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Antibiogram and molecular detection of beta-lactamase genes in *Proteus mirabilis* isolated from pork, chicken meat and chicken cloacal swabs

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Abstract
In the present study, a total of sixteen *Proteus mirabilis* isolates were recovered from 80 samples comprising of chicken (8 isolates from 25 samples), pork (2 isolate from 10 samples) and chicken cloacal swabs (6 isolates from 45 samples). The isolates were confirmed by standard cultural and biochemical tests. Antibiotic Sensitivity test (ABST) of *Proteus mirabilis* isolates performed by disc diffusion method revealed resistance pattern to broad spectrum of antibiotics *viz*. tetracycline (100%), amoxicillin-clavulanic acid (45%), cefotaxim (12.5%), gentamicin (38%) and ciprofloxacin (44%). The β-lactamase genes TEM (800 bp), CTXM-gp2 (404 bp), CTXM-gp9 (561 bp) and ACC (346 bp) were detected in six, seven, one and four isolates, respectively out of sixteen isolates of *Proteus mirabilis* by PCR. The high level of antimicrobial resistance of *Proteus mirabilis* isolated from pigs and chicken may pose a potential threat to food safety and public health.

Keywords: *Proteus mirabilis*, antimicrobial resistance, PCR, ESBL genes, pork, chicken

1. Introduction
Proteus mirabilis is a well-known zoonotic pathogen of public health importance found in soil, water, intestinal tracts of animals and birds (Nahar *et al*., 2014) [10]. *P. mirabilis* and other members of Enterobacteriaceae are the leading cause of community associated and nosocomial infections such as intra-abdominal and blood stream infection (Endimiani *et al*., 2005) [5]. They have also been isolated from pork, chicken and chicken droppings (Kim *et al*., 2005; Wong *et al*., 2013; Nahar *et al*., 2014) [8, 15, 10]. The ability of the organism to harbour both plasmids and integron mediated antimicrobial resistant determinant is one of the indicators of their public health threats in terms of possible transmission of antibiotic resistance factors to other pathogens (Kim *et al*., 2005) [8]. For instance, it has been reported that some extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases producing *Proteus mirabilis* could cause clonal spread resulting in intra-hospital, regional and even nationwide outbreaks (Nakano *et al*., 2012) [11].
P. mirabilis is an opportunistic pathogen that can cause wound and respiratory tract infections in immuno compromised individuals and is the most common cause of complicated urinary tract infection (UTI) and catheter-associated bacteriuria in long-term catheterized patients (Jacobsen *et al*., 2008) [7]. The emergence of multidrug-resistant (MDR) *P. mirabilis* has been increasingly reported in the last few years (Kim *et al*., 2005; Cheng *et al*., 2013) [8, 2]. The most significant concern is their increasing resistance to β-lactams and fluoroquinolones, since these antimicrobials are typical treatment options against clinical infections caused by *Proteus* (Wong *et al*., 2013) [15].
P. mirabilis are known to exist in different kinds of food products; in particular, meat products such as pork and chicken are implicated as vehicles (Kim *et al*., 2005) [8]. *P. mirabilis* can cause food poisoning (Wang *et al*., 2010) [14]. Therefore, its prevalence in food and the environment may contribute to clinical infections in humans. However, little is known about the contamination rate of *P. mirabilis* in meat products and their susceptibilities to antimicrobials, in particular the agents commonly used for treatment of clinical *P. mirabilis* infections. Hence, the objective of this study was to understand the occurrence of *P. mirabilis* in chicken, pork and chicken cloacal swabs, their susceptibility to major antimicrobials and the detection of beta-lactamase genes among the resistant isolates.

3. Materials and Methods

3.1 *P. mirabilis* isolation and confirmation

A total of 80 samples comprising of 10 samples of fresh pork from Dept. of LPT, NTR CVSc, 25 samples of chicken meat from local retail meat outlets at different locations in Gannavaram city and 45 chicken cloacal swab samples obtained from Rajasri breed of Dept. of ILFC, NTR CVSc were analysed for presence of *Proteus* spp. from February 1, 2017 to May 31, 2017. The isolation of *Proteus* spp. from the samples was carried out as per the method described by Wong *et al*., (2013) [15]. Only one colony from each sample was selected for further biochemical and molecular characterization. The motility test and biochemical tests *viz*. Triple Sugar Iron (TSI), urease and other sugar fermentation tests (*viz*. citrate utilization, arginine, lysine, H_2S production, ONPG, Arabinose, lactose, Maltose and Trihalose) were performed using HiMotility™ Biochemical kit (HiMedia, Mumbai). The isolates showing the typical morphology and biochemical test reaction were subjected to antimicrobial susceptibility testing and molecular detection of beta-lactamase genes in resistant *P. mirabilis* isolates.

3.2 Antimicrobial susceptibility testing

Antibiogram of *P. mirabilis* isolated in the present study was carried out against five different antibiotics viz. amoxicillin-clavulanic acid, cefotaxime, gentamicin, ciprofloxacin and tetracycline by Kirby Bauer disc diffusion method (Bauer *et al*., 1966) [1]. Susceptibility patterns of *P. mirabilis* were studied as per the zone of inhibition diameter interpretative breakpoints for Enterobacteriaceae as given in Clinical and Laboratory Standards Institute guidelines (CLSI, 2014) [3].

3.3 Molecular detection of beta-lactamase genes:

Three multiplex PCR assays were standardized for the detection of β-lactamase genes in *P. mirabilis* as described by Dallenne *et al*., (2010) [4] with slight modifications (Soma Sekhar, 2016) [13]. Thermal cycling parameters were standardized for multiplex PCR I, II and III using known positive DNA samples.

a. Multiplex PCR-I for detection of bla_{TEM}, bla_{SHV} and bla_{OXA} genes

All the isolates were subjected to multiplex PCR I for the amplification of bla_{TEM} (800 bp), bla_{SHV} (713 bp) and bla_{OXA} (564 bp) genes using oligonucleotide primers given in Table-3. Multiplex PCR I was optimized in 20 µl reaction mixture (Table-1) under standardized cycling conditions (Table-2).

b. Multiplex PCR-II for detection of bla_{CTX-M} Group 1, 2 and 9 genes

All the isolates were subjected to multiplex PCR II for the amplification of bla_{CTX-M} Group 1 (688 bp), Group 2 (404 bp) and Group 9 (561 bp) genes using primers given in Table-3. Multiplex PCR II was optimized in 20 µl reaction mixture (Table-1) under standardized thermal cycling conditions (Table-2).

c. Multiplex PCR-III for detection of *bla*$_{ACC, FOX, MOX and DHA}$ genes

All the isolates were subjected to multiples PCR for the amplification of plasmid-mediated AmpC b-lactamase genes including four groups based on percentage of similarity viz. bla_{ACC} (346 bp), bla_{FOX} (162 bp), bla_{MOX} (895 bp) and bla_{DHA} (997 bp) gene as described Dallenne *et al*., (2010) [4] using oligonucleotide primers given in Table-3. PCR was optimized in 20µl reaction mixture (Table-1) under standardized thermal cycling conditions (Table-2).

The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and bands were visualized under BIO-RAD gel documentation system.

Table 1: Optimized reaction mixtures for PCRs targeting β-lactamase genes

Components	Multiplex PCR I	Multiplex PCR II	Multiplex PCR III
PCR Master mix (2X)	10 µl	10 µl	10 µl
Forward primer (10 pmol/µl)	0.5 µl X 3 = 1.5 µl	0.5 µl X 3 = 1.5 µl	0.5 µl X 4 = 2.0 µl
Reverse primer (10 pmol/µl)	0.5 µl X 3 = 1.5 µl	0.5 µl X 3 = 1.5 µl	0.5 µl X 4 = 2.0 µl
Template DNA (50ng/µl)	1.0 µl	1.0 µl	1.0 µl
Nuclease free water	6.0 µl	6.0 µl	5.0 µl
Total volume	20.0 µl	20.0 µl	20.0 µl

Table 2: Standardized thermal cycling conditions for detection of β-lactamase genes

Steps	Multiplex PCR-I	Multiplex PCR-II	Multiplex PCR-III	Cycles
Initial denaturation	94 ˚C for 10 min	94 ˚C for 10 min	94 ˚C for 10 min	1
Denaturation	94 ˚C for 40 sec	94 ˚C for 40 sec	94 ˚C for 40 sec	30
Annealing	60 ˚C for 40 sec	60 ˚C for 40 sec	60 ˚C for 40 sec	
Extension	72 ˚C for 1 min	72 ˚C for 1 min	72 ˚C for 1 min	
Final extension	72 ˚C for 7 min	72 ˚C for 7 min	72 ˚C for 7 min	1

Table 3: Oligonucleotide primers used for the detection of β-lactamase genes

Primer	Target gene	Nucleotide sequence (5'-3')	Size (bp)
Multiplex PCR-I			
MultiTSO-T	*bla*$_{TEM}$	CATTTCCGTGTCGCCCTTATTC	800
		CGTTCATCCATAGTTGCCTGAC	
MultiTSO-S	*bla*$_{SHV}$	AGCCGCTTGAGCAAATTAAAC	713
		ATCCCGCAGATAAATCACCAC	
MultiTSO-O	*bla*$_{OXA}$	GGCACCAGATTCAACTTTCAAG	564
		GACCCCAAGTTTCCTGTAAGTG	
Multiplex PCR-II			
MultiCTXM- Gp1	*bla*$_{CTX-M}$ group 1	TTAGGAAATGTGCCGCTGTA	688

		CGATATCGTTGGTGGTACCAT	
MultiCTXM- Gp2	*bla*$_{CTX-M}$ group 2	CGTTAACGGCACGATGAC	404
		CGATATCGTTGGTGGTACCAT	
MultiCTXM- Gp9	*bla*$_{CTX-M}$ group 9	TCAAGCCTGCCGATCTGGT	561
		TGATTCTCGCCGCTGAAG	
Multiplex PCR-III			
MultiCase-ACC	*bla*$_{ACC}$	CACCTCCAGCGACTTGTTAC	346
		GTTAGCCAGCATCACGATCC	
MultiCase-FOX	*bla*$_{FOX}$	CTACAGTGCGGGTGGTTT	162
		CTATTTGCGGCCAGGTGA	
MultiCase-MOX	*bla*$_{MOX}$	GCAACAACGACAATCCATCCT	895
		GGGATAGGCGTAACTCTCCCAA	
MultiCase-DHA	*bla*$_{DHA}$	TGATGGCACAGCAGGATATTC	997
		GCTTTGACTCTTTCGGTATTCG	

4. Results and discussion

4.1 *P. mirabilis* isolation and confirmation

Out of 10 pork samples, 25 chicken samples and 45 chicken cloacal swabs analysed for *Proteus mirabilis*, 2 pork samples, 8 chicken meat samples and 6 chicken cloacal swabs were found to be positive for *Proteus mirabilis* by isolation in selective agars (XLD agar and BGA agar) and confirmed by various biochemical tests (Plate 1, 2, 3 and 4). The occurrence rate of *Proteus mirabilis* in pork, chicken meat and chicken cloacal swabs was found to be 20%, 32% and 13.3%, respectively. The overall prevalence among all the samples tested for *Proteus mirabilis* was calculated to be 20%. Nahar *et al*., (2014) [10] reported prevalence of *Proteus mirabilis* in chicken droppings to be 39% by cultural isolation. Kim *et al*., (2005) [8] and Wong *et al*., (2013) [15] also reported the prevalence of *Proteus mirabilis* in retail meat products including pork and chicken meat, respectively in their studies.

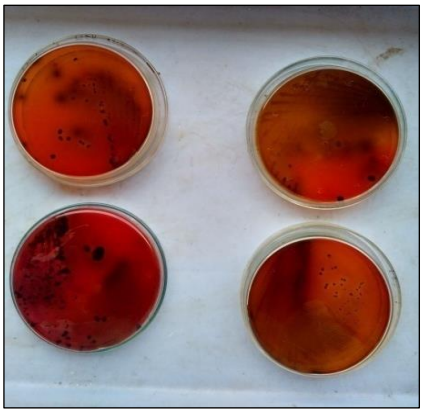


Plate 1: *Proteus mirabilis* on XLD agar

Plate 2: *Proteus mirabilis* on Brilliant green agar

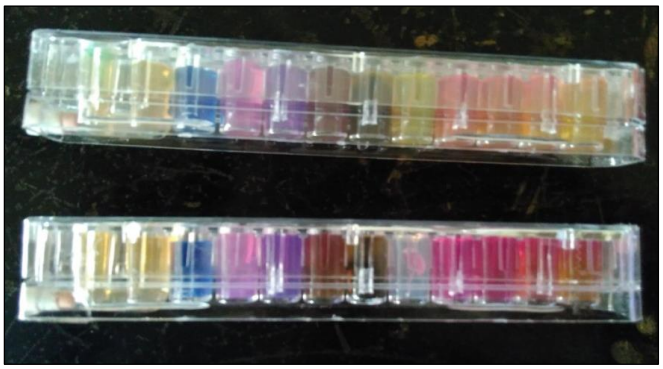


Plate 3: HiMotility^{TM} Biochemical kit showing motility and typical biochemical indicators for *Proteus mirabilis*

Plate 4: TSI and Urease test

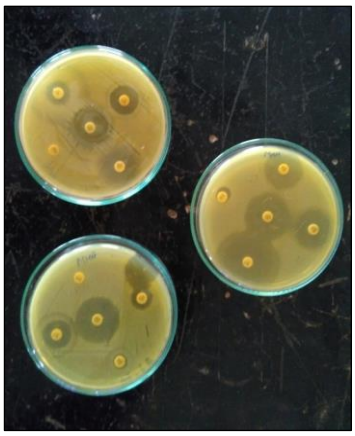


Plate 5: Antibiogram of *Proteus mirabilis* on Mueller-Hinton Agar

4.2 Antibiogram studies

Most of the *Proteus mirabilis* isolates showed sensitivity to cefotaxime (87.50%), gentamicin (56.25%) and Amoxiclav (50%). All the *Arcobacter* isolates were resistant to at least one of the antibiotics tested except cofotaxime. Higher resistance was observed for tetracycline (100%), ciprofloxacin and amoxiclav (43.75%) and gentamicin (37.50%). Notable percentages of isolates were intermediately resistant against cefotaxime and ciprofloxacin (12.50%). The details of the antibiogram patterns of *Proteus mirabilis* isolates from different sources are given in Table 4. Similar results were reported by Kim *et al*., (2005) [8], Wong *et al*., (2013) [15] and Nahar *et al*., (2014) [10] with slight variation in the percentage of resistant isolates for each of the antibiotic studied in their experiment. However, all the studies revealed higher resistance of *Proteus mirabilis* to tetracycline.

Table 4: Antibiogram patterns of *Proteus mirabilis* isolates from different sources

Isolate No.	Sample No.	Source of isolate	Antibiogram results				
			AMC 20/10 µg	CTX 30 µg	GEN 10 µg	CIP 5 µg	TE 30 µg
1.	2	Chicken meat	R	S	S	R	R
2.	4	Chicken meat	R	S	S	S	R
3.	5	Chicken meat	S	S	S	S	R
4.	6	Pork	R	I	R	R	R
5.	7	Chicken meat	R	S	R	R	R
6.	8	Chicken meat	S	S	S	S	R
7.	9	Chicken meat	R	S	R	S	R
8.	10	Pork	R	S	R	R	R
9.	PC-24	Chicken cloacal swab	S	S	I	S	R
10.	PC-25	Chicken cloacal swab	S	S	S	R	R
11.	PC-28	Chicken cloacal swab	S	S	S	S	R
12.	PC-29	Chicken cloacal swab	I	I	S	R	R
13.	PC-36	Chicken cloacal swab	S	S	R	R	R
14.	PC-37	Chicken cloacal swab	R	S	R	I	R
15.	C-1	Chicken meat	S	S	S	S	R
16.	C-2	Chicken meat	S	S	S	I	R

Note: AMX-Amoxiclav, CTX-Cefotaxime, GEN-Gentamicin, CIP-Ciprofloxacin, TE-Tetracycline, R-Resistant, I-Intermediate resistant, S-Sensitive.

4.3 Molecular detection of beta-lactamase genes in *P. mirabilis*

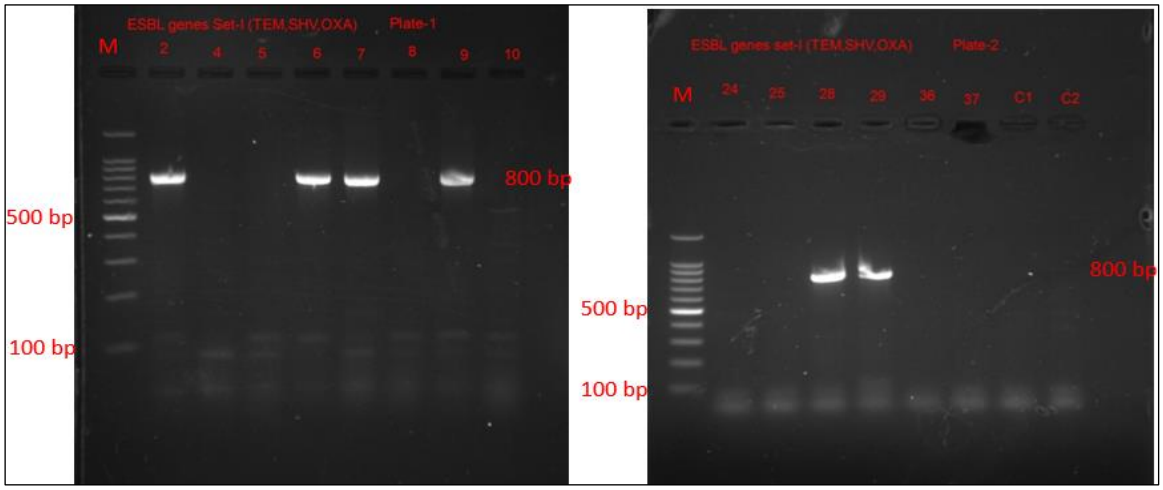
One or more β-lactamase genes were detected in a total of sixteen isolates of *Proteus mirabilis* by multiplex PCR.

In multiplex PCR-I (Fig.1), the β-lactamase gene *bla*$_{TEM}$ (800 bp) was detected in six isolates (37.5%) while the β-lactamase genes *bla*$_{OXA}$ (564 bp) and *bla*$_{SHV}$ (713 bp) were absent in all the isolates of *Proteus mirabilis.*

In multiplex PCR-II (Fig.2), the β-lactamase gene *bla*$_{CTX-M}$ group 2 (404 bp) was detected in seven isolates (43.75%), *bla*$_{CTX-M}$ group 9 (561 bp) and *bla*$_{CTX-M}$ group-1 (688 bp) was detected in one isolate (6.25%).

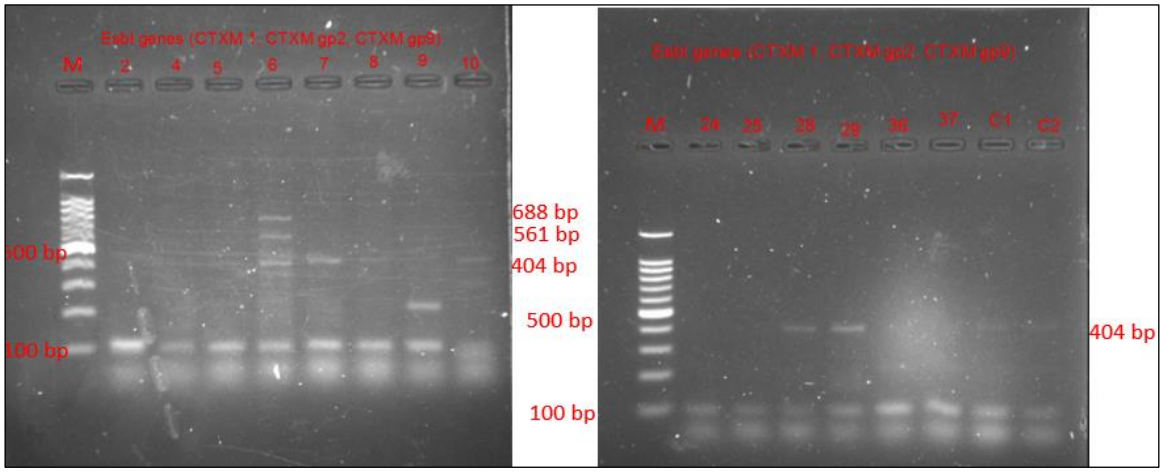
In multiplex PCR-III, the β-lactamase genes *bla*$_{ACC}$ (346 bp), *bla*$_{FOX}$ (162 bp), *bla*$_{MOX}$ (895 bp) and *bla*$_{DHA}$ (997 bp) were absent in all the isolates of *Proteus mirabilis.*

The β-lactamases TEM-1 and OXA-1, and extended-spectrum β-lactamases CTX-M-9 and CMY-2 were detected in β-lactam resistant isolates by Wong *et al*., 2013 [15].



Lane M-DNA ladder (100bp).
Lane 2 to10 and 24 to C2-Amplified DNA from *Proteus mirabilis*.

Fig 1: Gel photograph of multiplex PCR I targeting *bla*$_{TEM,}$ *bla*$_{SHV}$ and b*la*$_{OXA}$ genes in *Proteus mirabilis* species



Lane M-DNA ladder (100bp).
Lane 2 to10 and 24 to C2-Amplified DNA from *Proteus mirabilis*.

Fig 2: Gel photograph of multiplex PCR II targeting *bla*$_{CTX-M}$ Group 1 Group 2 and Group 9 genes in *Proteus mirabilis* species

5. Conclusions

The study concluded that the occurrence of *Proteus mirabilis* in pork, chicken meat and chicken cloacal swabs was 20%, 32% and 13.3%, respectively. The antimicrobial resistance was noticed against the commonly used antibiotics for treatment of urinary tract infections viz. ciprofloxacin and amoxiclav (43.75%) and gentamicin (37.50%). The β-lactamase resistant genes TEM, CTX-M group 2 and ACC genes were detected in higher numbers among the panel of ESBLs tested in this study. Therefore, *Proteus mirabilis* in meat could be a potential concern in food safety and may lead to spread of antimicrobial resistance among the other members of the Enterobacteriaceae family.

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