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V Prasastha Ram
M.V. Sc Scholar, Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

L Venkateswara Rao
Professor and Head, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Proddatur, Andhra Pradesh, India

T Srinivasa Rao
Associate Professor and Head, Department of Veterinary Public Health and Epidemiology. NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

KV Subramanyam
Professor and Head, Department of Microbiology, College of Veterinary Science, Proddatur, Andhra Pradesh, India

K Srinivas
M.V. Sc Scholar, Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Correspondence
V Prasastha Ram
M.V. Sc Scholar, Department of Veterinary Public Health and Epidemiology, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Prevalence and virulence gene profiles of *Proteus mirabilis* isolated from animal, human and water samples in Krishna District, Andhra Pradesh, India

V Prasastha Ram, L Venkateswara Rao, T Srinivasa Rao, KV Subramanyam and K Srinivas

Abstract
In the present study, a total of 507 samples comprising food of animal origin (215), faecal swab samples (188), human urine samples (65), human diarrhoeic stool samples (12) as well as water samples (27) were examined. Overall prevalence of *P. mirabilis* was found to be 34.51% (175/507) by species-specific PCR. Among foods of animal origin, the highest rate of *P. mirabilis* isolates were recovered from chicken samples (38.7%), followed by pork (37.5%) and mutton samples (28.9%). Among faecal swabs, the highest rate of *P. mirabilis* isolates were from poultry (49%), followed by pigs (37.8%). Human urine samples showed a prevalence rate of 10.7%. Water samples showed 7.4% prevalence. All the human diarrhoeic stool samples were negative for *P. mirabilis*. Some of the virulence factors genes were investigated using polymerase chain reaction technique. The genes *ureC* (80.5%), *ureA* (72.5%), *flaA* (28.5%), *hpmA* (60.5%) and *zapA* (50.28%) were detected in *P. mirabilis* isolates.

Keywords: *Proteus mirabilis*, prevalence, virulence genes, polymerase chain reaction

1. Introduction
The *Enterobacteriaceae* group of bacteria is the most challenging contaminant to meat and meat products worldwide. *E. coli*, *Salmonella*, *Proteus* and *Klebsiella* species are the most predominant species in all food poisoning cases associated with meat products. *Proteus* spp. are Gram negative rods measuring 1-3µm in length and 0.4-0.8µm in diameter, motile by peritrichous flagella, facultative anaerobic, non spore-forming and non-capsulated with most isolates having fimbriae. *Proteus* species are frequently found in soil, water and the intestinal tract of many animals, including humans (Drzewiecka, 2016 and Nahar *et al.*, 2014) [8, 13].
Many outbreaks of food poisoning have been attributed to organisms of the *Proteus* group and due to the rising incidence of food borne infections caused by *P. mirabilis*, there is an urgent need for control and/or prophylaxis for food poisoning outbreaks associated with meat products. It depends greatly on investigating such agents in foods eliminating them to ensure food safety and to protect public health from microbial contamination of food.
A few cases of food poisoning caused by *P. mirabilis* have been reported in the last several decades (Cooper *et al.*, 2005 and Zietze, 1984) [6, 20]. They have been isolated from chicken, beef, pork and poultry droppings (Nahar *et al.*, 2014; Wong *et al.*, 2013; Al-Mutairi 2011 and Kim *et al.*, 2005) [13, 18, 4, 10]. *Proteus* species are the causative agents of a variety of opportunistic nosocomial infections including those of the respiratory tract, ear, nose, skin, burns and wounds. They are important pathogens of the urinary tract and primary infectious agents in patients with indwelling urinary catheters (Jacobsen *et al.*, 2008 and Peerbooms *et al.*, 1986) [9, 15]. *P. mirabilis* causes 90% of *Proteus* infections and is mostly found in people with compromised immune system (Mandal *et al.*, 2015) [11]. Individuals suffering from urinary tract infections caused by *P. mirabilis* often develop cystitis, bacteruria, kidney and bladder stones, catheter obstruction due to stone encrustation and acute pyelonephritis (Alatrash and Al-Yaseen, 2017) [2].
Poor water quality is responsible for the death of an estimated 5 million children in the developing countries. The detection of bacteria belonging to the genus *Proteus* in drinking water disqualifies its suitability for consumption due to its fecal pollution, which would threaten with waterborne infections (Drzewiecka, 2016) [8].
The pathogenesis of these bacteria is associated with possessing many virulence factors which include the pili (fimbriae), flagella, urease, protease, hemolysin etc. The urease and protease

enzymes are produced by all strains of *Proteus* species and it is also considered as diagnostic and differential feature which characterizes the members of this genus from the rest of the intestinal family members (Ali and Yousif, 2015) [3].
Therefore, the present study was planned to perform the isolation of *P. mirabilis* from different sources and also to characterize their virulence gene profiles.

2. Materials and methods

2.1 Standard control and primers

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

2.2 Sample collection

A total of 507 samples were collected from different sources of raw meats including chicken samples (98), mutton (45) and pork (72) from retail shops and slaughter houses, poultry cloacal swabs (151) from Livestock Farm Complex (LFC), NTRCVSc, Gannavaram and pig rectal swabs (37) from Dept. of Livestock Products Technology, NTRCVSc, Gannavaram, human urine samples (65) and human diarrhoeic samples (12) from clinical laboratories and water samples (27) from areas in and around Gannavaram, Krishna district, Andhra Pradesh. The samples collected were inoculated into 5ml TSB and were incubated at 37 ºC for 24 h for enrichment. Enriched samples were streaked on MacConkey agar, nutrient agar and blood agar plates and incubated at 37 ºC for 24 h. Isolates were identified depending on morphological and biochemical tests and further confirmed by uniplex PCR.

2.3 DNA extraction from enriched broth samples

DNA was extracted by boiling and snap chilling method (Suresh *et al*., 2018) [17]. A 1.5 ml of enriched broths were taken into micro centrifuge tubes and centrifuged at 8000 rpm for 10 min. Supernatant was discarded, 50 µl of nuclease free water was added and placed in boiling water bath at 100 °C for 10 min then immediately snap chilled for 10 min and centrifuged at 10,000 rpm for 5 min. The supernatant was taken as template and subjected to PCR and the PCR products were subjected to gel electrophoresis using 1.5% agarose with ethidium bromide as fluorescent dye and visualized using Gel Documentation unit (BIORAD, USA).

2.4 Molecular confirmation of *Proteus mirabilis* by PCR

P. mirabilis isolated from different samples by cultural methods were confirmed using *P. mirabilis* specific PCR targeting *ureR* gene (Table-1). PCR assay was optimized in 25 µl reaction mixture containing 2 µl of DNA template, 2.5 µl of 10x master mix (Go Taq Green Master Mix, Promega), 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 s, 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) [19].

Table 1: *Proteus mirabilis* specific PCR primers and sequences (Zhang *et al*., 2013) [19]

Primer	Sequence (5'-3')	Amplicon size (bp)
UreR-F	GGTGAGATTTGTATTAATGG	225
UreR-R	ATAATCTGGAAGATGACGAG	

2.5 Detection of virulence genes in *P. mirabilis* isolates

All the confirmed *P. mirabilis* isolates from different sources were screened for the presence of virulence genes such as *ureA, ureC, hpmA, flaA* and *zapA* (Table-2). PCR assay was optimized in 25 µl reaction mixture containing 3 µl of DNA template**,** 2.5µl of 10x master mix (Go Taq Green Master Mix, Promega), 0.35µl 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 (Ali and Yousif, 2015) [3].

Table 2: Primers used for detection of *Proteus mirabilis* virulence genes (Ali and Yousif 2015) [3]

Primer	Target gene	Nucleotide sequence	Amplicon size
ureA:F	*ureA*	GATCTGGGCGACATAATCGT	362
ureA:R		TCACCGGGGATCATGTTATT	
ureC:F	*ureC*	GTTATTCGTGATGGTATGGG	317
ureC:R		TCGCCAGTTATCTTGACATTCTG	
flaA:F	*flaA*	AGGATAAATGGCCACATTG	417
flaA:R		CGGCATTGTTAATCGCTTTT	
hpmA:F	*hpmA*	GCATCATCAAGCGTACGTTCC	717
hpmA:R		AATGAGCCAAGCTTGTTAAGCT	
zapA:F	*zapA*	ACCGCAGGAAAACATATAGCCC	540
zapA:R		GCGACTATCTTCCGCATAATCA	

3. Results and Discussion

In the present study, a total of 507 samples from various sources viz. foods of animal origin (215), faecal samples (188), human clinical samples (77) and water samples (27) were analyzed for isolation and identification of *P. mirabilis*. Out of 507 samples, 175 were positive for *P. mirabilis* both by cultural and biochemical tests (Fig 1). The overall prevalence rate of *P. mirabilis* was 34.51%. Out of 215 meat food samples analyzed, the highest rate of *P. mirabilis* isolates was recovered from chicken samples (38.7%) followed by pork (37.5%) and mutton (28.9%). These prevalence rates were almost similar to the findings of Kim *et al*. (2005) [10] who reported an overall prevalence of about 20-35% in raw meat samples.

Fig 1: Characteristic swarming of *Proteus* spp. on nutrient agar as a consequence of the organisms active motility

Regarding human clinical samples, out of 65 urine samples screened, 7 were positive for *P. mirabilis* showing prevalence rate of 10.7%, which was slightly less when compared with findings of Alatrash and Al-Yaseen (2017) [2] who reported a prevalence rate of 19.3% in human urine samples and Ali and Yousif (2015) [3] who reported an overall prevalence of 17.6% (Fig 2). Less prevalence in our study can be attributed to the variation due to many factors such as geographic area, type of sampling, antibiotic usage etc.

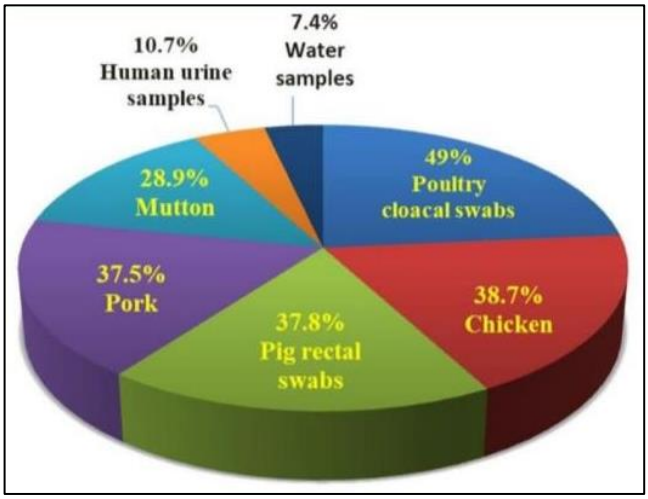

Fig 2: Prevalence of *P. mirabilis* in different samples

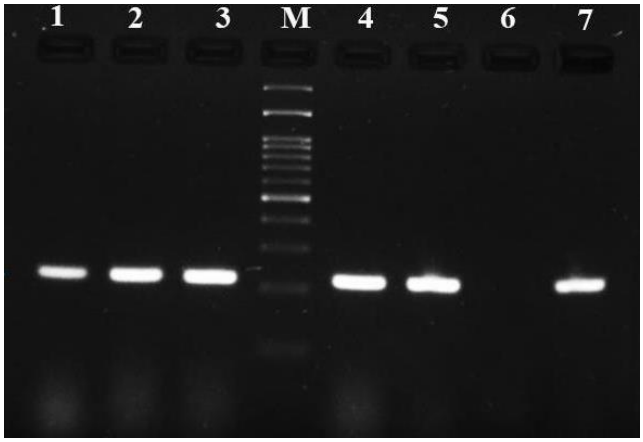

Fig 3: Gel photograph of *ureR* species-specific PCR for *P. mirabilis* Lane 1-3 Amplified DNA of *P. mirabilis* from chicken meat Lane M DNA ladder (100bp) Lane 4 & 5 Amplified DNA of *P. mirabilis* from poultry cloacal swabs Lane 6 Negative control Lane 7 Positive control of *P. mirabilis* (ATCC 12453) (225bp)

The investigation on virulence properties of potentially pathogenic bacteria in animals and foods of animal origin was essential to consumer's safety. For the detection of virulence genes, techniques have been developed to identify *ureA* and *ureC* genes responsible for urease production, *flaA* encoding flagella and *hpmA* responsible for production of hemolysin and *zapA* responsible for protease production. All the 175 *P. mirabilis* isolates recovered from different animal foods, faecal swabs and human urine samples by cultural, biochemical and molecular tests were further subjected to five uniplex PCR assays targeting *ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes for characterization of virulence gene profiles (Fig 4). Out of 175 *P. mirabilis* isolates, 141 showed presence of *ureC* (80.5%), 127 for *ureA* (72.5%), 106 showed *flaA* (60.5%), 88 showed *zapA* (50.2%) and 50 showed *hpmA* (28.5%).

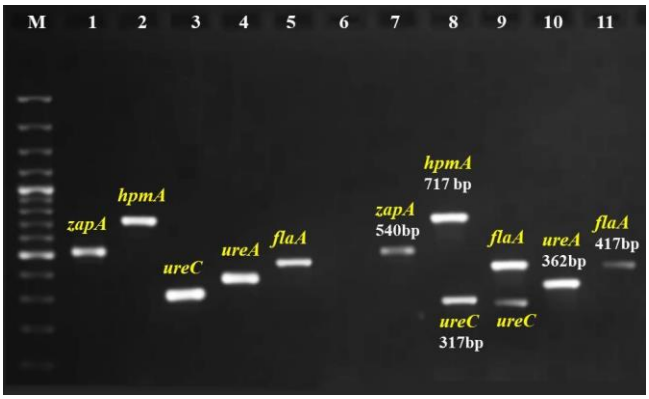

Fig 4: Gel photograph of PCR assay targeting putative virulence genes of *P. mirabilis* Lane M DNA ladder (100bp) Lane 1-5 Positive control of *P. mirabilis* (ATCC 12453) carrying all 5 putative virulence genes i.e., *zapA* (540bp), *hpmA (*717bp), *ureC* (317bp), *ureA* (362bp) and *flaA* (417bp) Lane 6 Negative control Lane 7 *P. mirabilis* isolate with *zapA* (540bp) gene Lane 8 *P. mirabilis* isolate with *hpmA (*717bp) and *ureC* (317bp) genes Lane 9 *P. mirabilis* isolate with *flaA* (417bp) and *ureC* (317bp) genes Lane 10 *P. mirabilis* isolate with *ureA* (362bp) gene Lane 11 *P. mirabilis* isolate with *flaA* (417bp) gene

The prevalence of *P. mirabilis* in chicken samples in the present study was 38.7%, which is in agreement with the findings of Kim *et al.* (2005) [10] who also reported 34% prevalence in chicken meat. Out of 38 *P. mirabilis* isolates recovered from chicken samples, all the five genes (*ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes) were found in twelve isolates (31.57%). Eight isolates (21.05%) showed *ureC*, *ureA*, *flaA* and *zapA* genes, eight isolates (21.05%) showed *ureA* and *ureC* genes, four isolates (10.52%) showed *ureC* and *flaA* genes.

The prevalence of *P. mirabilis* in chicken cloacal swabs was 49% in the present study, while 39% prevalence of *P. mirabilis* in poultry droppings was reported by Nahar *et al.* (2014) [13] in Bangladesh. The higher prevalence of *P. mirabilis* in chicken cloacal swabs might be due to unhygienic maintenance of broiler farms. Out of 74 *P. mirabilis* isolates recovered from poultry cloacal swabs, all the five genes (*ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes) were found in twenty isolates (27.02%). Seventeen isolates (22.97%) showed *ureC*, *ureA*, fla*A* and *zapA* genes, eight isolates (10.81%) showed *ureC* and *ureA* genes, five isolates (6.75%) showed *ureC*, *ureA* and *flaA* genes, three isolates (4.05%) showed *ureC* and *flaA* genes, two isolates (2.7%) showed *ureC* and *zapA* genes, five isolates (6.75%) showed only *ureC* gene, four isolates (5.4%) showed only *ureA* gene, one isolate (1.35%) showed only *hpmA* gene.

In the present study, the prevalence of *P. mirabilis* in pork and pig rectal swabs was found to be 37.5% and 37.8%, respectively. These results were higher when compared with the findings of Kim *et al.* (2005) [10] who reported only 20% prevalence of *P. mirabilis* in pork. The high prevalence rate of *P. mirabilis* in pork in the present study, may be due to unhygienic slaughter and processing or contaminated water used during slaughter of pigs. Out of 27 *P. mirabilis* isolates recovered from pork samples, all the five genes (*ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes) were found in eight isolates (29.62%). Three isolates (11.11%) showed *ureC*, *ureA*, *zapA* genes, one isolate (3.7%) showed *ureA*, *flaA* and *zapA* genes, two isolates (7.4%) showed *ureC* and *zapA* genes and one isolate (3.7%) showed only *ureC* gene. Out of 14 *P. mirabilis* isolates recovered from pig rectal swabs, all the five genes

(*ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes) were found in four isolates (28.57%). Five isolates (35.71%) showed *ureC*, *ureA* and *flaA* genes, three isolates (21.42%) showed *ureC* and *zapA* genes, one isolate (7.14%) showed *ureA* and *zapA* genes and one isolate (7.14%) showed only *ureA* gene.

The prevalence of *P. mirabilis* in mutton was 28.9%, which is in perfect correlation with the findings of Dabassa (2013) [7] and Begum (1984) [5] who reported a prevalence of 29.7% and 26.6% in mutton in Jimma town, Southwest Ethiopia and Islamabad, respectively. Out of 13 *P. mirabilis* isolates recovered from mutton samples, all the five genes (*ureC*, *ureA*, *flaA*, *hpmA* and *zapA* genes) were found in three isolates (23.07%). One isolate (7.69%) showed *ureC*, *flaA and zapA* genes, six isolates (46.15%) showed *ureC* and *ureA* genes and three isolates (23.07%) showed only *flaA* gene.

The prevalence of *P. mirabilis* in human urine samples in the present study was 10.7% and all human diarrhoeal samples were negative for *P. mirabilis.* The results were in agreement with the findings of Philips (2014) [16] who reported a prevalence of 14.59% of *P. mirabilis*. In contrast, Nachammai *et al*. (2015) [12] reported a lower prevalence of *P. mirabilis* (4.8%) in urine samples of humans. Out of 7 *P. mirabilis* isolates recovered from human urine samples, none of them showed all the five genes in a single isolate. Four isolates (57.14%) showed *ureC*, *ureA* and *flaA* genes, two isolates (28.57%) showed *ureA*, *ureC*, *zapA* and *hpmA* genes and one isolate (14.28%) showed only *flaA* gene.

The prevalence of *P. mirabilis* in water samples in the present study was 7.4%. These results were less when compared with findings of Adesoji and Ogunjobi (2016) [1] who reported an overall prevalence of 18.18% in selected drinking water distribution channels in Southwestern Nigeria. Less prevalence in the present study can be attributed to the variation due to many factors such as geographic area, type of sampling and disinfection of water.

Less prevalence rates of the target virulence genes was observed in the present study compared to the findings of Ali and Yousif (2015) [3] who isolated *P. mirabilis* from human clinical samples in Iraq and reported higher prevalence rates of the *ureC* (100%), *ureA* (96.66%), *flaA* (86.66%), *hpmA* (100%) and *zapA* (100%) genes and the prevalence rates of *ureC* and *zapA* genes in our study were in agreement with the findings of Pathirana *et al*. (2018) [14] who isolated *Proteus* spp. from humans and pet turtles and reported 52% and 91.7% of prevalence for *zapA* and *ureC* genes, respectively. Due to paucity of reports on virulence gene profiles of *P. mirabilis* isolated from animals and animal foods, the results could not be compared.

4. Conclusion

This study shows the presence of *P. mirabilis* and its distribution in livestock, foods of animal origin, humans and water samples in India. The presence of *P. mirabilis* in animal faeces could act as a potential source of contamination of carcass during slaughter and may pose threat to food safety and human health, as it has been associated with enteritis in humans. The results obtained in this study highlighted the virulence gene profiles of *P. mirabilis* of animal and human origin.

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