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Epidemiological surveillance, molecular characterisation and virulent gene expression of *Proteus mirabilis* and *Proteus vulgaris* from milk and meat samples

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Abstract
A study was carried out in Tirupati city to assess the *Proteus* species contamination in raw milk and meat, meant for human consumption and molecularly characterised the virulent gene profile of the isolates. A total of 741 samples including 347 raw milk and 394 raw meat samples were aseptically collected from different sampling points of 50 different wards of Tirupati, Andhra Pradesh for a period of 4 weeks. Out of 741 milk and meat samples, 189 (25.5%) isolates were positive for *Proteus* spp. (163 (41.4%) are from meat samples and 26 (7.5%) are from milk samples) based on cultural and biochemical characteristics. The biochemically confirmed isolates (189) were screened by using genus specific PCR targeting *16S rRNA*, 176 (93.1%) isolates were positive for genus specific gene of *Proteus* spp. (18 (69.2%) from milk samples and 158 (96.9%) from meat samples. Out of 176 *Proteus* spp. isolates 132 were confirmed as *P. mirabilis* (13 (50%) from milk and 119 (73%) from meat) by targeting *ureR* gene. Out of 176 *Proteus* spp. isolates 44 (25%) were confirmed as *P. vulgaris* (5 (19.2%) from milk and 39 (23.9%) from meat) by targeting *urease C* gene. The species confirmed isolates of *P. mirabilis* and *P. vulgaris* were screened for the presence of *rsbA*, *zapA*, *ureC and hpmA* virulent genes. 100% prevalence of *rsbA* and *zapA* genes were observed in *P. mirabilis* and *P. vulgaris* isolates of milk and meat samples. In multiplex PCR of *ureC* and *hpmA*, the prevalence of *ureC* gene is 100% and 92.44% and *hpmA* gene is 92.30% and 90.75%, respectively in milk and meat sample isolates of *P. mirabilis.* The prevalence of *ureC* gene of *P. vulgaris* is 100% and 82.05%and *hpmA* gene of *P. vulgaris* is 40% and 51.28%, respectively from milk and meat sample isolates.
The current study indicates higher prevalence of contamination of milk and meat samples with *Proteus species* and its virulence genes. It is suggested to create awareness among the farmers and butchers to take proper precautions while handling, collection and transport of the milk and meat products. The consumers are advised to consume the milk and meat products only after proper heating and cooking to prevent foodborne illness from milk and meat.

Keywords: Meat, milk, *Proteus mirabilis*, *Proteus vulgaris*, virulence genes

Introduction
Foodborne pathogens are responsible for causing a great number of diseases with significant effects on human health and economy. In 460 B.C. Hippocrates have recognised the relation between food poisoning outbreaks and consumption of spoiled or contaminated food (Hutt & Hutt, 1984) [25]. *Proteus* poses a significant challenge to both humans and animals throughout the world and is prevalent in several foods of animal origin including poultry (Lei *et al.*, 2014) [36]. All uncooked meats, fish, raw eggs, milk, fruits and vegetables, should be considered as probably being contaminated with *Proteus* species (Senior, 2010) [56].
Greek mythology describes *Proteus* as an early sea-god, noted for being versatile and capable of assuming many different forms (Drzewiecka, 2016) [16]. The genus *Proteus* was originally described and named by Hauser in 1885 for the Homer's Odyssey character "who has the power of assuming different shapes in order to escape being questioned" (Hoeniger, 1964) [23]. The *Proteus* genus is currently comprising *Proteus mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri*, *P. terrae*, and *P. cibarius* along with the unnamed genomospecies 4, 5, and 6 (6–10).
Both *P. mirabilis* and *P. vulgaris* are widely distributed in the environment and have been isolated from the intestinal tract of mammals, birds and reptiles. *P. mirabilis*, and to a lesser extent *P. vulgaris* are common inhabitants of the human gastrointestinal tract. *P. mirabilis* in particular, may also colonize the urinary tract under certain circumstances, an opportunistic

pathogen and one of the principal causes of urinary tract infections (UTIs) in hospitalised patients with indwelling urinary catheters, whereas, *P. vulgaris* is less commonly associated with UTIs (Manos and Belas, 2006) [37]. Colonization of the intestinal tract allows *Proteus* to establish reservoirs for transmission into the urinary tract by intermittent colonization of the periurethral region (Jacobsen *et al*., 2008) [27].

The virulence of the *Proteus* species is caused by several factors which are regulated and expressed by virulence genes encoded in operons (Pathirana *et al.*, 2018) [46]. These virulence genes increase the pathogenicity of *Proteus species* among which include urease (*ureC*) which is the most significant enzyme for kidney and bladder stone formation (Mohamed *et al.*, 2014) [60] and enables it to produce an environment in which it can survive (Aboh *et al.*, 2015) [2]. Swarming behaviour of *Proteus* species is mediated by *rsbA* gene which has been associated with biofilm formation and extracellular polysaccharide formation (Różalski *et al.*, 2012) [50]. Calcium independent hemolysin system is one of the main virulence factors in *Proteus* species and it consists of two proteins *hpmA* and *hpmB*. *hpmB* is responsible for the activation and extracellular secretion of the hemolysin *hpmA*. Activated *hpmA* once released into the external environment forms pores in and lyses the red blood cells (Ghaima *et al*., 2017) [20]. *ZapA* metalloprotease, is of distinctive significance, specifically expressed during differentiation of swimmer into swarmer cells (Pattanayak *et al.*, 2018) [47].

The present study was undertaken at Tirupati city to determine the prevalence of *Proteus* species and investigate the virulence gene expression in *P. mirabilis* and *P. vulgaris* isolated from milk and meat samples of all the 50 different wards in Tirupati.

Materials and Methods

Collection and processing of milk and meat samples

Meat and milk samples were collected from retail meat shops, local milk vendors, milk booths, cattle farms of all 50 municipal wards in Tirupati (Fig.-1). Sterile polythene zip lock bags and sterile sample collection bottles were used for collection of meat and milk samples respectively. Each bag and bottle were labelled with sample number and particulars about samples. Within 2 hours, all the samples were transferred in an ice box to the laboratory of Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati for further processing. All the procedures were carried out in a biosafety cabinet with due precautions.

Identification of all the isolates to the genus / species level was performed using conventional cultural and biochemical techniques as described by Al-Hamdani and Al-Hashimy, (2020) [4], Naidu *et al.*, (2021) [40], Nahar *et al.*, (2014) [39], Ali and Jasim, (2014) [6].

DNA extraction from enriched broth culture

DNA extraction was done by using boiling and snap chilling method with slight modifications (Naidu *et al.*, 2021) [40].

About 1.5 ml of the 18 hrs old enriched broth culture was pelleted (10,000 rpm for 10 min) and resuspended in 100 µl of sterile molecular grade water. It was then kept in a boiling water bath at 100 °C for 10 min and immediately chilled in an ice tub. After 20 min, the bacterial lysates were centrifuged at 10,000 rpm for 5 min and the supernatant was used as DNA template for PCR assays. The absorbance (A) ratio (A260/A280) of 1.8 to 2.0 pure DNA was stored at -20°C until further use.

Molecular confirmation of *Proteus genus* and *species* by PCR

All the biochemically confirmed *Proteus* isolates were subjected to PCR by targeting genus and species-specific genes (Table-1). The primers designed for the *16S rRNA*, *Urease C* and *UreR* genes were used for the detection of *Proteus species*, *P. mirabilis* and *P. vulgaris* respectively, were custom synthesized by Eurofin Genomics India Pvt. Limited, Bengaluru which are given in Table-2.

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. All the *Proteus* isolates were subjected to PCR by targeting *16SrRNA* for confirmation of the genus of *Proteus*. The cycling conditions were standardized at 4minute initial denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, initial extension at 72 °C for 10 sec and a 10 min final extension at 72 °C followed by maintenance at 4 °C. After confirming the genus of *Proteus*, all the isolates that were biochemically confirmed for *P. mirabilis* were subjected to PCR by targeting *ureR* gene. The cycling conditions were standardized at 4minute initial denaturation at 94 °C followed by 32 cycles of denaturation at 94 °C for 40 sec, annealing at 58 °C for 60 sec, initial extension at 72 °C for 20 sec and a 10 min final extension at 72 °C followed by maintenance at 4 °C. The isolates that were biochemically confirmed as *P. vulgaris* were subjected to PCR by targeting *ureaseC* gene. The cycling conditions were standardized at 4minute initial denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C for 60 sec, annealing at 60 °C for 60 sec, initial extension at 74 °C for 1 min and a 7 min final extension at 74 °C followed by maintenance at 4 °C.

Table 1: *Proteus* genus and species-specific PCR primers and sequences

Target gene	Primer sequence 5'-3'		Amplicon size (bp)	Reference
16S rRNA	F	GGAAACGGTGGCTAATACCGCATAAT	231	Latif *et al.*, (2017) [34]
	R	GCAGCGCTAGGTGAGCCTAATGGG		
urease C (*P. vulgaris*)	F	CGCTTT GCG ATG GCA AGT ACA AGT AAG	263	Kadhum & Muhaiesen *et al.*, (2015) [29]
	R	GCA AAT TGA GTG ACT TTG GCT GGA CC		
ureR (*P. mirabilis*)	F	GGTGAGATTTGTATTAATGG	225	Zhang *et al.*, (2013) [59]

2.4 Molecular detection of virulence genes of *Proteus species*

After the molecular characterization of *P. mirabilis and P. vulgaris*, all these isolates were subjected to PCR by targeting virulence genes of *Proteus species* (Table-2). All the confirmed *Proteus* isolates from different sources were

screened for the presence of virulence genes such as genes associated with extracellular protease production (*zapA*), urease production (*ureC*), hemolysin production (*hpmA*) and the genes facilitates biofilm and extracellular polysaccharide formation (*rsbA*) in the host.

The cycling conditions for *rsbA* gene were standardized at 5minute initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 60 sec, annealing at 58 °C for 45 sec, initial extension at 72 °C for 60 sec and a 7 min final extension at 72 °C followed by maintenance at 4 °C. The cycling conditions for *zapA* gene were standardized at 2minute initial denaturation at 95 °C followed by 31 cycles of denaturation at 95 °C for 30 sec, annealing at 59 °C for 30 sec, initial extension at 72 °C for 60 sec and a 5 min final extension at 72 °C followed by maintenance at 4 °C. The molecularly confirmed *P.mirabilis* and *P.vulgaris* were subjected to multiplex PCR for both *ureC* and *hpmA* genes which were standardized at 2-minute initial denaturation at 95 °C followed by 31 cycles of denaturation at 95 °C for 30 sec, annealing at 56.2 °C for 30 sec, initial extension at 72 °C for 20 sec and a 5min final extension at 72 °C followed by maintenance at 4 °C.

Table 2: Primers used for detection of virulence genes

Target gene	Primer	Primer sequence	Amplicon size (bp)	Reference
ureC	*ureC:F*	GTTATTCGTGATGGTATGGG	317	Owoseni *et al.*, (2021) [44], Ali & Yousif *et al.*, (2015) [5]
	ureC:R	TCGCCAGTTATCTTGACATTCTG		
hpmA	*hpmA:F*	GCATCATCAAGCGTACGTTCC	717	Ali & Yousif (2015) [5]
	hpmA:R	AATGAGCCAAGCTTGTTAAGCT		
rsbA	*rsbA:F*	TTGAAGGACGCGATCAGACC	467	Owoseni *et al.*, (2021) [44], Abbas *et al.*, (2015) [1].
	rsbA:R	ACTCTGCTGTCCTGTGGGTA		
zapA	*zapA:F*	ACCGCAGGAAAACATATAGCCC	540	Ali & Yousif (2015) [5]
	zapA:R	GCGACTATCTTCCGCATAATCA		

Results and Discussion

All the culturally and biochemically confirmed *Proteus* isolates were subjected to PCR by targeting *16SrRNA*for the confirmation of the genus of *Proteus*. The results revealed that out of 189 biochemically confirmed isolates targeted for *16SrRNA*, 176 (93.1%) isolates were confirmed as *Proteus spp* (Table-3). The results were higher to the results of Al-Mahemdy and Hamoody Abdullah *et al.*, (2020) [7]; Bakkar and Zubair (2019) [10]; Salih *et al*., (2019) [54]; Gwida *et al*., (2014) [22]; Fernandez-Delgado *et al.*, (2007) [8] who have reported the prevalence of 16SrRNA gene in their isolates of *Proteus* species at the rate of 14.16%, 50%, 41.4%, 60%, 0.33% respectively.

After confirming the genus of *Proteus*, all the isolates were subjected to PCR by targeting *ureR* and *ureaseC* gene. Out of 26 milk samples analysed, the percentage of *P. mirabilis* (*ureR*) and *P. vulgaris* (*ureaseC*) recovered from raw milk samples was 50% and 19.2% respectively (Table-3). The prevalence rate of *P. mirabilis* (50%) was higher to the findings of Olunrebi *et al*., (2021) [43]; Khater *et al*., (2021) [32]; Rudenko *et al*. (2021) [51]; Saad *et al*., (2017) [52]; Olivares-Pirez *et al*., (2015) [42]; Garedew *et al*., (2012) [19]; Goyal *et al*., (2009) [21]; Amany *et al*., (2014) [9]; Junaidu *et al*., (2011) [28] who have reported an overall prevalence of about 2.6%, 4.6%, 3.7%, 5.56%, 17.17%, 7.5%, 19% 2.33%, 3.26% respectively in raw milk samples. The prevalence rate of *P. vulgaris* (19.2%) was more or less similar to the findings of Saad *et al*., (2017) [52]; Olivares-Pirez *et al*., (2015) [42]; Goyal *et al*., (2009) [21] who reported an overall prevalence of about 17.17%, 7.5%, 19% respectively in raw milk samples. The prevalence rate of *P. vulgaris* (19.2%) was in contrast with the reports of Sadoon (2022) [53]; Danmallam and Pimenov (2019) [14]; Kavitha *et al*., (2016) [30]; Pirzada *et al*., (2016) [48]; Olivares-Pirez *et al*., (2015) [42]; Pathak *et al*., (2012) [48]; Enany *et al*., (2007) [17] who have reported 10.04%, 5.0%,1.5%, 37.5%, 9.21%, 1.79%, 1.4% respectively.

Out of 163 meat samples analysed, the percentage of *P. mirabilis* and *P. vulgaris* recovered from meat samples was 73% and 23.9% respectively. These prevalence rates of *P. mirabilis* (73%) were almost similar to the findings of Naidu *et al*., (2020) [40], Salih *et al*., (2019) [54], Barbour *et al*., (2012) [11] who have reported an overall prevalence of about 76.47%, 75.87%, 66% respectively in raw meat samples. The results were in contrast with the reports of Chinnam *et al*., (2020) [13], Ram *et al.*, (2019) [49], Nahar *et al.*, (2014) [39], Kim *et al.*, (2005) [33] who have reported 21.2%, 38.7%, 38.6%, 34% respectively. The prevalence rate *P. vulgaris* (23.9%) was similar to the findings of Owoseni *et al.*, (2021) [44]; Salih *et al*., (2019) [54]; Sudhakar *et al.*, (2010) [57] who have reported an overall prevalence of about 21.6%, 24.13% and 23.6% respectively in raw meat samples. The prevalence rate of *P. vulgaris* (23.9%) in the present study was found higher with the reports of Ogunleye and Carlson (2016) [41] who have reported 4.34% prevalence. A higher prevalence (44%) than the present study was reported by Gwida *et al*., (2014) [22].

Table 3: Showing the results of genus and species-specific PCR

Type of sample	Total No. of culturally positive samples	PCR positive samples for *Proteus* spp.		PCR positive samples for *P. mirabilis* (*ureR*)		PCR positive samples for *P. vulgaris* (*urease C*)	
		Number	%	Number	%	Number	%
Milk	26	18	69.2	13	50	5	19.2
Meat	163	158	96.9	119	73	39	23.9
Total	189	176	93.1	132	69.8	44	23.28

3.1 Prevalence of virulence genes in raw milk samples

All the 176 *Proteus* isolates recovered from raw milk and meat samples by cultural, biochemical and molecular tests were further subjected to PCR by targeting *ureC*, *hpmA*, *rsbA* and *zapA* genes for characterization of virulence gene profiles (Table-4 & Table-5). Out of 13 *P. mirabilis* isolates from milk, 13 (100%) isolates have shown presence of *ureC* gene, 12 (92.30%) isolates have shown presence of *hpmA* gene, 13 (100%) isolates have shown the presence of *rsbA* gene and 13(100%) isolates have shown the presence of *zapA* gene. Out of 5*P. vulgaris* isolates from milk, 5(100%) isolates have shown the presence of *ureC* gene, 2(40%) isolates have shown the presence of *hpmA gene*, 5(100%) isolates have shown the presence of *rsbA*gene and 5(100%) isolates have shown the presence of *zapA* gene.

The frequency of occurrence of *ureC g*ene was100% among the *P. mirabilis* and similar type of results were reported by Bunyan and Albakery (2020) [12]; Hussein *et al.*, 2020 [24]; Ali and Yousif (2015) [5] who have reported 100%, 95.2%, 100% prevalence respectively. The results were higher than the reports of Ghaima *et al.*, (2019) [20] and Alsheers *et al.*, (2016) [8] who have reported 88.23% and 33.5% respectively.

The prevalence of *hpmA* gene in the study was 92.30% among the *P. mirabilis* isolates and these results were almost agreed with the frequency reported by Bunyan and Albakery (2020) [12]; Lazm *et al.*, (2018) [35]; Hussein *et al.*, 2016 [24]; Ali and Yousif (2015) [5] who have reported 100%, 100%, 98.4%, 100% prevalence respectively. Our findings were higher than the reports of Ghaima *et al.*, (2019) [20] who have reported 87.3% prevalence.

The *rsbA* gene was amplified in *P. mirabilis* with a frequency of 100% in the present study which was found similar with the frequency reported by Bunyan and Albakery (2020) [12] and, Hussein *et al.*, (2016) [24] who have reported 100% prevalence. The results were in contrast with the reports of Dehnavi *et al.*, (2017) [15] who have reported 67% prevalence.

The frequency of occurrence of *zapA* gene was 100% among the *P. mirabilis* isolates and the results were similar to the results reported by Bunyan and Albakery (2020) [12], Ali and Yousif (2015) [5] who have reported 100% prevalence. The results were in contrast with the reports of Alsheers *et al.* (2016) [8] who have reported 44.5%.

The frequency of occurrence of *ureC* gene was 100% among the *P. vulgaris* isolates and the results were in agreement with the findings of Algammal *et al.*, (2021) [3]; Sun *et al.*, (2020) [58]; Ram *et al.*, (2019) [49]; Keisam *et al.*, (2019) [31]; Pathirana *et al.*, (2018) [46] who have reported 100%, 90.91%,96.60%, 80.5%, 100% of prevalence respectively. The results were in contrast with the reports of Owoseni *et al.*, (2021) [44] who have reported 40% prevalence.

The frequency of occurrence of *hpmA* gene was 40% among the *P.* vulgaris isolates and the results were less compared to the findings of Jabur *et al.*, (2013) [26] who have reported 100% prevalence.

The *rsbA* gene was amplified in *P. vulgaris* with a frequency of 100% which was higher than the frequency of 26.7% and 80% reported by Owoseni *et al.*, (2021) [44] and Pathirana *et al.*, (2018) [46]. The frequency of the *rsbA* gene was almost similar to the findings of Algammal *et al.*, (2021) [3] who have reported 94.3% of *rsbA* gene in *P. vulgaris* isolated from ducks. The frequency reported was in contrast to the reports of Abbas *et al.*, (2015) [1], who have reported that *rsbA* gene could not be amplified in *P. vulgaris*.

The frequency of occurrence of *zapA* gene was 100% among the *P. vulgaris* isolates which was found higher than the findings of Jabur *et al.*, (2013) [26] who have reported 100% prevalence.

Table 4: Showing the results of PCR for virulence genes

Type of sample	Total No. of PCR positive samples for *Proteus* spp.	*zapA*		*rsbA*		*ureC*		*hpmA*	
		Number	%	Number	%	Number	%	Number	%
Milk	18	18	100	18	100	18	100	14	77.7
Meat	158	158	100	158	100	152	96.2	128	84.2
Total	176	176	100	176	100	170	96.5	142	80.7

Table 5: Showing the results of PCR for virulence genes of *P. mirabilis*

Type of sample	Total No. of PCR positive samples for *P. mirabilis*	*zapA*		*rsbA*		*ureC*		*hpmA*	
		Number	%	Number	%	Number	%	Number	%
Milk	13	13	100	13	100	13	100	12	92.30
Meat	119	119	100	119	100	110	92.44	108	90.75
Total	132	132	100	132	100	123	96.22	120	91.53

Table 6: Showing the results of PCR for virulence genes of *P. vulgaris*

Type of sample	Total No. of PCR positive samples for *P. vulgaris*	*zapA*		*rsbA*		*ureC*		*hpmA*	
		Number	%	Number	%	Number	%	Number	%
Milk	5	5	100	5	100	5	100	2	40
Meat	39	39	100	39	100	32	82.05	20	51.28
Total	44	44	100	44	100	37	84.1	22	50

Prevalence of virulence genes in raw meat samples

Out of 119 *P. mirabilis* isolates from meat, 110 (92.44%) isolates have shown the presence of *ureC* gene , 108 (90.75%) isolates have shown the presence of *hpmA* gene, 119 (100%) isolates have shown the presence of *rsbA* gene *and* 119 (100%) isolates have shown the presence of *zapA* gene .Out of 39 *P. vulgaris* isolates from meat, 32 (82.05%) isolates have shown the presence of *ureC* gene, 20 (51.28%) isolates have shown the presence of *hpmA* gene, 39 (100%) isolates have shown the presence of *rsbA* gene and39 (100%) isolates have shown the presence of *zapA* gene.

The frequency of occurrence of *ureC* gene (92.44%) among the *P. mirabilis* was similar to the findings of Sun *et al.*, (2020) [58] and Ram *et al.*, (2019) [49], who have reported

90.91% and 96.60% respectively. A higher prevalence (100%) than the present study was reported by Algammal *et al.*, (2021) [3] and Pathirana *et al.*, (2018) [46]. A lower prevalence of 80.5% and a very low prevalence of 40% than the present study was reported by Keisam *et al.*, (2019) [31] and Owoseni *et al.*, (2021) [44] respectively.

The prevalence of *hpmA* gene in the study was 90.75% among the *P. mirabilis* isolates which was lower than the reports (100%) of Sanches *et al.*, (2019) [55] and Keisam *et al.*, (2019) [31]. Our findings were higher than the reports of Sun *et al.* (2020) [58] and Ram *et al.*, (2019) [49] who have reported a prevalence of 70.45% and 60.5% respectively. The frequency of *hpmA* gene reported in the study was in contrast to the report of Naidu *et al.*, (2020) [40], who have reported that the *hpmA* gene was not detected in samples of animal origin.

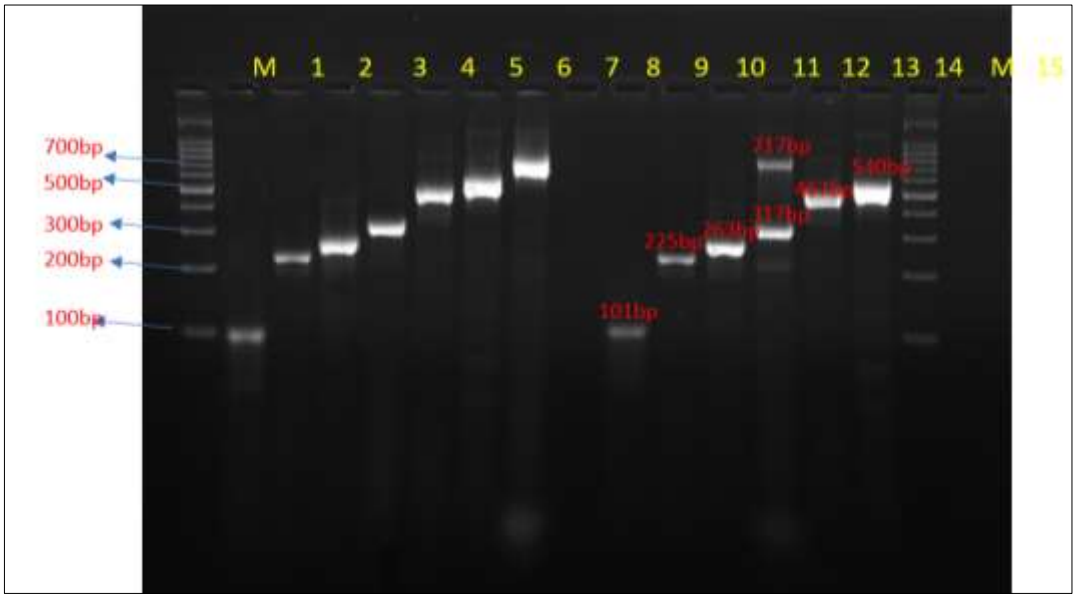
The *rsbA* gene was amplified in *P. mirabilis* with a frequency of 100% which was higher than the frequency of 26.7% and 80% reported by Owoseni *et al.*, (2021) [44] and Pathirana *et al.*, (2018) [46] respectively. The frequency was almost similar to the findings of Algammal *et al.*, (2021) [3] who have reported 94.3% of *rsbA* gene in *Proteus species* isolated from ducks. The frequency of *rsbA* gene reported in the current study was in contrast to the reports of Abbas *et al.*, (2015) [1], who have reported that *rsbA* gene could not be amplified in *P. mirabilis*.

The frequency of occurrence of *zapA* gene was 100% among the *P. mirabilis* isolates which was found similar to the findings of Naidu *et al.*, (2020) [40]; Sanches *et al.*, (2019) [55]; Keisam *et al.*, (2019) [31] who have reported 100%,100%, 96.59% respectively. The frequency of *zapA* gene reported was higher to the findings of Algammal *et al.*, (2021) [3]; Ram *et al.*, (2019) [49]; Pathirana *et al.*, (2018) [46] who have reported 91.4%, 50.28 and 73.3% respectively.

The frequency of occurrence of *ureC* gene was 82.05% among the *P. vulgaris* was lower when compared with the frequency reported by Pathirana *et al.*, (2018) [46] who have reported 100% whereas it was higher than the reports of Owoseni *et al.*, (2021) [44] who have reported 26.6%.

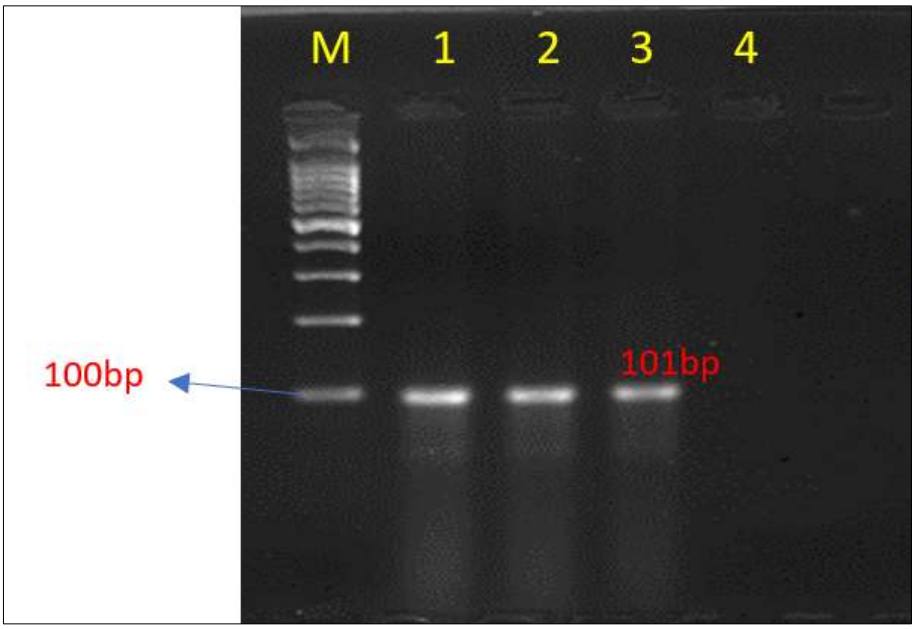
The *hpmA* gene was amplified in *P. vulgaris* with a frequency of 51.28% which was lower than the findings of Jabur *et al.*, (2013) [26] who have reported a frequency of 100%

The *rsbA* gene was amplified in *P. vulgaris* with a frequency of 100% which was in contrast to the reports of Owoseni *et al.*, (2021) [44] who have reported a frequency of 6.6% . The results were in contrast to the reports of Pathirana *et al.*, (2018) [46] and Abbas *et al.*, (2015) [1], who have reported that *rsbA* could not be amplified in *P. vulgaris.* The *zapA* gene was amplified in *P. vulgaris* with a frequency of 100% which was in contrast to the reports of Pathirana *et al.*, (2018) [46], who have reported that *zapA* gene could not be amplified in *P. vulgaris.*



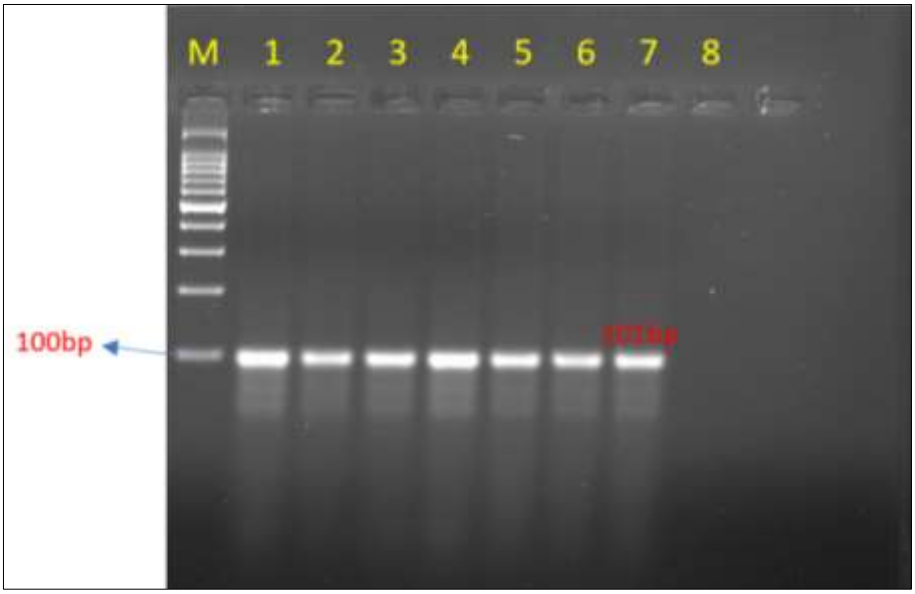
Lane M: Molecular weight marker (100 bp)
Lane 1 and 9: Positive control for *16SrRNA* (101 bp) and Isolate carrying (101 bp)
Lane 2 and10: Positive control for *UreR* (225 bp) and Isolate carrying *UreR* (225 bp)
Lane 3 and 11: Positive control for *UreaseC* (263 bp) and the isolate carrying *UreaseC* (263 bp)
Lane 4 and 12: Positive control for *UreC* (313 bp) and Isolate carrying *UreC* (313 bp)
Lane 5 and 13: Positive control for *rsbA* (467 bp) and Isolate carrying *rsbA* (467 bp)
Lane 6 and 14: Positive control for *ZapA* (540 bp) and Isolate carrying *ZapA* (540 bp)
Lane 7 and 12: Positive control for *hpmA gene* (717bp) and the isolate carrying *hpmA gene*
Lane 8 and 15: Negative control

Fig 1: Gel photograph of PCR targeting all the genes in the current study



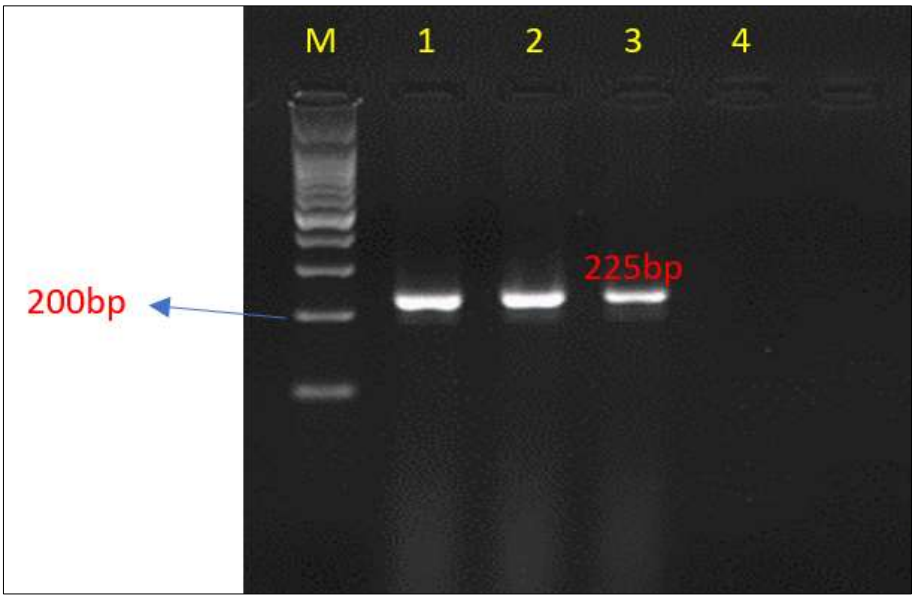
Lane M: Molecular weight marker (100 bp)
Lane 1 to 3: Positive control for 16S rRNA (101bp)
Lane 4: Negative control

Fig 2: Standardization of genus specific PCR for the detection of 16S RNA gene for confirmation of *Proteus* spp.



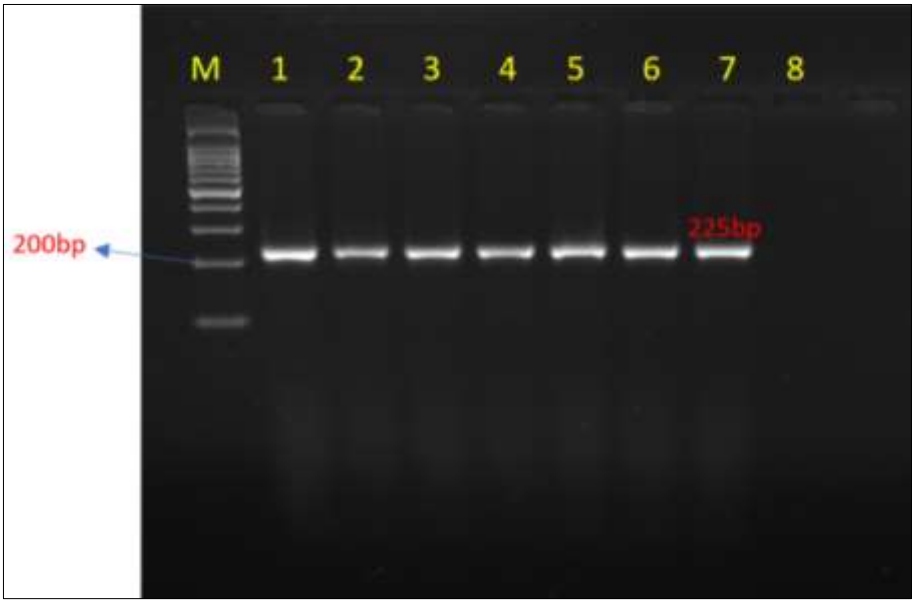
Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for 16S rRNA (101bp)
Lane 2 to 4: Isolates of milk carrying 16S rRNA
Lane 5 to 7: Isolates of meat carrying 16S rRNA
Lane 8: Negative control

Fig 3: Detection of 16S RNA gene for confirmation of *Proteus* spp.



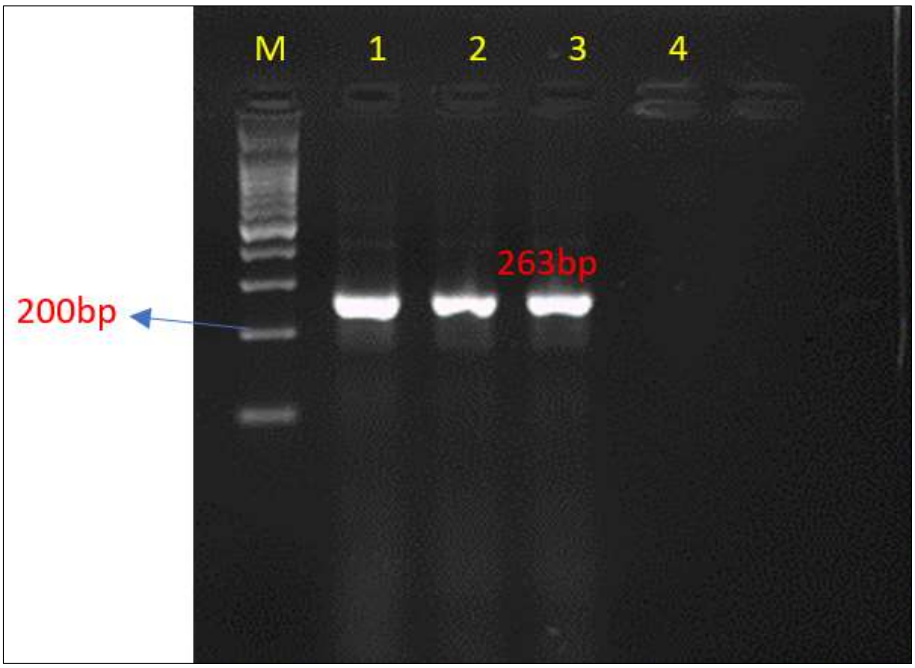
Lane M: Molecular weight marker (100 bp)
Lane 1 to 3: Positive control for *UreR* gene (225 bp)
Lane 4: Negative control

Fig 4: Standardization of species-specific PCR for the detection of *UreR* gene for confirmation of *P. mirabilis.*



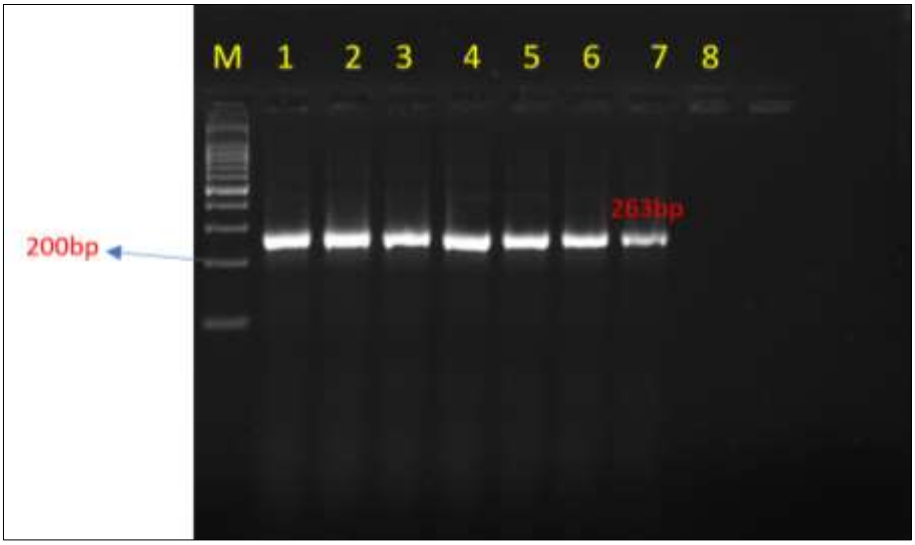
Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for *UreR* gene (225 bp)
Lane 2 to 4: Isolates of milk carrying *UreR* gene
Lane 5 to 7: Isolates of meat carrying *UreR* gene
Lane 8: Negative control

Fig 5: Detection of *UreR* gene for confirmation of *P. mirabilis.*



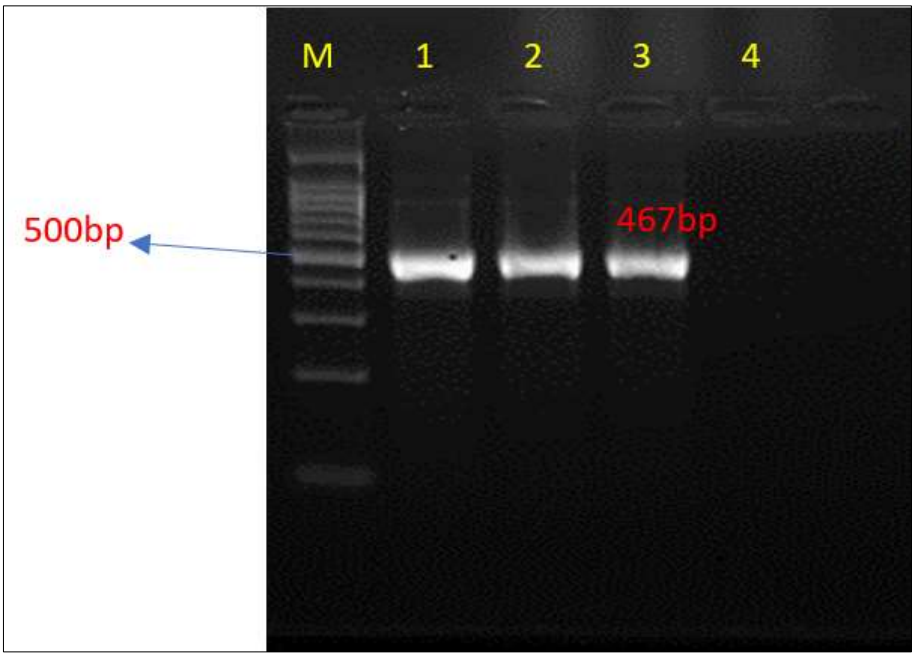
Lane M: Molecular weight marker (100 bp)
Lane 1 to 3: Positive control for *Urease* gene (263 bp)
Lane 4: Negative control

Fig 6: Standardization of species-specific PCR for the detection of *Urease C* gene for confirmation of *P. vulgaris.*



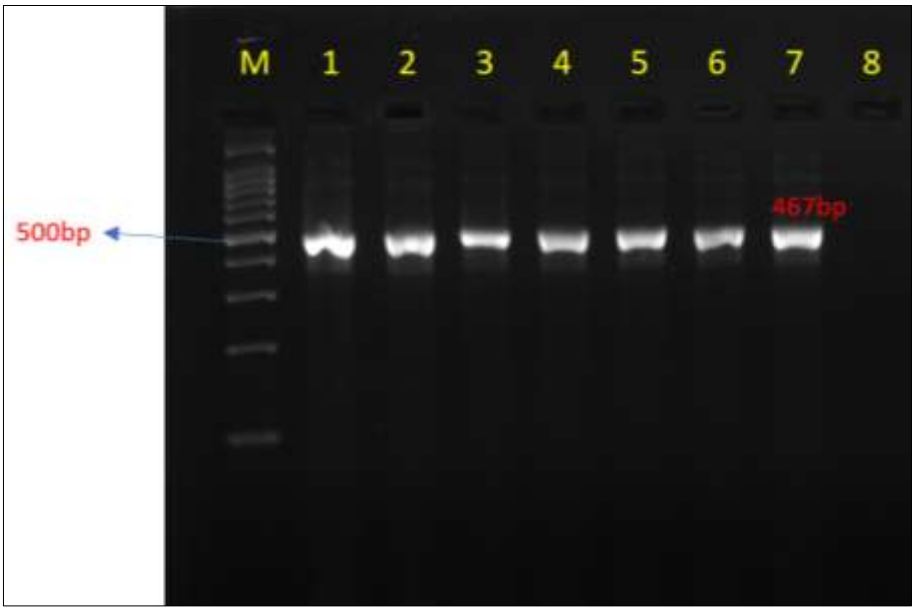
Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for *Urease C* gene (263 bp)
Lane 2 to 4: Isolates of milk carrying *Urease C* gene
Lane 5 to 7: Isolates of meat carrying *Urease C* gene
Lane 8: Negative control

Fig 7: Detection of *Urease C* gene for confirmation of *P. vulgaris.*



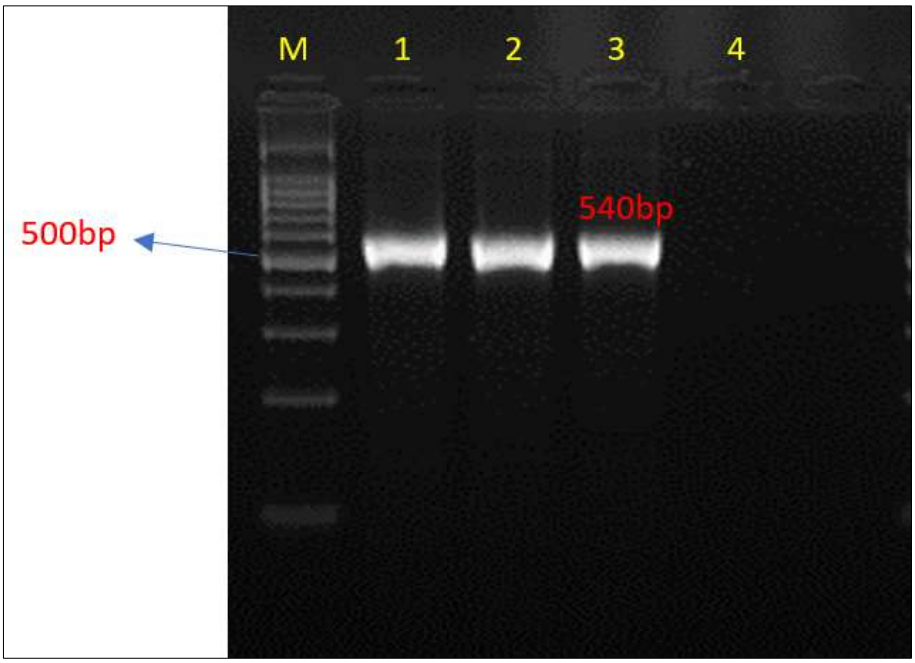
Lane M: Molecular weight marker (100 bp)
Lane 1 to 3: Positive control for *rsbA* gene (467 bp)
Lane 4: Negative control

Fig 8: Standardization of uniplex PCR for the detection of *rsbA* gene



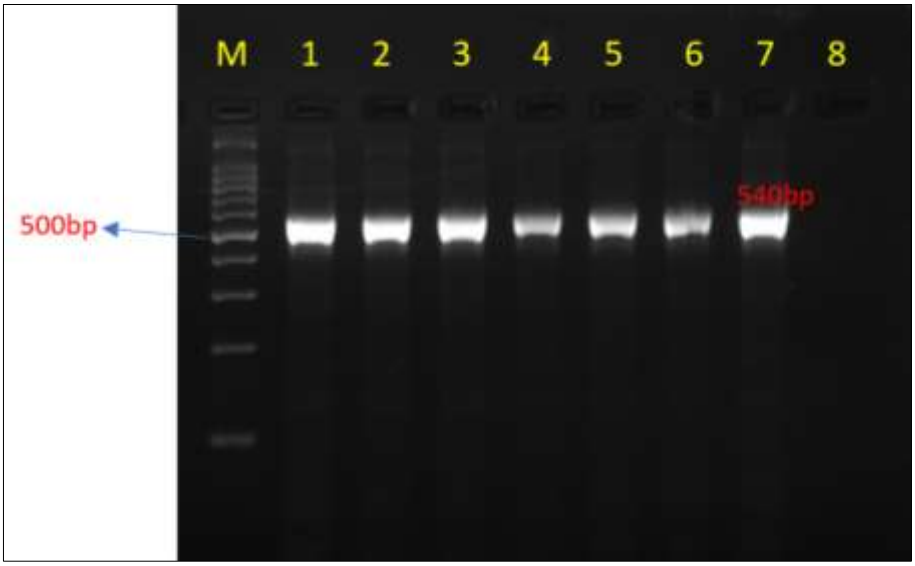
Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for *rsbA* gene (467 bp)
Lane 2 to 4: Isolates of milk carrying *rsbA* gene
Lane 5 to 7: Isolates of meat carrying *rsbA* gene
Lane 8: Negative control

Fig 9: Detection of *rsbA* virulence gene in the isolates



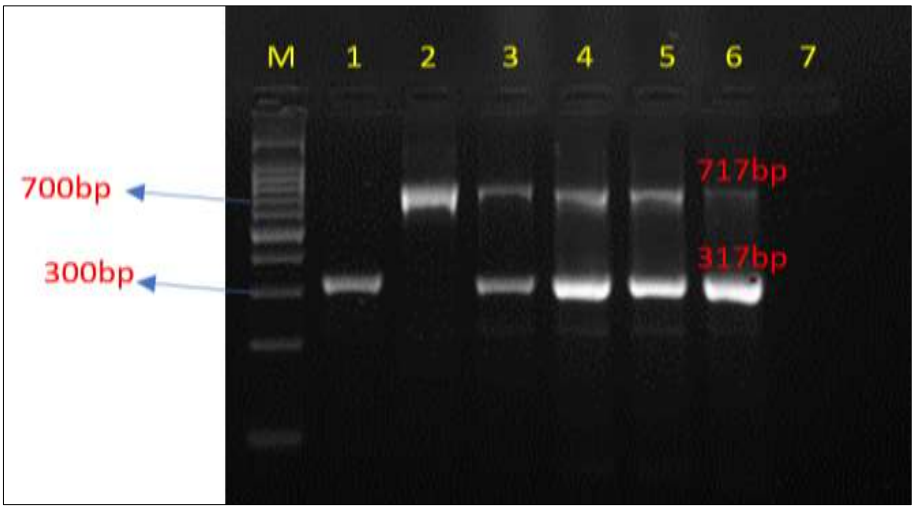
Lane M: Molecular weight marker (100 bp)
Lane 1 to 3: Positive control for *ZapA* gene (540 bp)
Lane 4: Negative control

Fig 10: Standardization of uniplex PCR for the detection of *ZapA* gene



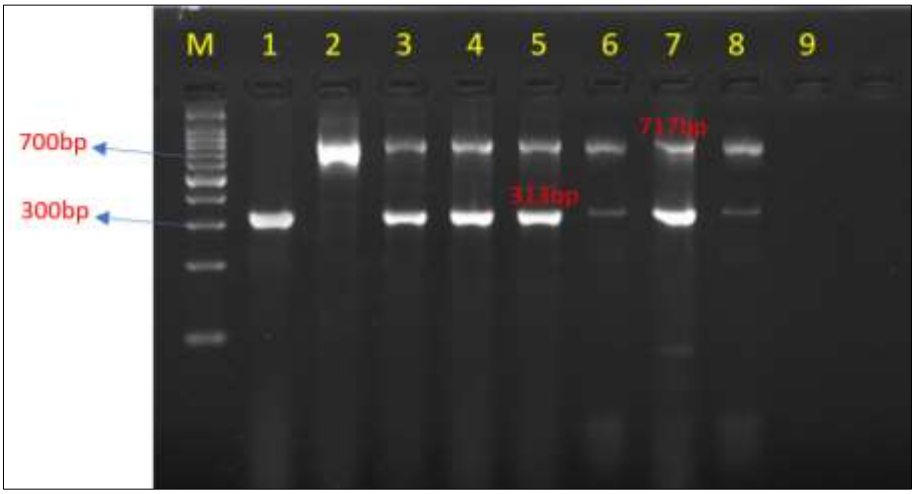
Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for *ZapA* gene (540 bp)
Lane 2 to 4: Isolates of milk carrying *ZapA* gene
Lane 5 to 7: Isolates of meat carrying *ZapA* gene
Lane 8: Negative control

Fig 11: Detection of *ZapA* virulence gene in the isolates



Lane M: Molecular weight marker (100 bp)
Lane 1 and 2: Positive control for *UreC* (317 bp) and *hpmA gene* (717 bp)
Lane 3 to 6: Positive isolates
Lane 7: Negative control

Fig 12: Standardization of multiplex PCR for the detection of *UreC* and *hpmA gene*



Lane M: Molecular weight marker (100 bp)
Lane 1 and 2: Positive control for *UreC* (317 bp) and *hpmA gene* (717 bp)
Lane 3 to 5: Isolates of milk carrying *UreC* and *hpmA gene*
Lane 6 to 8: Isolates of meat carrying *UreC* and *hpmA gene*
Lane 9: Negative control

Fig 13: Detection of *UreC* and *hpmA* virulence gene in the isolates

Conclusion

Higher prevalence of *Proteus spp* and virulence factors in the present study may be due to the variation in the hygienic practices followed. All the milk samples were collected from the local milk vendors who have collected the milk from the nearby villages to Tirupati and also from the dairy farmers in different wards of Municipal Corporation, Tirupati who have very little knowledge about the hygienic precautions to be taken while transportation and handling of milk. Hence unhygienic practices followed while collection, storage and transportation of the milk may be the important reasons for the increased prevalence of *Proteus* species in milk. In addition, the hygienic practices followed by the retail meat shops were very poor, and probably lack of awareness among the meat handlers may be the most important factor for the increased prevalence of *Proteus species* in meat.

Acknowledgement

I extend my thanks to the Department of Veterinary Public Health and Epidemiology, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India.

Conflicts of interest

The authors have no conflicts of interest to declare. All the co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Ethical committee approval

The ethical committee approval is not needed for the current study as it was done by collecting the meat samples from meat shops which are kept for sale and the milk samples were collected from local milk vendors and farms. In this study no invasive procedure was used on animals and no animal was harmed in the study.

Impacts

- Emerging food borne pathogens like *Proteus species* are of major public health concern and are responsible for causing outbreaks of food poisonings.
- This study revealed the prevalence of 50% and 73% of *Proteus mirabilis* from the isolates of milk and meat samples as well as 19.2% and 23.9% prevalence of *Proteus vulgaris* from the isolates of milk and meat samples.
- During this study, all the isolates of *Proteus mirabilis* and *Proteus vulgaris* from milk and meat were screened for the presence of *rsbA, zapA, ureC and hpmA* virulent genes and identified the highest prevalence of virulent genes in the isolates.

References

1. Abbas KF, Jawad K, Khafaji AL, Maysaa S, Shukri AL. Molecular detection of some virulence genes in *Proteus mirabilis* isolated from Hillaprovince. Int. J. Res. Stud. in Biosci.(IJRSB), 2015;3(10):85-89.
2. Aboh EA, Giwa FJ, Giwa A. Microbiological assessment of well waters in Samaru, Zaria, Kaduna, State, Nigeria. Annals of African Medicine. 2015;14(1):32.
3. Algammal AM, Hashem HR, Alfifi KJ, Hetta HF, Sheraba NS, Ramadan H, *et al*. atp D gene sequencing, multidrug resistance traits, virulence-determinants, and antimicrobial resistance genes of emerging XDR and MDR-Proteus mirabilis. Scientific reports. 2021;11(1):1-15.
4. Al-Hamdani H, Al-Hashimy A. Molecular detection of *UreC*, *HpmA*, *rsbA* and *mrpA* genes of *proteus mirabilis* urinary tract infection in patient with rheumatoid arthritis. The Iraqi Journal of Agricultural Science. 2020;51:245-251.
5. Ali HH, Yousif MG. Detection of some virulence factors genes of *Proteus mirablis* that isolated from urinary tract infection. IJAR. 2015;3(1):156-163.
6. Ali TH, Jasim HM. Detection of Virulence Factors of Local Isolates of Proteus mirabilis. Al-Nahrain Journal of Science. 2014;17(4).
7. Al-Mahemdy, Hamoody Abdullah. Molecular detection of *Proteus vulgaris* isolated from urine of cow in Al-Anbar province of Iraq. Plant Archives. 2020;20:1253-1257.
8. Alsherees HAA, Abdzaid AJ, Talib R. Molecular study of Proteus mirabilis bacteria isolated from urine and wounds in hospitals Al-Najaf province. International Journal of Advanced Research in Res. Biol. Sci. 2016;3(6):99-105.
9. Amany MI, Hisham SN, Ahmed AE. Molecular characterization of antibiotic resistance in gram–negative bacteria isolated from dairy products. Kafrelsheikh Veterinary Medical Journal, 2014;12(2):39-61.
10. Bakkar FA, Zubair M. Molecular study based on Proteus mirabilis contamination in chicken meat in selected districts of Kerala, India. International Journal of Development Research. 2019;09(06):28367-28369.
11. Barbour EK, Hajj ZG, Hamadeh S, Shaib HA, Farran MT, Araj G, *et al*. Comparison of phenotypic and virulence genes characteristics in human and chicken isolates of Proteus mirabilis. Pathogens and global health. 2012;106(6):352-357.
12. Bunyan IA, Albakery SA. Molecular Detection of Some Virulence Factors of Uropathogenic Proteus mirabilis. Annals of Tropical Medicine & Public Health. 2020;24(6):536-545.
13. Chinnam BK, Nelapati S, Tumati SR, Bobbadi S, Peddada VC, Bodempudi B. Detection of β-lactamase producing *Proteus mirabilis* of animal origin in Andhra Pradesh, India and their genetic diversity. Journal of Food Protection. 2021;84(8):1374-1379.
14. Danmallam FA, Pimenov NV. Study on prevalence, clinical presentation, and associated bacterial pathogens of goat mastitis in Bauchi, Plateau, and Edo states, Nigeria. Veterinary World. 2019;12(5):638-645.
15. Dehnavi S, Zarif BR. RsbA Gene's Confirmation in *P. mirabilis* With PCR Method in Patients With Urinary Tract Infection in Province of Qorveh. Biosciences Biotechnology; c2017.
16. Drzewiecka D. Significance and roles of *Proteus* spp. bacteria in natural environments. Microbial ecology. 2016;72(4):741-758
17. Enany ME, EL-Attar AA, Khafagy AAR, EL-Gammal AM. Bacterial and mycotic causes of subclinical bovine mastitis. Suez Canal Veterinary Medicine Journal. 2007;12:107-133.
18. Fernández-Delgado M, Contreras M, García-Amado MA, Gueneau P, Suárez P. Occurrence of *Proteus mirabilis* associated with two species of Venezuelan oysters. Revista do Instituto de Medicina Tropical de São Paulo. 2007;49:355-359.
19. Garedew L, Berhanu A, Mengesha D, Tsegay G. Identification of gram-negative bacteria from critical control points of raw and pasteurized cow milk consumed at Gondar town and its suburbs, Ethiopia. BMC public health. 2012;12(1):1-7.
20. Ghaima KK, Latteef NS, Abdulhussein AM, Hasan SF. Molecular Study of Hemolysin genes in *Proteus mirabilis* isolated from patients with urinary tract infections. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 2017;10(1):2149.
21. Goyal N, Dhewa T, Bhandari BS, Khare S. Emergence of extended spectrum beta-lactamase (ESBLs) producing Proteus in raw milk of Doon Valley. Journal of Applied and Natural Science. 2009;1(2):129-131.
22. Gwida M, Hotzel H, Geue L, Tomaso H. Occurrence of Enterobacteriaceae in raw meat and in human samples from Egyptian retail sellers. International scholarly research notices; c2014.
23. Hoeniger JFM. Cellular changes accompanying the swarming of Proteus mirabilis: I. Observations of living cultures. Canadian journal of microbiology. 1964;10(1):1-9.
24. Hussein EI, Al-Batayneh K, Masadeh MM, Dahadhah FW, Al Zoubi MS, Aljabali AA, *et al*. Assessment of pathogenic potential, virulent genes profile, and antibiotic susceptibility of Proteus mirabilis from urinary tract infection. International journal of microbiology, 1-5.
25. Hutt PB, Hutt PBI. A history of government regulation of adulteration and misbranding of food. Food Drug Cosm. LJ. 1984;39:2.
26. Jabur MH, AL-Saedi EA, Trad SJK. Isolation of *Proteus mirabilis and Proteus vulgaris* from different clinical sources and study of some virulence factors. Journal of Babylon University/Pure and Applied Sciences. 2013;21(1):1-6.

27. Jacobsen SM, Stickler DJ, Mobley HLT, Shirtliff ME. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clinical microbiology reviews. 2008;21(1):26-59.
28. Junaidu AU, Salihu MD, Tambuwal FM, Magaji AA, Jaafaru S. Prevalence of mastitis in lactating cows in some selected commercial dairy farms in Sokoto metropolis. Adv Appl Sci Res. 2011;2(2):290-294.
29. Kadhum SJ, Muhaiesen SH. Isolation of Uropathogens from Pediatric Associated UTI, with Special Focus on the Detection of Proteus Vulgaris. Iraqi journal of biotechnology. 2015;14(1):77-84.
30. Kavitha KL, Rao KS, Rajesh K, Satheesh K. Isolation of bacterial pathogens from clinical cases of mastitis in buffaloes. Buffalo Bulletin. 2016;35(4):745-748.
31. Keisam S, Tuikhara N, Ahmed G, Jeyaram K. Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India. International Journal of Food Microbiology. 2019;296:21-30.
32. Khater DF, Lela RA, El-Diasty M, Moustafa SA, Wareth G. Detection of harmful foodborne pathogens in food samples at the points of sale by MALDT-TOF MS in Egypt. BMC Research Notes. 2021;14(1):1-6.
33. Kim SH, Wei CI, An H. Molecular characterization of multidrug-resistant *Proteus mirabilis* isolates from retail meat products. Journal of food protection. 2005;68(7):1408-1413.
34. Latif B, Aleabdyn SZ, Ahmed I. A Comparative bacteriological and molecular study on some virulence factor of *Proteus spp* isolated from clinical and environment specimens. Int. J. Curr. Res. Acad. Rev. 2017;5(5):26-33.
35. Lazm AM, Jebur MS, Alomashi GB. Sequencing of HpmA Gene in *Proteus mirabilis* of UTIs among rheumatoid arthritis patients. Journal of Pharmaceutical Sciences and Research. 2018;10(2):265-271.
36. Lei CW, Zhang AY, Liu BH, Wang HN, Guan ZB, Xu CW, *et al*. Molecular characteristics of *Salmonella* genomic island 1 in *Proteus mirabilis* isolates from poultry farms in China. Antimicrobial agents and chemotherapy. 2014;58(12):7570-7572.
37. Manos J, Belas Robert. The genera *proteus, providencia, and morganella*. Prokaryotes. 2006;6:245-269.
38. Mohammed SO, Elshahaby OA, Hafez EE, Mohammed AK, Ahmed E. Characterization and purification of urease enzyme from new *Proteus mirabilis* strain. J. Adv. Sci. Res. 2014;5(4):08-11.
39. Nahar A, Siddiquee M, Nahar S, Anwar KS, Ali SI, Islam S. Multidrug Resistant-*Proteus mirabilis* isolated from chicken droppings in commercial Poultry Farms: Bio-security concern and emerging public health threat in Bangladesh. Biosafety and Health Education. 2014;2(2):1.
40. Naidu S, Bodempudi B, Chinnam B, Nelapati S, Tummati S, Cherukuri N, *et al*. Study of Virulence Genes, Antibiotic and beta-Lactamase Profiles of *Proteus mirabilis* Isolated from Livestock and Livestock Products. International Journal of Livestock Research. 2020;10(12):201-209.
41. Ogunleye AO, Carlson S. Drug resistant *Proteus mirabilis* and *Proteus vulgaris* isolated from rats captured from some poultry houses in Ibadan, Oyo State, Nigeria and their Public health importance. African Journal of Biomedical Research. 2016;19(3):261-266.
42. Olivares-Pérez J, Kholif AE, Rojas-Hernández S, Elghandour MMMY, Salem AZM, Bastida AZ, *et al*. Prevalence of bovine subclinical mastitis, its etiology and diagnosis of antibiotic resistance of dairy farms in four municipalities of a tropical region of Mexico. Tropical animal health and production. 2015;47(8):1497-1504.
43. Olunrebi BA, Onaolapo JA, Bolaji RO, Otaru S. Antimicrobial Susceptibility Pattern of Enteric Bacteria from Fresh cow milk and handlers in Zaria Metropolis, Kaduna State Nigeria. AROC in Pharmaceutical and Biotechnology. 2021;1(2):35-42.
44. Owoseni MC, Oyigye O, Sani B, Lamin J, Chere A. Antimicrobial resistance and virulence genes profiling of *Proteus species* from poultry farms in lafia, Nigeria. *BioRxiv*; c2021.
45. Pathak A, Verma K, Anand Soni K. Physico-chemical and Microbial quality of boiled milk. Int. J. Food, Agri. Veter. Sci. 2012;2:10-15.
46. Pathirana HNKS, De Silva BCJ, Wimalasena SHMP, Hossain S, Heo GJ. Comparison of virulence genes in *Proteus species* isolated from human and pet turtle. Iranian journal of veterinary research. 2018;19(1):48.
47. Pattanayak S, Kumar PR, Sahoo MK, Paul A, Sahoo PK. First field-based evidence of association of Proteus mirabilis causing large scale mortality in Indian major carp farming. Aquaculture. 2018;495:435-442.
48. Pirzada M, Malhi KK, Kamboh AA, Rind R, Abro SH, Lakho SA, Bhutto KR, *et al*. Prevalence of subclinical mastitis in dairy goats caused by bacterial species. J. Anim. Health Prod. 2016;4(2):55-59.
49. Ram P, Rao V, Rao S, Subramanyam KV, Srinivas K. Prevalence and virulence gene profiles of *Proteus mirabilis* isolated from animal, human and water samples in Krishna District, Andhra Pradesh, India. Pharma Innov. J. 2019;8(6):19-23.
50. Różalski A, Torzewska A, Moryl M, Kwil I, Maszewska A, Ostrowska K, *et al*. Proteus sp.–an opportunistic bacterial pathogen–classification, swarming growth, clinical significance and virulence factors. Acta Universitatis Lodziensis. Folia Biologica et Oecologica. 2012;8:1-17.
51. Rudenko P, Sachivkina N, Vatnikov Y, Shabunin S, Engashev S, Kontsevaya S, *et al*. Role of microorganisms isolated from cows with mastitis in Moscow region in biofilm formation. Veterinary World. 2021;14(1):40.
52. Saad A, Ibrahim G, Ismail T, Mohamed A. Microbiological Studies on some Spoilage and Food-Poisoning Microorganism in Raw Milk. Suez Canal Veterinary Medical Journal. SCVMJ. 2017;22(2):43-54.
53. Sadoon AS. Clinical and subclinical mastitis in buffalue in Mosul area, Iraq. Iraqi Journal of Veterinary Sciences. 2021;36(1):177-186.
54. Salih SS, Mohammed SJ, Noori IM, Mohammed LM, Soor TAH. Prevalence and molecular characterization of Beta-lactamase resistance gene in multidrug resistance bacteria, Proteus spp. Kurdistan Journal of Applied Research, 2019, 20-28.
55. Sanches MS, Baptista AAS, de Souza M, Menck-Costa MF, Koga VL, Kobayashi RKT, *et al*. Genotypic and phenotypic profiles of virulence factors and antimicrobial

resistance of *Proteus mirabilis* isolated from chicken carcasses: potential zoonotic risk. Brazilian Journal of Microbiology. 2019;50(3):685-694.
56. Senior BW. Proteus, morganella, and providencia. Topley & Wilson's Microbiology and Microbial Infections; c2010.
57. Sudhakar B, Ashish MP, Vikas SW, Ravindra JZ. Prevalence of microorganisms of hygienic interest in an organized abattoir in Mumbai, India. J Infect Dev Ctries. 2010;4(7):454-458.
58. Sun L, Sun Y, Jiang M, Luo L, Yu X, Yao W, *et al*. Isolation, identification and pathogenicity of Proteus vulgaris from moribund common carp (Cyprinus carpio) farmed in China. Aquaculture. 2020;525:735294.
59. Zhang W, Niu Z, Yin K, Liu P, Chen L. Quick identification and quantification of Proteus mirabilis by polymerase chain reaction (PCR) assays. Annals of microbiology. 2013;63(2):683-689.
60. Mohamed EB, Souissi MN, Baccar A, Bouri A. CEO's personal characteristics, ownership and investment cash flow sensitivity: Evidence from NYSE panel data firms. Journal of Economics Finance and Administrative Science. 2014 Dec 1;19(37):98-103.