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Original Research

Study of Virulence Genes, Antibiotic and β-Lactamase Profiles of Proteus mirabilis Isolated from Livestock and Livestock Products

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Abstract

Proteus mirabilis is an emerging foodborne pathogen and has been associated with food poisoning outbreaks, urinary tract infections and causes serious diseases. The present study was undertaken to characterize P. mirabilis species from foods of animal origin based on cultural isolation, PCR confirmation, virulence profiles, antibiogram and production of β-lactamases. Among the 183 samples (135 foods of animal origin and 48 chicken cloacal swabs), 61 (33.33%) isolates were found to be P. mirabilis by species-specific PCR. All the P. mirabilis isolates carried different combinations of putative virulence genes. β-lactamase genes were detected in a total of 37 isolates out of 61(60.65%) with blaTEM being the predominant gene detected (89.18%) followed by blaOXA (10.81%), blaSHV (5.40%) and DHA (2.70%). The findings of this study revealed that there may be a danger of transmission of drug resistant P. mirabilis to consumers through consumption of improperly cooked meat which can lead to treatment failure.

Keywords: P. mirabilis, Virulotyping, Multiple antibiotic resistance index, β-lactamases, ESBLs, AmpC β- lactamases

Introduction

P. mirabilis is a Gram-negative, rod-shaped, motile, facultative anaerobic, non-spore forming and non-capsulated organism belonging to the family Enterobacteriaceae. This is one of the most important bacteria that spreads in hospitals. Wide distribution of *Proteus* species in soil, water and the intestinal tract of many animals, including humans have also been reported (Nahar *et al*., 2014 and Drzewiecka, 2016). *P. mirabilis* is an opportunistic pathogen that causes infections like urinary tract, respiratory tract, ear, nose, skin, burns and wound infections resulting in septicaemia (Wong *et al*., 2013). It also causes food poisoning when we consume contaminated foods such as meat, vegetables and seafood. Over the past few years, some cases of *P. mirabilis* food poisoning have been reported (Cooper *et al*., 2005).

The pathogenesis of *P. mirabilis* is due to virulence factors which include the pili (fimbria), flagella (*flaA*), urease (*ureA, ureC*), protease (*zapA*), hemolysin (*hpmA*) and multi-sugars adipose (lipopolysaccharide) called endotoxin. *P. mirabilis* occurring around the periurethral area can colonize and bind to receptors on the bladder epithelium via fimbriae then highly flagellated ones ascend to the kidneys through ureters. MR/P (mannose-resistant/Proteus) fimbriae can bind to the renal epithelium and leads to formation of biofilm then urease hydrolyzes urea in the urine which leads to rising of urine p^H and forms struvite or apatite stones (urolithiasis) by initiating the precipitation of supersaturated polyvalent cations and anions. Hemolysin is a potent cytotoxin in vitro for epithelial cell and causes cytolysis of renal cells only after a period of brief contact (Mobley and Belas, 1995). The protease which protects the bacteria from the host defence mechanism and urease are produced by all strains of *Proteus* species and these are used to differentiate the members of this genus from the Enterobacteriaceae family (Ali and Yousif, 2015).

Wild-type strains *of P. mirabilis* are commonly susceptible to ampicillin and different β-lactams, but the production of acquired β-lactamases cause a progressive increase of β-lactam resistance in this species (Pagani *et al*., 2002). Production of extended-spectrum β-lactamases (ESBLs) active on expanded-spectrum cephalosporins have also been seen in *P. mirabilis*, recently (Mariotte *et al.*, 1994). No β-lactamases are as common as TEM penicillinases in *P. mirabilis* species and TEM-1 is the most common enzyme produced by penicillinase-producing *P. mirabilis* isolates (Bonnet *et al*., 1999 and Chanal *et al*., 2000). Production of *ampC* β-lactamases results in resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftriaxone, and ceftazidime (CAZ), but often remains susceptible to the advanced-spectrum cephalosporins (ASCs), cefepime and cefpirome (Tenover *et al*.,2009*)*

Although many studies have been conducted on *P. mirabilis* nosocomial infections occurring in different parts of India, there is a lack of systematic studies providing prevalence of *P. mirabilis* in livestock and livestock products. Therefore, the present study was carried out to isolate the *P. mirabilis* from livestock and livestock products and also to characterize their virulence gene profiles and production of β-lactamase genes.

Materials and Methods

Standard Control and Primers

ATCC (American Type Culture Collection) culture of *Proteus mirabilis* (ATCC 12453) was used as a standard positive control and *E. coli* (ATCC 25922) as a negative control. Oligonucleotide primers were custom synthesized from M/s. Bioserve Biotechnologies Pvt. Ltd. (Hyderabad).

Sample Collection

A total of 183 samples consisting of 135 food samples of animal origin (17 chicken, 35 mutton, 15 beef, 20 pork and 48 fish) and 48 poultry cloacal swabs were collected from places in and around Krishna district, Andhra Pradesh. Ten gram of meat samples were inoculated into 90 ml of Tryptose soya broth (TSB) and homogenized, while cloacal swabs were directly inoculated into 10 ml TSB broth test tubes and incubated at 37^{o}C for 24h. After incubation, the samples were streaked on XLD and Mac Conkey agar plates and incubated at 37^{o}C for 24h. Isolates were identified depending on colony morphology (XLD - black colonies, Mac Conkey - pale or colorless colonies) and further confirmed by species-specific PCR.

Confirmation of *P. Mirabilis* by Species-Specific-Uniplex PCR

Extraction of DNA was done by boiling and snap chilling method (Suresh *et al*., 2018). Molecular confirmation of presumptive colonies of *P. mirabilis* was done by species-specific genes (Table-1). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 12.5 µl of 2x master mix (EmeraldAmp GT PCR Master Mix, Takara), 1.5 µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease-free water. The cycling conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 40 sec, 58°C for 1 min and 72°C for 20 sec and a final elongation step at 72°C for 10 min (Zhang *et al*., 2013). PCR products were subjected to gel electrophoresis using 1% agarose with ethidium bromide as fluorescent dye (Sambrook & Russell, 2001) and visualized using Gel Documentation unit (BIORAD).

Table 1: Proteus *Mirabilis* specific PCR primer sequences (Zhang *et al*., 2013)

PRIMER	SEQUENCE (5'-3')	AMPLICON SIZE (BP)
UreR-F	GGTGAGATTTGTATTAATGG	225
UreR-R	ATAATCTGGAAGATGACGAG	

Detection of Virulence Genes In *P. Mirabilis* Isolates

DNA from all the confirmed *P. mirabilis* isolates was screened for the presence of virulence genes. PCR assay was optimized in 25 µl reaction mixture containing 2.5µl of DNA template, 12.5µl of 2x master mix (Emerald Amp GT PCR Master Mix, Takara), 0.3 µl each of forward and reverse primers (10 pmol/µl) and the rest of the volume is made by adding nuclease-free water, under different standardized cycling conditions for different virulence genes as per Ali and Yousif (2015).

Antimicrobial Resistance Profile of *P. Mirabilis* Isolates

Antibiogram profile of confirmed *P. mirabilis* isolates was carried out against 14 different antibiotics like cefepime, ceftriaxone, amikacin, tetracycline, ceftazidime, gentamicin, piperacillin/tazobactam, ceftazidime+clavulanic acid, ofloxacin, chloramphenicol, amoxicillin+clavulinic acid, ampicillin, cefotetan, imipenem on Muller Hinton agar by using Kirby Bauer disc diffusion method as described by Bauer *et al*., 1966 following the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2018)

Detection OF ESBL Production in *P. Mirabilis* Isolates by Phenotypic Methods

P. mirabilis isolates were screened for resistance against third-generation cephalosporins like cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg) and monobactams like aztreonam (30 µg) by disc diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar using commercial discs (HiMedia, Mumbai). Resistance to at least one of the four antibiotics used was considered as a positive screening test (PST) for ESBL production (Drieux *et al*., 2008 and CLSI, 2018). Isolates that were found positive in PST were further subjected to phenotypic confirmation test (PCT) by combination disc method (CDM), where discs of cephalosporins along with combination disc containing cephalosporin plus clavulanate/sulbactam were used i.e. 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 sulbactam (CIS, 30/10 µg). An increased inhibition zone diametre of ≥ 5 mm in case of combination discs confirms ESBL production (Drieux *et al*., 2008 and CLSI, 2018).

Identification of ESBL Genes by M-PCR

DNA from all the PCT positive *P. mirabilis* isolates was subjected to two m-PCR assays for the detection of different ESBL genes (blaTEM, blaSHV, blaOXA and blaCTX-M). PCR assays were optimized in 25 µL reaction mixture containing 2 µL of DNA, 12.5 µL of 2X master mix (EmeraldAmp GT PCR Master Mix, Takara), 0.5 µL of forward and reverse primers (10 pmol/µL) and the rest of the volume is made by adding nuclease-free water. The primers used and PCR conditions followed were according to Dallenne *et al*., 2010.

Detection of *AMPC* B-Lactamase Genes in *P. Mirabilis* by M-PCR

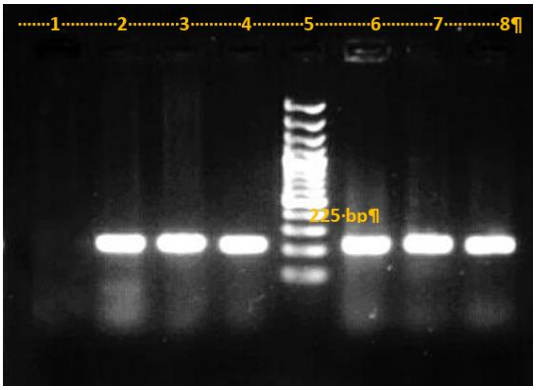
All the PST positive isolates were subjected to m-PCR assays for the detection of blaAmpC genes as PST positive and PCT negative isolates also might be positive for *ampC* β-lactamases. PCR was optimized in 25 µL reaction mixture containing 2 µL of DNA, 12.5 µL of 2X master mix (EmeraldAmp GT PCR Master Mix, Takara), 0.2 µL 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 as described by Manoharan *et al.*, 2012.

Results and Discussion

In the present study, the overall prevalence of *P. mirabilis* was found to be 33.33% (61/183) (Table 2 & Fig. 1) by using PCR. Among different samples, the highest prevalence of *P. mirabilis* isolates was observed in chicken samples (76.47%), followed by pork (60%), beef (46.66%), mutton (45.71%), fish (14.58%) and cloacal swabs (12.5%). The prevalence rates of *P. mirabilis* in beef, mutton and pork were higher than the findings of Bradeeba and Siva Kumar (2013) who reported 12%, 28% and 32% of prevalence rate, respectively. High prevalence in our study may be due to several factors like variation in the hygienic husbandry practices in different geographical area (low hygiene at study location), convenient sampling and variation in antibiotic usage pattern. Prevalence in cloacal swabs was lesser than the findings of Nahar *et al.* (2014) who reported 39% of prevalence in poultry droppings. Less prevalence may be due to the hygienic maintenance of broiler farms in the study area.

Table 2: Prevalence of *P. mirabilis* in poultry cloacal swabs and different foods of animal origin

Sample	Positives	Prevalence (%)
Chicken (17)	13	76.47
Pork (20)	12	60
Beef (15)	7	46.66
Mutton (35)	16	45.71
Fish (48)	7	14.58
Cloacal swabs (48)	6	12.5
Total (183)	**61**	**33.33**



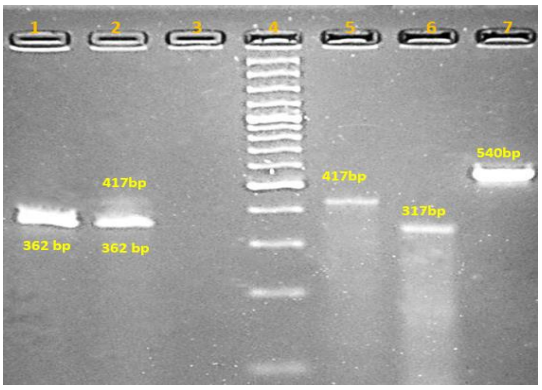
Lane 1: Negative control**; Lane 2:** Positive control of *P. mirabilis* (ATCC 12453) (225 bp); **Lane 5:** DNA ladder (100 bp); **Lane 3, 4, 6, 7, 8:** *P. mirabilis* (225 bp) isolated from different samples

Figure 1: Gel Photograph of Species-Specific PCR for *P. mirabilis*

As consumer safety mainly depends on the virulence properties of pathogenic bacteria in animals and foods of animal origin (Ram *et al*., 2019), investigation on virulence properties was carried out which revealed that all the *P. mirabilis* isolates carried a combination of putative virulence genes in which *zapA* (65.57%) was the predominant gene followed by *ureA* (59.01%), *flaA* (40.98%), *ureC* (32.78%) and no *hpmA* gene was detected (Table-3 & Fig-2). Less prevalence rate of *ureA* and *ureC* and high prevalence rate of *zapA* and *flaA* virulence genes was observed compared to the findings of Ram *et al*. (2019) who reported higher prevalence rates of *ureA* (72.5%), *ureC* (80.5%) and *hpmA* (60.5%) and less prevalence of *zapA* (50.28%) and *flaA* (28.5%) genes.

Table 3: Prevalence of putative virulence genes in *P. mirabilis* isolated from different samples

Samples	*P. mirabilis* Positive samples	*Urea A (%)*	*Urea C (%)*	*Hpma A (%)*	*Fla A (%)*	*ZapA (%)*
Chicken	13	11 (84.61)	6(46.15)	0 (0)	9(69.23)	13(100)
Beef	7	3(42.8)	0(0)	0(0)	2(28.57)	5(71.42)
Mutton	16	9(56.25)	6(37.5)	0(0)	7(43.75)	10(62.5)
Fish	7	5(71.4)	3(42.85)	0(0)	2(28.57)	4(57.14)
Pork	12	4(33.33)	2(16.66)	0(0)	2(16.66)	4(33.33)
Cloacal swabs	6	4(66.66)	3 (50)	0(0)	3 (50)	4(66.66)
Total	61	36(59.01)	20(32.78)	0(0)	25(40.98)	40(65.57)



Lane 1: *P. mirabilis* isolate with *ureA* (362 bp) gene; **Lane 2:** *P. mirabilis* isolate with *ureA* (362 bp) and *flaA* (417 bp) gene; **Lane 3:** Negative control; **Lane 4:** DNA ladder (100 bp); **Lane 5:** *P. mirabilis* isolate with *flaA* (417 bp); **Lane 6:** *P. mirabilis* isolate with *ureC* (317 bp) gene; **Lane 7:** *P. mirabilis* isolate with *zapA* (540 bp) gene

Figure 2: Gel photograph of PCR assay targeting *P. mirabilis* putative virulence genes

Virulotyping of 61 *P. mirabilis* isolates, resulted in 13 virulotypes of which, maximum number of virulence genes i.e., 4 genes were detected in six isolates belonging to virulotype Pm1. Lowest number of virulence genes i.e., only one gene was detected in 24 isolates belonging to virulotypes Pm10, Pm11, Pm12 and Pm13. The discriminatory power of virulotyping for *P. mirabilis* was found to be 0.9011(Table 4), indicates good distinction among the isolates.

Table 4: Virulotypes of *P. mirabilis*

Virulotype Pattern	Isolate No.	N0. of isolates	Virulence Gene (9)	No. of genes
Pm1	KC 1, 4, 7, 12, KM 14 & 27	6	*Urea A*, *Urea C*, *Fla A* and *ZapA*	4
Pm2	KC 2, 3, 10, 13, 16, KB7 & KM19	7	*Urea A*, *Fla A* and *ZapA*	3
Pm3	KM 15	1	*Urea A*, *Urea C* and *ZapA*	3
Pm4	KM 5, KCC13 &39	3	*Urea A*, *Urea C* and *Fla A*	3
Pm5	KC 5 &KCC 34	2	*Urea C* and *ZapA*	2
Pm6	KC 11, KF 11, 33, KCC 14 & 45	5	*Urea A*and*ZapA*	2
Pm7	KB 15	1	*Urea A*and*Fla A*	2
Pm8	KF 5, 27, KM 10, 13, 17 & KCC1	6	*Fla A*and*ZapA*	2
Pm9	KF 12, 24, 31, KM 4, 29 & 33	6	*Urea A*and *Urea C*,	2
Pm10	KC 8, KB 1,4, 10, 12, KP 7, 9, 16, 18, KM 15, 20, 31 & 34	13	*ZapA*	1
Pm11	KB 11, KP 3, 8,10, 13, KM 21 & 25	7	*Urea A*	1
Pm12	KP 4 & 12	2	*Urea C*	1
Pm13	KP 14 & 19	2	*Fla A*	1

Number of unrelated strains: 61; Number of types: 13; Discriminatory power: 0.9011

All the *P. mirabilis* isolates (61) were tested for antimicrobial susceptibility against 14 antibiotics. Among these, highest levels of resistance was seen against tetracyclines followed by ceftazidime+clavulanic acid, amoxyclav, ampicillin, chloramphenicol, ceftazidime, ceftriaxone, gentamicin, ofloxacin, amikacin, cefepime, piperacillin/ tazobactam and imipenem (Table 5). In this study, highest resistance was observed against tetracyclines (85.24%) which was in agreement with findings of Nahar *et al*. (2014) who reported tetracycline was the highest individually-resistant (94%, 34/36) antibiotic. In our study, resistance to tetracyclines among *P. mirabilis* is mainly due to the reason that these are commonly suggested drugs for the treatment of livestock diseases by the veterinarians in the study area. All isolates from chicken samples and chicken cloacal swabs were found to be resistant to tetracyclines as these are commonly used as growth promoters and also widely used drugs for infections in poultry industry. No isolate was found to be resistant to imipenem as resistance to carbapenems has been extremely rare in veterinary medicine (Papich, 2002).

Table 5: Details of antibiotic sensitivity test of *proteus mirabilis* by phenotypic method

S. No.	Drug	Sensitive	Intermediate	Resistant
1	Cefepime	37 (60.65%)	21(34.42%)	3 (4.91%)
2	Ceftriaxone	46 (75.40%)	9 (14.75%)	6 (9.8%)
3	Amikacin	45 (73.77%)	13(21.31%)	3 (4.91%)
4	Tetracycline	6(9.83%)	3(4.91%)	52(85.24%)
5	Ceftazidime	48 (78.68%)	6 (9.8%)	7 (11.47%)
6	Gentamicin	54 (88.52%)	1(1.6%)	6 (9.8%)
7	Piperacillin/ Tazobactam	53 (86.88%)	6 (9.8%)	2 (3.27%)
8	Ceftazidime+Clavulanic acid	11 (18.03%)	16 (26.22%)	34 (55.73%)
9	Ofloxacin	45 (73.77%)	12(19.67%)	4 (6.55%)
10	Chloramphenicol	28 (45.90%)	17 (27.86%)	16 (26.22%)
11	Amoxyclav	15 (24.59%)	19(31.14%)	27(44.26%)
12	Ampicillin	34 (55.73%)	6(9.83%)	21(34.42%)
13	Cefotetan	55 (90.16%)	4 (6.55%)	2 (3.27%)
14	Imipenem	41(67.21%)	20 (32.78%)	0

Multiple antibiotic resistance index (MAR index) values for 61 *P. mirabilis* isolates ranged between 0.07 and 0.50. MAR index values for 28 *P. mirabilis* isolates were found to be >0.2. MAR index of more than 0.2 is an evidence of high-risk source contamination (Krumperman, 1983). Fourteen isolates were found to be resistant to 5-7 antibiotics with MAR index higher than 0.28 ranging from 0.36-0.50. Depending on combination of antibiotic resistance pattern, 61 *P. mirabilis* isolates were divided to 7 MAR index groups with average MAR index of 0.21 (Table 6). A significant number of *P. mirabilis* isolates from this study had a MAR index > 0.2 which indicates that the isolates were from the sources where antibiotics were commonly used or previous exposure of the organism to antimicrobial agents.

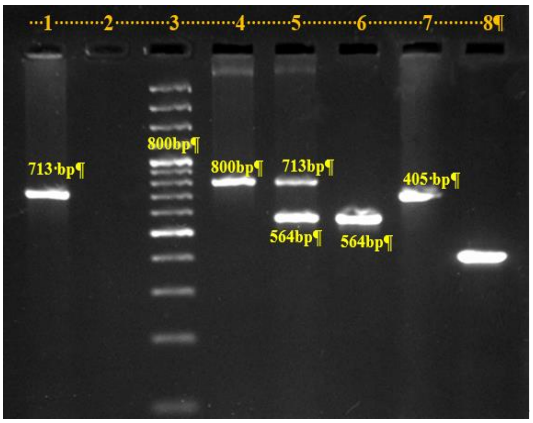
Table 6: Detection of MAR Index Value of *P. Mirabilis* isolates

MAR index label	Sample ID	No. of isolates (A)	Total no. antibiotics the isolate was resistant to (B)	Total no. of antibiotics (C)	MAR index value/each isolate (B/C)	Total MAR index (A*B/C)
MARp1	KP8, 13, KM13, 19 & KF27	5	7	14	0.5	2.5
MARp2	KC7, KB7, KM4, KM15, KF11	5	6	14	0.42	2.1
MARp3	KC10, 11, KM14 & 24	4	5	14	0.36	1.44
MARp4	KC1, 3, KB4, KP7, KM5, 10, 20 &21	8	4	14	0.28	2.24
MARp5	KC2, KB11, KP10, KM17, KF24, KCC34	6	3	14	0.21	1.26
MARp6	KC4, 5, 8, 12, 13, KB1, KP12, 14, KM25, 29, 31, KF5, 12 & 31	14	2	14	0.14	1.96
MARp7	KC15, 16, KB10, 12, 15, KP3, 4, 9, 16, 18, 19, KM33, 34, KF33, KCC1, 13, 14, 45 & 39	19	1	14	0.07	1.33
TOTAL		**61**				**12.83**

All 61 *P. mirabilis* isolates were subjected to phenotypic detection of β-lactamases out of which 53 isolates were positive for PST and 46 isolates for CDM. All the 53 PST positive *P. mirabilis* isolates were subjected to m-PCR assays for the presence of different β-lactamase genes. Of the 61 *P. mirabilis* isolates, 37 isolates (60.65%) were found to possess at least one of the β-lactamase genes (Fig. 3). *bla*TEM was the predominant gene detected (89.18, 33/37) followed by *bla*OXA (10.81%, 4/37), *bla*SHV (5.40%, 2/37) and *bla*AmpC gene DHA (2.7%, 1/37). In our study 30 isolates were positive for *bla*TEM (5-chicken, 3-beef, 5-mutton, 3-fish, 8-pork, 6-cloacal swabs), one for *bla*OXA (pork isolate), two for *bla*SHV (beef isolates), one for *bla*AmpC gene DHA (pork isolate) and three mutton isolates were carrying both *bla*TEM and *bla*OXA genes (Table 7). In this study, *bla*TEM was the predominant gene among β-lactamase genes which is in perfect agreement with the reports from Italy and France which stated that ESBL-producing *P. mirabilis* strains are mainly TEM-type ESBL producers (Tonkic *et al*., 2010; Spanu *et al*., 2002; Biendo *et al*., 2005). All six *P. mirabilis* isolates obtained from cloacal swabs of poultry showed *bla*TEM since β -lactams are often typical choice in treating a wide range of *Proteus* infections in poultry (Nahar *et al*., 2014) which leads to development of resistance to β–lactams by producing β –lactamases.

Table 7: Prevalence of B-lactamase genes in *P. mirabilis* isolated from different samples

Samples	No. of samples collected	*P. mirabilis* Positive samples	*TEM*	*OXA*	*SHV*	*DHA*
Chicken	17	13	5	-	-	-
Beef	15	7	3	-	2	-
Mutton	35	16	8	3	-	-
Fish	48	7	3	-	-	-
Pork	20	12	8	1	-	1
Cloacal Swabs	48	6	6	-	-	-
Total	**183**	**61**	**33**	**4**	**2**	**1**



Lane 1: Positive control (*Klebsiella pneumoniae* ATCC 700603) showing bla_{SHV} (713 bp) gene; **Lane 2:** Negative control **Lane 3:** DNA ladder (100 bp); **Lane 4:** *P. mirabilis* isolate with bla_{TEM} (800 bp) gene; **Lane 5:** *P. mirabilis* isolate with bla_{TEM} (800 bp) and bla_{OXA} (564 bp) genes; **Lane 6:** *P. mirabilis* isolate with and bla_{OXA} (564 bp) genes; **Lane 7:** *P. mirabilis* isolate with bla_{AmpC} gene DHA (405 bp) gene

Figure 3: Gel photograph of M-PCR assays targeting ESBL and *AMPC* B-lactamase genes in *P. Mirabilis*

Conclusion

The present study revealed the presence of drug resistant *P. mirabilis* in chicken cloacal swabs and foods of animal origin collected from in and around Krishna district of Andhra Pradesh. Indiscriminate use of antibiotics for treatment of sick animals and also as a growth promoter lead to development of resistant bacteria. These resistant bacteria will be excreted through faeces and enter into environment. These bacteria may spread to humans through consumption of contaminated meat, inhalation of dust. Emergence and spread of antibiotic resistant strains particularly ESBL and *AmpC* β-lactamase producing strains cause infections that are difficult to treat. It is therefore important to take an action on judicious use of antibiotics in livestock production in order to protect and promote public health by ensuring food safety.

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Conflict of Interests

There is no conflict of interest.

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