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Occurrence of *Klebsiella pneumoniae* and *Salmonella* spp. in beef and cow milk from central Kerala[#]

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Abstract

*Klebsiella pneumoniae is a clinically important opportunistic pathogen and Salmonella spp. is a major food-borne pathogen causing gastroenteritis worldwide. The present study was conducted to determine the occurrence of K. pneumoniae and Salmonella spp. in beef and cow milk collected from central Kerala. The study was conducted in a span of 10 months from, January 2022 to October 2022. A total of 400 samples, comprising of 200 raw cow milk samples and 200 beef samples, were collected from different milk societies and retail shops of Thrissur and Palakkad districts. The K. pneumoniae and Salmonella spp. were isolated by conventional culture techniques on MacConkey agar and Xylose lysine deoxycholate agar, respectively. When conventional culture technique was used, 57.50 per cent and 10.25 per cent samples were positive for K. pneumoniae and Salmonella spp., respectively. The molecular confirmation of K. pneumoniae was carried out by PCR targeting genus specific gyr*A *followed by species specific rpo*B *gene. Salmonella spp. was confirmed on PCR by amplifying species specific inv*A *gene and spv*C *gene was targeted to evaluate the virulent nature of the isolates. Out of the 400 milk and beef samples analysed 50.75 per cent were positive for K. pneumoniae and 10.25 per cent were positive for Salmonella spp. Implementation of standard hygiene practices during production, distribution chain are required to avoid the contamination of milk and beef samples indented for human consumption.*

Keywords: *Klebsiella pneumoniae, Salmonella spp., cow milk, beef*

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In India, every year 100 million foodborne illness and 120,000 death related to foodborne illness occur. A review of records of foodborne illness in India from 1980 to 2016 shows *Klebsiella* and *Salmonella* to be two important foodborne pathogens (Kumar, 2021). *Klebsiella pneumonia* is a clinically important opportunistic pathogen that colonise the upper respiratory tract and gastrointestinal tract of human as well as animals. The organism is responsible for causing respiratory tract infection such as pneumonia and a variety of urinary tract infections. It accounts for up to 10 per cent of all nosocomial bacterial infections, and is critical in patients admitted to intensive care units (Struve and Krogfelt, 2004). The organism is ubiquitous in nature and is prevalent in dairy farm environment, milking equipments, sites like bulk tank, milking parlour and hind limbs of animals. The bacteria enter the animal body and colonise various organs and are later secreted in milk and meat (Langoni *et al*., 2015). *Salmonella* spp. is one of the major causes of foodborne illness across the globe. The organism has a wide variety of animal reservoirs. The majority of *Salmonella* infections in human are caused by the consumption of contaminated foods of animal origin such as meat, milk and eggs (Scallan *et al*., 2011). The clinical illness is characterised by acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. Symptoms are usually self-limiting, but can be life-threatening in young children, old and immune-compromised individuals. Beef and cow milk are the two important sources of protein in the human diet. India is the world's largest milk producer and fifth largest beef producer which accounts for 22 per cent and 6.21 per cent of global production, respectively. Both beef and milk are susceptible to the contamination by pathogenic and deteriorative bacteria. Contamination of milk and meat can occur at any point in the production process. The present study aimed to assess the presence of *K. pneumoniae* and *Salmonella* spp. in beef and raw cow milk samples from central Kerala.

Materials and methods

Collection of beef and milk samples

A total of 200 each of beef and cow milk samples were collected from various retail shops and milk societies of Thrissur and Palakkad districts. Milk samples were collected from five different milk societies in Thrissur and four different milk societies in Palakkad district. Beef samples were collected from five retail outlets in Thrissur district and three retail outlets in Palakkad districts. Approximately 250g of beef samples were sliced from the whole carcass and transferred into aseptic polythene bags. The milk samples were collected aseptically after thorough mixing of milk in individual cans, with a plunger and by transferring approximately 250 mL of milk to sterile sample containers.

Isolation and identification of K. pneumoniae and *Salmonella spp. by culture techniques*

All the samples collected were subjected to the isolation and identification of *K. pneumoniae* by conventional culture technique (Salfinger and Tororell, 2015). Milk samples (0.1 mL) were enriched overnight in nutrient broth (9.9 mL). In case of beef samples, 25g of each sample was placed in 225mL of buffered peptone water (BPW) in sterile stomacher bags and processed for 120 sec. in a stomacher (Smasher, AES, France) and incubated at 37°C overnight. The samples in nutrient broth were streaked onto MacConkey agar (MCA) and incubated at 37°C for 24 h. Characteristic large, pink mucoid colonies were selected for further confirmation by biochemical tests.

The isolation of *Salmonella* spp. was carried out by conventional culture techniques (Salfinger and Tororell, 2015). Pre-enrichment was done for both beef and milk samples separately. A 25 mL of milk sample was added to 225 mL buffered peptone water (BPW). Approximately 25 g portion of meat was removed from different regions of the beef sample using sterile scissors and forceps. Each of the collected samples was added to 225 mL of BPW and pre-enriched at 37ºCfor 18h. A 0.1mL of pre-enriched sample of beef and milk were transferred to 9.9 mL each of Rappaport Vassiliadis (RV) broth for enrichment and incubated at 37°C for 24 h. The samples in RV broth were streaked onto Xylose Lysine Deoxycholate (XLD) agar and incubated at 37°C for 24 h. Characteristic red colonies with black

centres were selected for further confirmation by biochemical tests.

Polymerase chain reaction

The DNA was extracted from the isolates by snap chill method (Ram *et al*., 2019). Molecular confirmation of *Klebsiella* spp. was carried out by PCR targeting *gyr*A gene, and *K. pneumoniae* organisms were identified by amplification of *rpo*B gene (Swetha, 2022). The thermal cycling conditions for amplification of genes included an initial denaturation 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min.

The *inv*A gene was targeted by PCR for the molecular confirmation of *Salmonella* spp. (Radhika, 2022) and *spv*C gene was targeted to detect the virulent nature of isolates. The cycling conditions for amplification of genes were standardised with initial denaturation 94°C for 1 min, followed by 35 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min, followed by final extension at 72°C for 10 min. The primers used in this study are listed in Table 1.

Statistical analysis

The results obtained in this study were statistically analysed using SPSS version 24.0 software. Chi-square test was used to analyse the statistical difference in the occurrence of *K. pneumoniae* and *Salmonella* spp. between Thrissur and Palakkad districts.

Results and discussion

Occurrence of K. pneumoniae in milk and beef samples by culture techniques

A total of 200 cow milk samples and 200 beef samples (100 each from both districts) were analysed for the presence of *K. pneumoniae.* Out of the total of 200 milk samples (100 each) analysed from Thrissur and Palakkad districts, *K. pneumoniae* was detected in 69 and 86 samples, respectively. Among the 200 beef samples (100 each), the samples collected from Thrissur were more contaminated with *K. pneumoniae* (45 per cent) than samples from Palakkad (30 per cent). The statistical analysis by *Chi-square* revealed that, there was significant difference (p=0.006) between the occurrence of *K. pneumoniae* in milk from both districts. Similarly, there was significant difference between (p=0.04) the occurrence of *K. pneumonia* in beef samples from Thrissur and Palakkad districts (Table 2).

From a total of 400 beef and raw cow milk samples (200 each) analysed by conventional culture techniques, 230 (57.50 per cent) samples were positive for *K. pneumoniae.* Maximum number of isolates were detected in milk samples (77.50 per cent) compared to beef samples (37.50 per cent). The result of the study is similar to Uddin *et al*. (2010) who recorded a prevalance of 63.63 per cent in raw cow milk from Bangladesh. A study from South Africa conducted by Montso *et al*. (2019) who reported a similar occurrence of 44.01 per cent of *K. pneumonia* in beef samples. On the

Table 1. Primers used for identification of *gyr*A, *rpo*B, *inv*A and *spv*C genes

Genes	Primers	Primer sequences	Amplicons (bp)	Reference
*gyr*A	F	5'CGCGTACTATACGCCATGAACGTA 3'	441	Bobbadi *et al.* (2020)
	R	5'ACCGTTGATCACTTCGGTCAGG 3'		
*rpo*B	F	5'CAACGGTGTGGTTACTGACG 3'	108	
	R	5'TCTACGAAGTGGCCGTTTTC 3'		
*inv*A	F	5'ACAGTGCTCGTTTACGACCTGAAT3'	244	Zadernowska and Wierzchowska (2020)
	R	5'AGACGACTGGTACTGATCGATAAT 3'		
*spv*C	F	5'ACTCCTTGCACAACCAAATGCGGA3'	571	
	R	5'TCTCTTCTGCATTTCGCCACCATCA 3'		

Table 2. Occurrence of *K. pneumonia* isolates by culture techniques

Sl. No.	Districts	Milk Samples analysed	Positive milk Samples		Beef Samples Analysed	Positive beef Samples	
			Number	Per cent		Number	Per cent
1.	Thrissur	100	69	69.00	100	45	45.00
2.	Palakkad	100	86	86.00	100	30	30.00
			Chi- square 8.287** p-value0.006			*Chi*- square 4.8* p-value- 0.04	
Total		**200**	**155**	**77.50**	**200**	**75**	**37.50**

**Significant at 1% level ; *Significant at 5% level

Table 3. Occurrence of *Salmonella spp.* isolates by culture techniques

Sl. No.	Districts	Milk Samples Analysed	Positive Samples		Beef Samples Analysed	Positive Samples	
			Number	Per cent		Number	Per cent
1.	Thrissur	100	5	5.00	100	17	17.00
2.	Palakkad	100	7	7.00	100	12	12.00
			Chi- square 0.355 ns p-value 0.76			*Chi*- square 1.008 ns p-value 0.42	
Total		**200**	**12**	**6.00**	**200**	**29**	**14.50**

ns - non significant at 5% level

contrary, a higher occurrence of *K. pneumoniae* in milk (85.58 per cent) was reported by Badri *et al*. (2017) from Sudan. Likewise, a higher occurrence of *K. pneumoniae* in beef (76 per cent) was reported by Klaif *et al*. (2019) in Iraq. Jindal *et al*. (2021) in Punjab and Abdaslam *et al*. (2014) in Saudi Arabia reported a lower occurrence of *K. pneumonia* in milk (19.6 per cent) and beef (6.60 per cent), respectively. In present study, while collecting the samples, it was observed that, majority of the milkers and animal handlers were unaware of practices such as clean milk production and importance of maintaining hygienic environment in animal house. Similarly, beef handlers were using bare hands during handling the meat and whole beef carcasses were found to be hanging from roofs of the shops without any covering. This may be the reason for high occurrence of *K. pneumonia* organisms in this study.

Occurrence of Salmonella spp. in milk and beef samples by culture techniques

On conventional culture technique, seven per cent of milk samples from Palakkad and five per cent of milk samples from Thrissur district were positive for *Salmonella* spp. In beef, presence of *Salmonella* spp. was noted in 17 per cent of samples from Thrissur and 12 per cent of samples from Palakkad district. The statistical analysis by *Chi-square* test revealed that, there were no significant differences ($p>0.05$) between the occurrence of *Salmonella* spp. in milk as well as beef samples from Thrissur and Palakkad districts (Table 3).

From a total of 400 beef and raw cow milk samples (200 each) analysed by conventional culture techniques, 41 (10. 25 per cent) samples were positive for *Salmonella* spp. The occurrence of *Salmonella* spp. in milk was recorded as six per cent and that from beef was recorded as 14.5 per cent. Kaushik *et al*. (2014) in Bihar reported a similar occurrence of 7.7 per cent of *Salmonella* spp. in milk and Dallal *et al*. (2009) in Iran, reported a similar occurrence of 10.02 of *Salmonella* spp. in beef samples. However a higher occurrence of *Salmonella* spp. in milk (25.88 per cent) and beef (58.3 per cent) was reported by Qamar *et al*. (2020) in South Punjab-Pakistan and Sohail *et al*. (2021) in Karnataka, respectively.

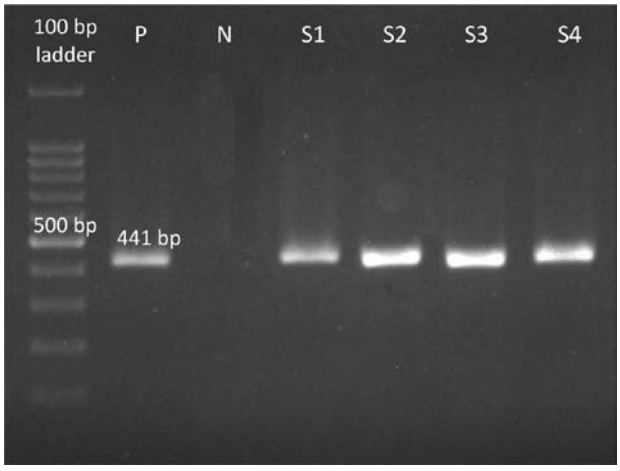
Most of the beef stalls from which the samples were collected were located in common markets, where other meat such as chicken, pork and fish were also sold. There were no separations between the stalls, which might lead to cross-contamination between

species. Unhygienic practices during handling of meat and milk might have contributed to the occurrence of *Salmonella* spp. in the samples analysed in the study.

Molecular confirmation of K. pneumoniae by PCR

For the molecular confirmation of *K. pneumonia*e isolates, the PCR targeting the gene *gyr*A was standardised with an amplicon size of 441 bp (Fig.1) and that targeting *rpo*B gene was standardised with an amplicon size of 108 bp ((Fig.2) . Out of the 230 culture positive isolates of *Klebsiella* spp. 223 isolates amplified *gyr*A gene on PCR (Fig. 1). The gene *rpo*B was detected in a total of 203 culture positive *K. pneumoniae* isolates in this study (Fig. 2).

Most of the milk samples collected from both Thrissur (91.30 per cent) and Palakkad (95.18 per cent) districts were contaminated with *K. pneumoniae*. A similar observation was made by Masse *et al*. (2019) with an occurrence of 93.02 per cent for *rpo*B gene in *K. pneumoniae* from Canada. Two independent studies conducted by Hasan *et al*. (2021) in Iraq and Das *et al*. (2020) in India reported amplification of *rpo*B gene of *K. pneumoniae* in cent per cent isolates. However, in a study performed by Chander *et al*. (2011) in Minneapolis, a lower occurrence of 48 per cent in detection of *rpo*B *gene* was recorded. The gene *rpo*B is a house keeping gene, which is universally present in all *K. pneumoniae* isolates, and this gene can be targeted to differentiate isolates at species level and even at sub species level (He *et al*.,

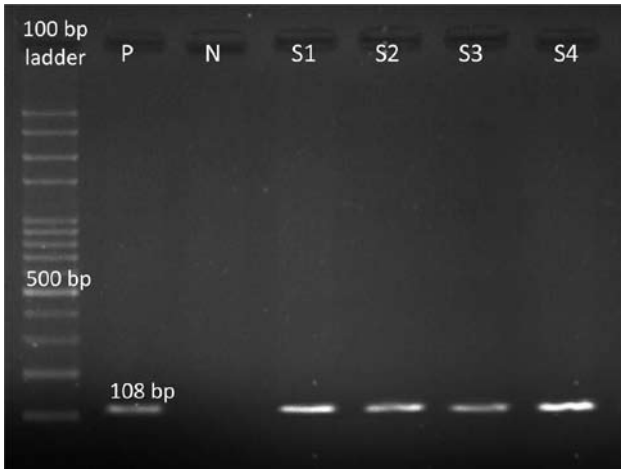


P- Positive Control

N- Negative Control

S1, S2, S3, S4 - Samples

Fig.1. Amplicons of *gyr*A gene



P- Positive Control

N- Negative Control

S1, S2, S3, S4 - Samples

Fig.2. Amplicons of *rpo*B gene

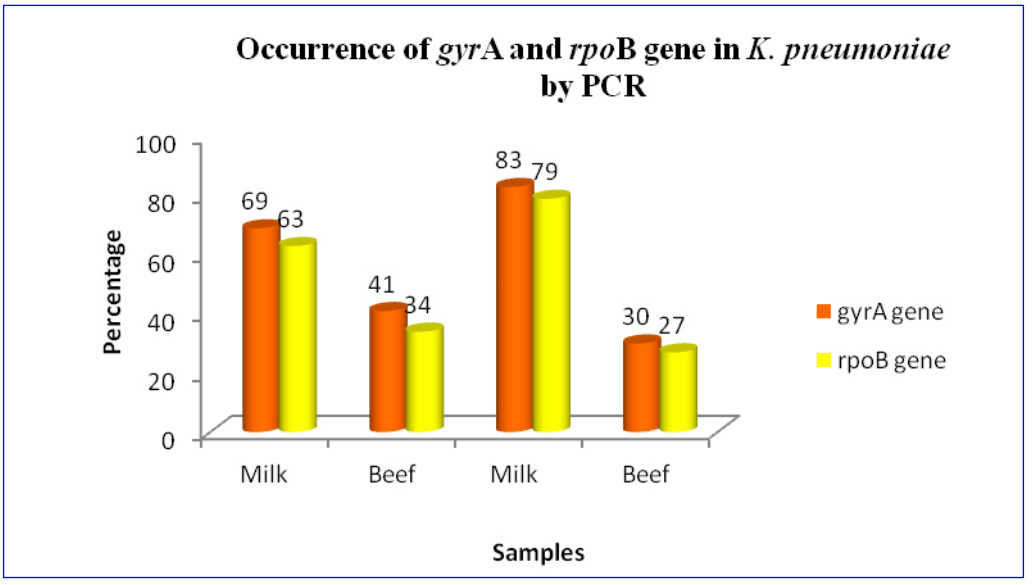
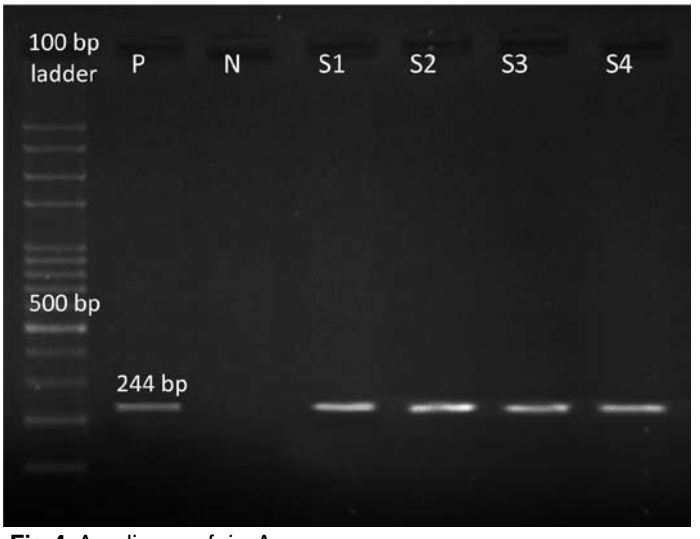


Fig. 3. The occurrence of *gyr*A and *rpo*B gene in *K. pneumoniae* by PCR



P- Positive Control

N- Negative Control

S1, S2, S3, S4 - Samples

Fig.4. Amplicons of *inv*A gene

2016). The occurrence of *gyr*A and *rpo*B gene in *K. pneumonia* by PCR is represented in Fig. 3.

Molecular confirmation of Salmonella spp.

Molecular confirmation of *Salmonella* spp. obtained by culture techniques was done by PCR targeting *inv*A gene with an amplicon size of 244 base pairs (Fig.4) All the 41 culture positive isolates amplified *inv*A gene on PCR (Fig. 4).

Naik *et al*. (2015) from Chhattisgarh and Olufunke *et al*. (2014) in Nigeria had detected the *inv*A gene in all the *Salmonella* spp. isolates obtained in the respective studies. A lower level of detection was reported by Singh *et al*. (2018) from Jabalpur, India, where only 31.25 per cent of isolates had *inv*A gene. The virulence character of *Salmonella* spp. was studied by targeting the *spv*C gene in the confirmed isolates. In this study, none of the isolates amplified *spv*C gene on PCR. This was in accordance with findings of Oueslati *et al*.

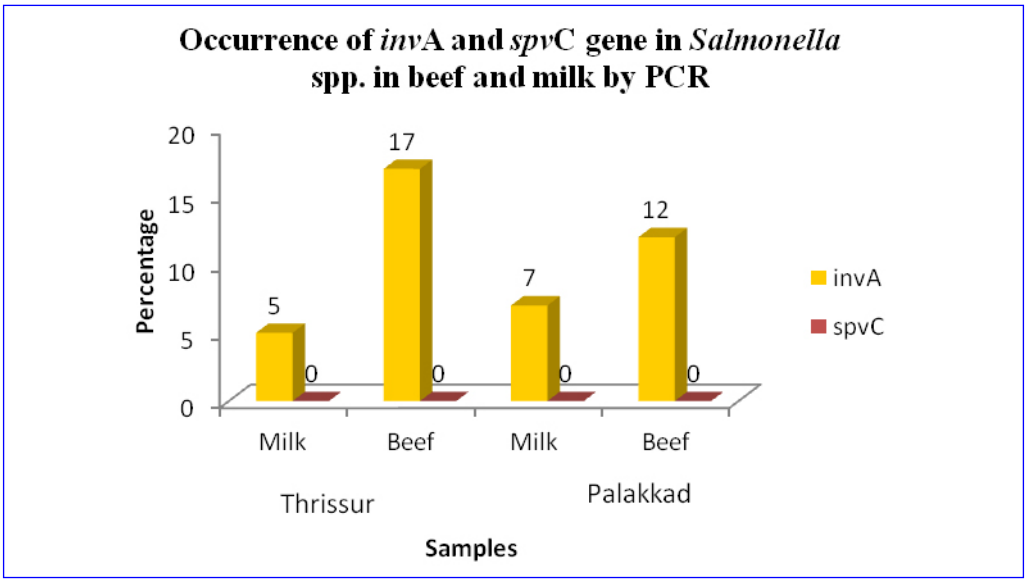


Fig. 5. Occurrence of *inv*A and *spv*C gene in *Salmonella* spp. in beef and milk by PCR

(2016) in Tunisa, where *inv*A gene was amplified in cent per cent of *Salmonella* spp. isolates, but *spv*C gene was not amplified in any samples. The virulence nature in *Salmonella* spp. is attributed to a set of chromosomal genes and plasmid mediated genes. The chromosomal genes are mostly located on regions termed as Salmonella pathogenicity islands (SPIs). The genes located on SPIs promote invasion and rapid multiplication of the host cells (Andesfha *et al*., 2019). The genes located on Salmonella plasmid virulence (spv) region are responsible for suppression of host's immune system. According to Mkangara *et al*. (2019) not all serovars of *Salmonella* harbour *spv*C gene. The occurrence of *inv*A *and spv*C gene in *Salmonella* spp.by PCR is represented in Fig. 5.

Conclusion

The present study revealed a high occurrence of *K. pneumoniae* and *Salmonella* spp. in milk and beef samples. Microbiological contamination of milk and beef can occur at any stage of the production, processing and distribution chain. Both *K. pneumoniae* and *Salmonella* spp. are highly prevalent in dairy farm environment. Hence, good management practices like clean milk production, proper housing of food animals and hygienic handling of food products are very critical in reducing the risk of potent infection in animals and human. Creating awareness among the food handlers regarding hygienic practices focussing on a'farm to fork concept'is the need of the hour to ensure food safety and public health.

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Conflict of interest

The authors declare that they have no conflict of interest.

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