitivity towards ciprofloxacin and gentamicin, respectively. Latif *et al*. (2017) reported a lower resistance (21%) to amikacin among *P. mirabilis* isolated from human clinical samples which was in agreement with the present study (28%). The lower resistance values towards Ampicillin, Erythromycin and Tetracycline may be attributed to reduced exposure of the isolates to antibiotic rich environment. However, genotypic resistome characterization of isolates is required to arrive at a solid conclusion.

As a part of first step recommended by CLSI, *P. mirabilis* isolates in this study were screened for resistance to indicator cephalosporins (cefotaxime, ceftriaxone, ceftazidime and aztreonam), which showed resistance to aztreonam in 42 (24.0%) isolates, cefotaxime in 43 (24.57%), ceftriaxone in 41 (23.42%) and ceftazidime in 40 (22.85%) isolates. As ESBLs vary in their hydrolysis of these cephalosporins as substrates, resistance to atleast one of them can be considered as positive (CLSI, 2018). Hence, a

Table 3: Antibiotic sensitivity/resistance pattern of *P. mirabilis* isolates.

Antimicrobial agent (dose)	Pattern of antibiogram (n=175)					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Amikacin 30 µg	106	60.57	6	3.42	63	36.00
Ampicillin 10 µg	113	64.57	6	3.42	56	32.00
Cefoxitin 30 µg	57	32.57	32	18.28	86	49.14
Ciprofloxacin 5 µg	59	33.71	6	3.42	110	62.85
Colistin 10 µg	80	45.70	0	0	95	54.28
Erythromycin 15 µg	14	8.00	36	20.57	125	71.42
Gentamicin 10 µg	134	76.57	20	11.42	21	12.00
Kanamycin 30 µg	107	61.14	26	14.85	42	24.00
Nalidixic acid 30 µg	48	27.42	17	9.71	110	62.85
Polymixin-B 300 U	70	40.00	0	0	105	60.00
Tetracycline 10 µg	53	30.28	17	9.71	105	60.00
Streptomycin 30 µg	76	43.42	58	33.14	41	23.42

total of 43 (24.57%) out of 175 *P. mirabilis* isolates were considered to be positive in the screening test, which includes 12 from chicken samples, 9 from pork samples, 20 from poultry cloacal swabs and 2 from human samples.

The level of resistance to third generation cephalosporins and monobactams in *P. mirabilis* isolates seen in the present study is more when compared with the findings of Passat (2016). Although cefotaxime and ceftazidime (third generation cephalosporins) have important role as a drug of choice to treat most of the *Proteus* infections, resistance to these drugs is increased progressively in recent years, so the susceptibility of *Proteus* to these drugs was investigated. It was noticed that 24.57% *P. mirabilis* isolates were resistant to cefotaxime and 22.85% were resistant to ceftazidime. These results were in contrast with the findings of Passat (2016) who reported 66.6% resistance towards ceftazidime and were in agreement with 20% resistance towards cefotaxime.

Drug resistance in this commonly overlooked species has been increasingly reported (Sturenburg and Mack, 2003). ESBL production was confirmed in *P. mirabilis* isolates using combination discs of β-lactam antibiotics and β-lactamase inhibitors (Rana *et al*., 2016). As a part of second step recommended by CLSI, isolates that were positive in the screening test in our study were further tested for confirmation of ESBL production by using β-lactamase inhibitor based test, using three combination discs *i.e.* cefotaxime and cefotaxime clavulanic acid (CTX/CEC), ceftazidime and ceftazidime + clavulanic acid (CAZ/CAC) and cefpodoxime and cefpodoxime + clavulanic acid (CPD/CCL).

Of the 43 isolates that were positive in screening test, 23 isolates were confirmed phenotypically as ESBL producers by CDM (with an increase in inhibition zone diameter by a minimum of 5 mm). All these 23 isolates were resistant to at least one of the indicator cephalosporin in screening test, but susceptible to combination of indicator cephalosporin with clavulanic acid in confirmatory test. As clavulanic acid or sulbactam were β-lactamase inhibitors, it was concluded that, the cephalosporin resistance mechanism could be mediated by β-lactamase production in these 23 *P. mirabilis* isolates. In the remaining 20 *P. mirabilis* isolates, β-lactamase inhibitor synergy (*i.e.* 5 mm principle) was not detected which may be due to co-production of other non-ESBL β-lactamases such as MBLs that confer resistance to β-lactamase inhibitors, ultimately masking the synergy in the confirmatory test. Another explanation could be the existence of other resistance mechanisms conferring resistance to β-lactam antibiotics, like presence of porin proteins or efflux pumps, which are unaffected by the β-lactamase inhibitors used in the confirmatory test (Drieux *et al.,* 2008).

In the present study, CPD/CCL discs were able to detect ESBL production in 52.17% (12/23) of confirmed ESBL producers. CTX/CEC discs were able to detect ESBL production in 56.52% (13/23) and CAZ/CAC discs in 47.82% (11/23) of confirmed ESBL producers. Hence, in the present study CPD/CCL and CTX/CEC discs were found to be more sensitive in the detection of ESBL production in *P. mirabilis* species. However, if CPD/CCL discs alone were to be used, 11 out of 23 confirmed ESBL producers detected in the present study would have been missed. Similarly, 11 out of 23 confirmed ESBL producers and 13 out of 23 confirmed ESBL producers would have been missed if only CTX/CEC discs and CAZ/CAC discs were to be used alone, respectively.

Elucidation of antimicrobial resistance genes in bacteria using nucleic acid- based techniques have been of great use as they complement well with phenotypic results. The overall prevalence of β-lactamase genes in *P. mirabilis* isolates was found to be 13.14% (23/175). These results showed a lower prevalence of ESBLs compared to the findings of Passat (2016) who reported an overall prevalence of 30.7%. Prevalence rates of β-lactamase genes among different samples is 23.68%, 11.1%, 10.8% and 42.85% among the *P. mirabilis* isolates obtained from chicken, pork, poultry cloacal swabs and human urine samples, respectively. Similar prevalence rate of ESBLs in chicken meat samples was observed by Shrestha *et al.* (2017) who reported a prevalence of 26.3% Ismaeil (2017) reported a similar prevalence of 44.44% among urinary isolates and a higher value of 66.67% among chicken faeces. *bla*TEM was found to be the predominant gene (69.56%, 16/23) followed by *bla*OXA (26.08%, 6/23), *bla*AmpC gene FOX (13.04%, 3/23), *bla*CTX-M group I (4.34%, 1/23), *bla*SHV (4.34%, 1/23) and *bla*AmpC gene CIT (4.34%, 1/23) among all the tested *P. Mirabilis* isolates (Fig 1, 2 and 3).

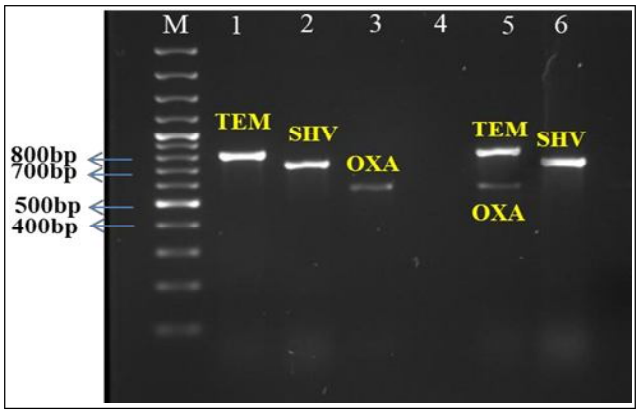

Fig 1: Gel photograph of m- PCR targeting *bla*TEM, *bla*OXA and *bla*SHV genes in. ESBL-producing *P. mirabilis* isolates.

Lane M Molecular weight marker (100 bp).
Lane 1 Known DNA standard for *bla*TEM (800 bp).
Lane 2 Known DNA standard for *bla*SHV (713 bp).
Lane 3 Known DNA standard for *bla*OXA (564 bp).
Lane 4 Negative control.
Lane 5 *P. mirabilis* isolate carrying *bla*TEM and *bla*OXA genes.
Lane 6 *P. mirabilis* isolate carrying *bla*SHVgene.

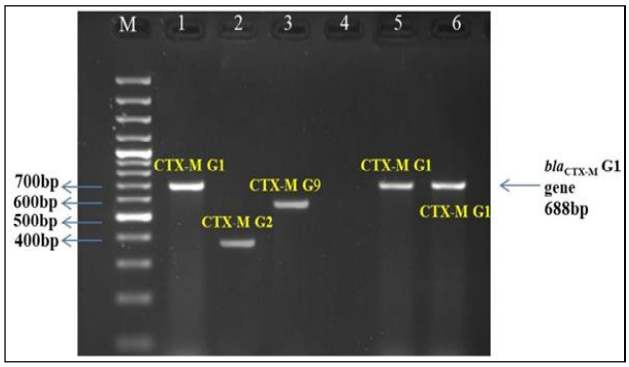

Fig 2: Gel photograph of m-PCR targeting *bla*CTX-M group 1, group 2 and group 9 genes in ESBL- producing *P. mirabilis* isolates.

Lane M Molecular weight marker (100 bp).
Lane 1 Known DNA standard for *bla*CTX-M group 1gene (688 bp).
Lane 2 Known DNA standard for *bla*CTX-M group 2 gene (404 bp).
Lane 3 Known DNA standard for *bla*CTX-M group 9 gene (561 bp).
Lane 4 Negative control.
Lane 5 *P. mirabilis* isolate carrying *bla*CTX-M group 1gene (688 bp).
Lane 6 *P. mirabilis* isolate carrying *bla*CTX-M group 1gene (688 bp).

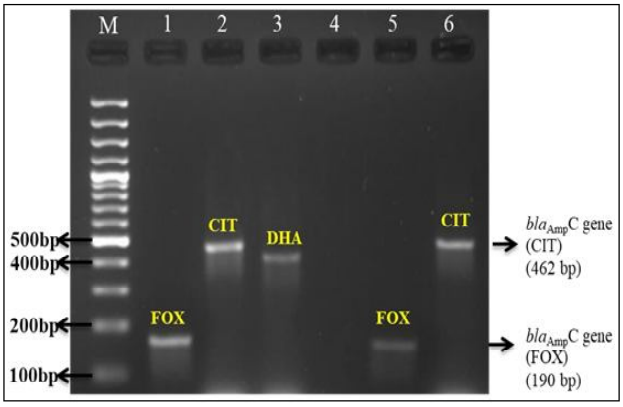

Fig 3: Gel photograph of PCR targeting *bla*AmpC genes in *P. mirabilis* species.

Lane M Molecular weight marker (100 bp).
Lane 1 Known DNA standard for *bla*AMPC gene (FOX) (190 bp).
Lane 2 Known DNA standard for *bla*AMPC gene (CIT) (462 bp).
Lane 3 Known DNA standard for *bla*AMPC gene (DHA) (405 bp).
Lane 4 Negative control.
Lane 5 *P. mirabilis* isolate carrying *bla*AMPC gene (FOX) (190 bp).
Lane 6 *P. mirabilis* isolate carrying *bla*AMPC gene (CIT) (462 bp).

CONCLUSION

The results from this study showed alarming resistance frequencies of *P. mirabilis* obtained from raw meats, fecal swabs of animals and human urine samples. Antibiotic resistance patterns in this study also revealed a clear variation among resistance patterns between human isolates and foods. Beta-lactam resistance profiles of *P. mirabilis* of animal and human origin detected in the present study may pose threat to food safety, animal and human health in this region. Hence, there is a dire need for adequate monitoring of antibiotic use by promoting their rational use particularly the extended-spectrum beta-lactams to prevent increased prevalence of ESBL-positive MDR *P. mirabilis* in this region.

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