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Studies on Risk of Listeriosis from Carabeef and Slaughter House Environment

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A B S T R A C T

Food of animal origin is frequently associated with Listeriosis caused by *L. monocytogenes*. The present study was carried out to examine the contamination of carabeef samples and the slaughterhouse environment of *Listeria spp*., especially *L. monocytogenes*. A total of 305 carabeef samples (275 from Punjab and 30 from Delhi) were collected. In addition to that, a total of 213 environmental samples (hand swab, knife, chopping boards/table-tops, floor, and hanger) were also examined for *Listeria* contamination. Slaughterhouses for carabeef and environmental samples were different. Samples were processed for isolation of *Listeria* spp. using standard protocol and isolates were subjected to biochemical and molecular testing. Only two samples (0.65%, 2/305) of carabeef yielded *Listeria* spp., which were identified as *L.innocua*. Both the isolates were obtained from carabeef samples of Punjab (0.72%, 2/275). The present study did not yield any *L. monocytogenes* isolates, but the presence of *L.innocua* pointed to the potential presence of pathogenic *L. monocytogenes*. None of the environmental samples were positive for *Listeria* spp. The *L.innocua* isolates were non-hemolytic and CAMP negative, they were non-pathogenic on ALOA chromogenic media. Although contamination of carabeef and environmental samples with *Listeria* spp. was low, it is important to aware occupational workers and consumers about the hygienic practices that need to be followed in relation to the food of animal origin to reduce the burden of foodborne diseases.

Introduction

*Listeriosi*s is an important bacterial zoonosis caused by pathogenic strains of *Listeria monocytogenes* reported worldwide and reported in a variety of animal species and man. World Health Organisation (WHO) has characterised *Listeriosi*s as a bacterial zoonotic disease with a severe threat to consumer's safety with a high mortality rate (Adak *et al.*, 2002). It is responsible for the second-highest case fatality (28%) among all the food-borne illnesses (Mead *et al.*, 1999) and accounts for a 25-50% mortality rate (Pal *et al*, 2017). Two species of *Listeria*are considered as pathogenic namely, *L. monocytogenes* and *L. ivanovii*.*L. monocytogenes* is recognised as an "emerging

food-borne pathogen" as the infection is generally transmitted through food (Bille and Rocourt, 1996). Listeriosis leads to severe invasive illness in humans, characterized by septicemia, encephalitis, meningitis, meningoencephalitis, stillbirth, abortion, perinatal infections, and gastroenteritis especially in pregnant women, newborn, elderly, and immunocompromised individuals (Posfay-Barbe and Wald, 2004).

Ingestion of contaminated foods of animal origin such as meat (chicken, chevon, mutton, pork, beef, carabeef) dairy products, processed meat products either raw or partially cooked, RTE, and vegetables are considered as a primary source of transmission of foodborne illness in sporadic cases as well as outbreaks (Farber and Peterkin, 1991). Occurrence of *Listeria* within meat processing facilities and slaughter houses has also been associated with environmental colonisation because of its ability to adapt and survive on equipment, floors, and rooms (Lunden *et al.,* 2000). There are two main routes by which this diseases can spread through the meat to humans. Firstly, by direct contact with the contaminated carcass or intestine rupture during evisceration (Skovgaard and Norrung, 1989). These sources are mainly confined to workers in slaughterhouses and meat shops (Aitken, 1996). Secondly, by ingestion of contaminated, under cooked, and RTE foods, which causes illness in the consumer and affectsa huge number of the population (Johnston, 1990 and Sockett, 1995). Slaughtering place, the environment of the slaughterhouse, air, floor, and vehicle for meat transportation to retail outlets also adds to the contaminant (Bhandare *et al.,* 2009).

As per the Food Safety and Standards Authority of India (2012), *L. monocytogenes* should be absent in 25g of frozen or canned meat and meat products.

India stands 5th in the world's meat production and accounts for 3% of the total meat production. Carabeef in India contributes about 31% of total meat production. Punjab contributes 3.57% of the total meat production in India, the contribution of carabeef being 8.58% of total meat production in Punjab, third after Uttar Pradesh, and Maharashtra (APEDA 2013). However, the prevalence of *Listeria* spp. in carabeef has not been studied to a large extent. Due to its immense public health significance, there is a need to assess the presence of *Listeria* spp. in the carabeef and slaughterhouse environment. As has been *Listeria*recognized as a food-borne pathogen, there was developing interest in understanding the risk related to this organism in the foods of animal origin. Therefore, keeping in view the above facts and its zoonotic potential the present study was aimed to determine the prevalence and phenotypic characterization of *Listeria* spp. from carabeef and slaughterhouse environment and its prevalence and phenotypic characterization of *Listeria* spp. from carabeef and slaughterhouse environment and virulence profiling and genotypic characterization of *Listeria* spp., especially *L. monocytogenes* isolates.

Materials and Methods

Calculation of sample size

A total of 151 swab samples (place of collection as per availability) and 151 swab samples from slaughterhouse environment of Punjab were estimated using an expected prevalence of 6.7% (Nayak *et al.,* 2010)with 95% confidence interval and 4% precision. Environmental samples were collected from registered slaughterhouses in different districts *viz*. Hoshiarpur, Bhatinda, Ludhiana, Patiala, Jalandhar, and SAS Nagar from Punjab, India. For the slaughterhouse

environment, swab samples were collected from butcher's hands, slaughterhouse floor, knives, chopping blocks/boards, cutting tables, and hangers, etc. There were two different investigations. In one study microbiological quality of carabeef samples (from Delhi slaughterhouse and Punjab slaughterhouse) was carried out in reference to *Listeria* spp. whereas in the second part of the study contamination of the slaughterhouse environment (Punjab) was studied. The slaughterhouses from where environmental samples were taken were different from the slaughterhouses from where carabeef samples were picked.

Finally a total of 305carabeef from Delhi and Punjab (as per the availability) were collected (Table 1). The carabeef samples were collected aseptically in sterilized sampling bags with proper labeling. In addition, a total of 213 swab samples including 76 hand swabs, 62 knife swabs, and 06 chopping board swabs, 40 floor swabs and 29 Hanger swabs were also collected (Table 2). For swab sample collection sterile swab (Hi-Media) was used. Before the collection of the sample, the sterile swab was dipped in sterile 2 ml of 0.9% normal saline.

Isolation and identification

Isolation of *Listeria spp.* from the carabeef and swab samples was done using protocol as described by Alka *et al.,* (2019). Carabeef sample (25 gm) was triturated in sterile mortar using 1 gm of sterile sand with 5-10ml of sterile University of Vermont Medium (UVM)-I broth (Hi-media Labs, Mumbai, India).The sample was then triturated with a sterile pestle.

The mixture was then transferred to the flask with the remaining volume of 225 ml of UVM-I and incubated at 30^{o}C for 24 hrs. After that, 0.1 ml of the UVM-I from incubated flask was transferred to 9 ml of UVM-II (Hi-media Labs, Mumbai, India) and incubated at 30^{o}C for 24 hrs.

Enriched sample from UVM-II was streaked on polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar plate (Hi-media Labs, Mumbai, India) and incubated at 37^{o}C for 24-48 hrs. The plates with diffuse black zones of aesculin hydrolysis with typical green-yellow, glistening, iridescent, pointed colonies were considered as presumptive *Listeria* spp. The colonies were subjected to gram staining, biochemical tests, molecular detection and *in-vitro* pathogenicity tests as described below (Table 4 and 6).

Confirmation of isolate

Colonies of *Listeria* from PALCAM agar showing Gram-positive coccobacilli morphology were subculture in brain heart infusion (BHI) broth and incubated at 37^{o}C for 12 hrs. The isolates which were catalase-positive, methyl red, and Voges-Proskauer-positive, and with nitrate-negative reactions as well characteristic tumbling motility at 20-25°C were considered as "presumptive" *Listeria* isolates.

The isolates were further put through biochemical characterisation by sugar fermentation tests (xylose, lactose, rhamnose, dextrose, mannitol, and alpha-methyl-D-Mannoside; Hi media, Lab, Mumbai). The isolates were also subjected to *in-vitro* pathogenicity tests *viz.*, haemolysis test on 7% sheep blood agar (SBA), Phosphatidylinositol-specific phospholipase C (PI-PLC) activity using Agar *Listeria* according to Ottaviani and Agosti (ALOA; Hi media, Lab, Mumbai) and Christie, Atkins, Munch-Peterson (CAMP) test (Seeliger and Jones, 1986).

Molecular detection of Genus *Listeria*

The genomic DNA was prepared by the snap chill method (Shakuntala *et al.,* 2006) and used as template in molecular detection.

After performing conventional biochemical tests for the detection of *Listeria* spp. and sugar fermentation, additional confirmation was done by molecular technique especially, PCR. The PCR reaction for the detection of *Listeria* genus targeting genus-specific gene, putative phosphoribosyl pyrophosphate synthetase (*prs*) was done. The amplification reaction yielded isolates yielded a 370 bp product which was indicative of *Listeria* genus (Fig. 1). All the primers were procured from Eurofins Genomics India Pvt. Ltd (India).

Detection of virulence genes

Detection of virulence gene of *L.monocytogenes* was standardised on standard strain of *L.monocytogenes* (ATCC 19115). The protocol as used by Alka *et al.*, (2019) was used. The virulence genes *viz.*, haemolysin (*hly*A), PI-PLC (*plc*A), actin (*act*A), p60 (*iap*), and regulatory (*prf*A) were targeted. Primers used are mentioned in Table 3. As none of isolate was *L.monocytogenes*, detection of virulence genes on our isolate was not attempted.

Genoserotyping

For genoserotyping of *L. monocytogenes*, *lmo*0737, *lmo*1118, ORF2819, ORF2110 and *prs* genes are generally targeted. As none of the isolates in the present study were *L. monocytogenes*, therefore genoserotyping was not attempted.

Results and Discussion

In this study contamination of carabeef samples from slaughterhouses of Punjab and Delhi area was examined for *Listeria* spp., especially *L. monocytogenes*. A total of 305 carabeef samples (275 from Punjab and 30 from Delhi) were subjected to isolation of *Listeria* spp. using a double enrichment process. The enriched samples were plated on selective agar (PALCAM). The colonies from PALCAM agar showing visible aesculin hydrolysis were processed for further confirmation. The colonies that showed Gram-Positive coco-bacillary rods, morphologically on Gram staining were further subjected to biochemical tests.

Based on the selective isolation and Gram Staining two isolates showed characteristics of *Listeria* spp.

Biochemical and molecular detection of *Listeria* isolates

Two- presumptive *Listeria* spp. isolates based on selective plating and Gram staining were further subjected to biochemical tests for species identification. Based on catalase, nitrate- reduction, and Methyl red (MR), Voges-Proskauer's (VP), and motility test. The isolates which were catalase positive, oxidase negative, nitrate reduction negative, MR-VP positive, and motile were considered *Listeria* spp.

The two *Listeria* spp. isolates that showed typical *Listeria* spp. reactions on cultural and biochemical characterisation were also confirmed through molecular detection method using Polymerase chain reaction (PCR) targeting genus-specific gene, putative phosphoribosyl pyrophosphate synthetase (*prs*). The two isolates yielded a 370 bp product which was indicative of *Listeria* spp. (Fig. 1).

For speciation of these *Listeria* isolates, they were subjected to sugar fermentation tests. The isolates fermented sugars Xylose, alpha-

Methyl-D-Mannoside, Rhamnose, Glucose (Dextrose), and Mannitol and therefore were designated as *L. innocua* (Table 4).

Based on the cultural, biochemical characterisation two samples were found positive for *Listeria* spp. i.e. *L.innocua* giving an overall prevalence of 0.65% (2/305).

Out of 275 carabeef samples collected from slaughterhouses in Punjab, only two samples were positive for *Listeria* spp. i.e. *L.innocua* with a prevalence of 0.72 % (2/275) from Punjab only. None of the carabeef samples from Delhi slaughterhouse were positive for *Listeria* spp. (Table 5).

Contamination of food with zoonotic pathogens is a serious public health concern worldwide. Among different foodborne infections, food associated listeriosis is a problem in food quality and safety. Listeriosis caused by *L.monocytogenes* is considered among the leading cause of food associated infections. Foodborne Listeriosis is further a problematic area because of the ability of the organism to survive at refrigeration temperature in food products and in the food processing environment (Rocourt and Cossart, 1997 and Borucki *et al*., 2003).

In the present study contamination of carabeef samples with *Listeria* spp., especially *L.monocytogenes* was explored.Varying prevalence of *Listeria* spp. and that of *L.monocytogenes* from carabeef has been reported from India and abroad. Kumar *et al*.,(2014) found none of the carabeef samples (zero % prevalence)from Municipal slaughterhouse and retail meat shops of Hyderabad, Karnataka India, positive for *Listeria* spp. which was lower than the contamination reported in the current study. In another study from India, in the state of Gujarat, Nayak *et al.,* (2012) found a slightly higher 2.7 % prevalence of *Listeria* spp. from carabeef samples as compared to the current study. Barbuddhe *et al.,* (2002) found a 5.4 % prevalence of *Listeria* spp. from carabeef samples collected from slaughtered buffaloes in Uttar Pradesh. In another study from India in the state of Gujarat, Nayak *et al.,* (2010) found a 6.7% level of *Listeria* spp. from carabeef sold in the retail meat market, as compared to the current study.

Further, higher contamination of carabeef samples with *Listeria* spp. to the extent of12 % has been reported from carabeef collected from buffalo slaughtered at the Municipal Corporation Slaughterhouse, Bareilly (U.P) India (Chaudhari *et al.,* 2004).

In some of the studies done abroad also, observed varying levels of *Listeria* contamination in carabeef samples. Zarei *et al.,* (2013) found 2.8% of carabeef samples from Iran positive for *Listeria* spp. In another study from the same country, Rahimi *et al.,* (2012) found 29.2% of carabeef samples positive for *Listeria* spp.

As mentioned before none of the carabeef samples yielded *L. monocytogenes*, however, it has been isolated from carabeef samples in other investigations. Brahmbhatt and Anjaria (1993) reported 6% of buffalo meat samples positive for *L. monocytogenes*. Chaudhari, (1997) found 3.08 % out of 130 buffalo meat samples contaminated with *L. monocytogenes*. In a similar study done by Chaudhari *et al.,* (2004) found even higher contamination of *L. monocytogenes*, (12 %,15/125) in buffalo meat samples collected from buffalo slaughtered at the Municipal Corporation Slaughterhouse, Bareilly, India. In another study done in Gujarat, India, by Nayak *et al.,* (2010) found *L. innocua* with a prevalence of 1.3% from buffalo meat samples, comparatively higher than the prevalence reported in the current study.

Variations in contamination of carabeef samples with *Listeria spp*. and *L. monocytogenes* may be attributed to different factors. The variations may be accounted as a result of the amount of sample processed, method of isolation, enrichment media used, type of samples collected, and prevailing environmental conditions under which animals were slaughtered (Nightingale *et al.*, 2006). *Listeria* spp. are occasionally carried in the gastrointestinal tract of clinically healthy ruminants and are shed asymptomatically in their faeces contaminating their surroundings (Esteban *et al.,* 2009). The animals shed *Listeria* spp. in their faeces can also get soiled with contaminated faeces, when such animals are slaughtered without proper hygiene they can contaminate meat, environment, another meat samples through cross-contamination (Autio *et al.,* 2003, Rocourt and Seeliger, 1985)

The low contamination of carabeef samples in the present study can be attributed to its actual lower contamination of slaughtered animals or their meat samples. It may also be attributed to the fact that *Listeria spp*. are slow-growing organisms as compared to some well-known enteric commensal organisms, which may be competing with *Listeria spp.*, present in low numbers. The organisms may also be present in an injured physiological state in the meat samples becoming difficult to revive and isolate

Prevalence of *Listeria* spp. in slaughterhouse environment of different districts of Punjab

In another part of the study contamination of the slaughterhouse environment with *Listeria* spp. was explored by taking swab samples from butcher's hand, knife, chopping-board (table-top), floor, and hangers. The swab samples were processed for *listeria* spp.as per standard protocol. None of the swab samples from the slaughterhouse environment of Punjab were found contaminated with *Listeria* spp. The swab samples collected in the study were not from the same slaughterhouses from where these meat samples were collected.

Hygiene and quality of the slaughterhouse environment or meat processing environment also play a major role in the contamination of meat samples. The animals slaughtered may be healthy and clean but if slaughtering or the processing environment is not clean then the meat from healthy animals may get contaminated with foodborne pathogens through cross-contamination. Quality of the equipment (knife, chopping-board/tabletops) used during slaughtering are other important contributors to the cross-contamination of the meat samples.

In our study none of the samples from the slaughterhouse environment were positive for *Listeria spp.,* pointing to the fact that these important components in the meat slaughtering environment were not contaminated with at least *Listeria spp*. This may also be attributed to the presence of a very low number or injured *Listeria* spp. in the surrounding which couldn't be revived at the pre-enrichment step and the inhibition/competition provided by other commensal microflora could have also led to the no isolation of *Listeria* spp. from environmental samples.

On the contrary, in some studies, it has been observed that *Listeria* spp. survive in the slaughterhouse environment/food processing environment for a longer period and become persistent source of contamination of food through cross-contamination (Lin *et al* 2006)

Nayak *et al.,* (2012) found 2% (1/50) prevalence of *L. monocytogenes* in the meat processing environment. In a recently conducted study in the department, Alka *et*

al., (2019) found butcher's hand, knife, and chopping-board / cutting table, used in the retail meat shops contaminated with *L. monocytogenes*. Gowda *et al.,* (2017) found a knife and cutting table surfaces positive for *Listeria* spp. in a cattle slaughterhouse of Kerala, India. In their study, they also isolated *L.monocytogenes* from the water reservoir used to wash carcasses, is underscoring the importance of safe and clean water for washing carcasses. In another study from Kerala, Vasu *et al.,* (2014) examined the meat processing facility and retail market for *Listeria* spp. Even though they didn't find *L.monocytogenes* in them eat processing facility and retail market environment but they did find the presence of *L.innocua* in the processing facility. They suggested to the potential presence of *L.monocytogenes* as *L.innocua* and *L.monocytogenes* share the same ecological niche.

Contamination of meat processing environment, equipment, and food contact surfaces has also been observed in studies done abroad. Saludes *et al.,* (2015) found the presence of *L. monocytogenes* on the food handler's hand and food processing plant. Mulu and Pal, (2016) observed the presence of *L.monocytogenes* to be 6.7%, 7.5%, and 8.9% from equipment, knives, and cutting tables respectively, in the raw meat market and abattoir of Addis Ababa, Ethiopia.

Haemolysis on sheep blood agar (SBA)

Haemolysis produced by haemolysin of *Listeria* spp. has been considered as an important characteristic of its pathogenicity, whereas its non-haemolytic character is considered as non-pathogenic (Courtieu, 1991).

In this study, the two *Listeria* isolates i.e. *L. innocua* isolated from carabeef were non-haemolytic on 5-7% sheep blood agar (Table 6).

Haemolytic *Listeria* spp. especially *L.monocytogenes* have been reported in several studies Kaur *et al.,* (2017) found *L.monocytogenes* from the food of animal origin to be haemolytic on sheep blood agar. Similarly, haemolytic *L.monocytogenes* has also been reported in studies from different parts of India (Barbuddhe *et al.,* 2000, Vaidya *et al.,* 2018, Chaudhari *et al.,* 2004, Jallewar *et al.,* 2007). The existence of non-haemolytic strains of *L.monocytogenes* has also been documented in various studies (Mathakiya *et al.,* 2011, Rodriguez *et al.,* 1986).

Christie, Atkins, Munch-Petersen (CAMP) test

The CAMP test is done to differentiate *Listeria* spp. such as *L. ivanovii* and *L. monocytogenes*. *L. monocytogenes* during the CAMP test gives an enhanced zone of haemolysis towards *S. aureus* on sheep blood agar, whereas, *L.ivanovii* gives towards *R. equi.* None of the isolates in our study were *L. monocytogenes* or *L.ivanovii* therefore they did not give any enhanced zone of haemolysis either towards *S. aureus* or *R. equi* (Table 6).

Table.1 Carabeef samples collected from different regions

Source	Number of Samples
Delhi	030
Punjab	275
Total	305

Table.2 Swabs samples collected from different districts of Punjab

Districts	No. of swab samples					
	Hand	Knife	Chopping Board	Floor	Hanger	Total swabs
Moga	12	10	03	00	00	**025**
Patiala	12	08	03	06	00	**029**
Amritsar	10	10	00	05	05	**030**
Bhatinda	12	10	00	08	02	**032**
Hoshiarpur	12	08	00	08	07	**035**
SAS Nagar	12	11	00	08	09	**040**
Ludhiana	06	05	00	05	06	**022**
Total	**076**	**062**	**006**	**040**	**029**	**213**

Table.3 Primers used for detection of virulence marker genes of *L. monocytogenes*

Primer names	Primer sequence		Product Size	Reference
***hly*A**	Forward	5'- GCA GTT GCA AGC GCT TGG AGT GAA - 3'	456bp	Pazaik-Domanska *et al.*, (1999)
	Reverse	5'- GCA ACG TAT CCT CCA GAG TGA TCG - 3'		
***plc*A**	Forward	5'-CTG CTT GAG CGT TCA TGT CTC ATC CCC C-3'	1484 bp	Notermans *et al.*, (1991)
	Reverse	5'- CAT GGG TTT CAC TCT CCT TCT AC - 3'		
***act*A**	Forward	5'- CGC CGC GGA AAT TAA AAA AAG A - 3'	836 bp	Suarez and Vazquez-Boland (2001)
	Reverse	5'- ACG AAG GAA CCG GGC TGC TAG - 3'		
iap	Forward	5'- ACA AGC TGC ACC TGT TGC AG - 3'	131 bp	Furrer *et al.*, (1991)
	Reverse	5'- TGA CAG CGT GTG TAG TAG CA -3'		
***prf*A**	Forward	5'- CTG TTG GAG CTC TTC TTG GTG AAGCAA TCG -3'	1060 bp	Notermans *et al.*, (1991)
	Reverse	5'- AGC AAC CTC GGT ACC ATA TAC TAA CTC - 3'		

Table.4 Biochemical characteristics of *Listeria* spp. isolates from Carabeef samples

Isolate	Place	Biochemical Test						Sugar Fermentation						Species
		M	C	O	MR	VP	NR	X	L	MM	R	G	M	
RCB-212	Punjab	+	+	-	+	+	-	+	-	+	+	+	-	*L.innocua*
RCB-216	Punjab	+	+	-	+	+	-	-	-	+	+	+	-	*L.innocua*

M; Motility, C; Catalase test, O; Oxidase test, MR; Methyl red test, VP; Voges-Proskauer's test, NR; Nitrate Reduction test, X; Xylose, L; Lactose, MM; alpha-Methyl-D-Mannoside, R; Rhamnose, G; Glucose (Dextrose), M; Mannitol

Table.5 Prevalence of *Listeria* spp. and *L. monocytogenes* in carabeef samples

Source of samples (Place)	No. of samples	Positive *Listeria*Spp. (%)	Positive *L. monocytogenes* Isolates (%)	Positive *L.innocua* Isolates (%)
Delhi	030	0	0	0
Punjab	275	2(0.72)	0	2(0.72)
Total	**305**	**2 (0.65%)**	**0**	**2 (0.65%)**

Table.6 Characterization of *Listeria* isolates by haemolysis, CAMP test and PI-PLC assay

Isolate	Place of isolation	Tests			
		Haemolysis on SBA	CAMP test		PI-PLC Assay
			S. aureus	*R. equi*	
RCB-212	Punjab	-	-	-	-
RCB-216	Punjab	-	-	-	-

Fig.1

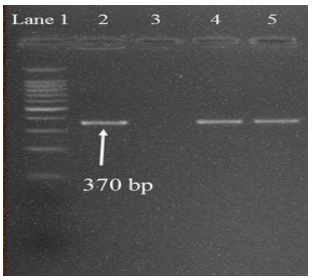
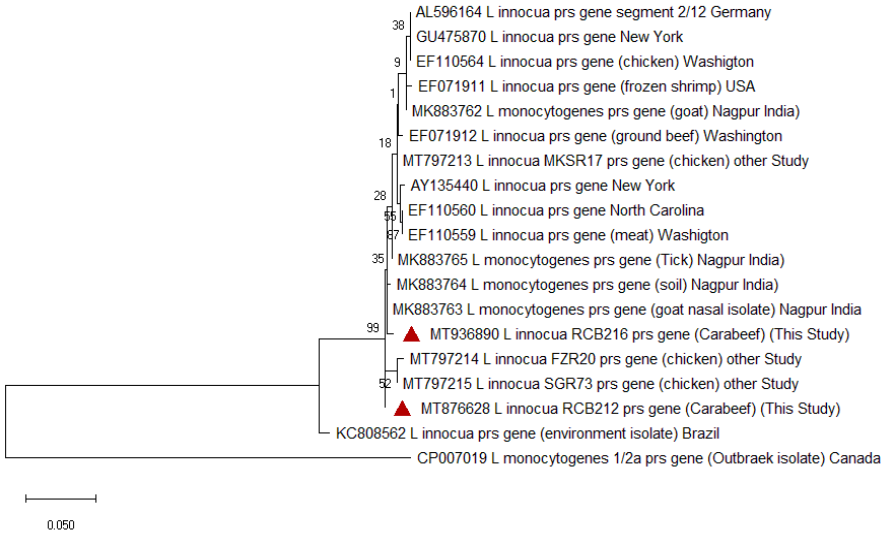


Fig. 1: Genus specific PCR for detection of *Listeria* genus; gene, *prs* (putative phosphoribosyl pyrophosphate synthetase); yielding a product of 370 bp;
Lane 1; Ladder (Ldr) 100bp
Lane 2; Positive Control (S) Standard (*Listeria monocytogenes*, ATCC 19115)
Lane 3; No template control (NTC)
Lane 4; Isolate (RCB-212),
Lane 5; Isolate (RCB-216)

Fig.2



Alka *et al.,* (2019) found all their 18 *L.monocytogenes* isolates from the food of animal origin and food processing environment positive in the CAMP test.

Similarly, Darshan, (2019) found their six *L.monocytogenes* isolates from pork and food processing environments to be positive for the CAMP test. There are other studies that have documented CAMP positivity of *L.monocytogenes* isolates from different sources (Dudhe *et al.,* 2012, Sran *et al.,* 2015)

Phosphatidylinositol-specific phospholipase C (PI-PLC) assay

The Phosphatidylinositol-specific phospholipase C (PI-PLC) assay is used to examine the pathogenicity of *Listeria* spp. especially *L. monocytogenes* using Agar *Listeria* according to Ottaviani and Agosti (ALOA) this is an *in-vitro* pathogenicity test that uses chromogenic selective media for differentiating pathogenic *Listeria* spp. Colonies showing typical blue-green colour with opaque halo are considered as pathogenic while colonies without halo as non-pathogenic. The isolates in our study produced blue-green colour without halo and were considered as non-pathogenic *Listeria* spp. (Table 6).

Alka *et al.,* (2019) found their *L.monocytogenes* isolates from mutton, chevon, and swabs from butchers' shops and slaughterhouses in Punjab, with typical blue-green colonies and halo on ALOA media and were designated as pathogenic. Similarly, Darshan, (2019) found their *L.monocytogenes* isolates from pork samples from Punjab, to be pathogenic on ALOA media.

Virulence gene detection of *Listeria* spp.

The PCR was standardised in the department for the detection of virulence genes associated with *L.monocytogenes viz*. *hly*A, *prf*A, *plc*A, actA, and *iap* using a standard strain of *L. monocytogenes* (ATCC 19115). The amplification of these genes yielded products of size 456bp, 1060bp, 1484 bp, 839 bp, and 131bp, respectively in the standard strain.

As none of the isolates in our study were *L.monocytogenes*, therefore detection of virulence genes in the study was not attempted. However, several studies have found pathogenic *L.monocytogenes* from the food of animal origin based on virulence genes.

Alka *et al.,* (2019) in their study found *L.monocytogenes* from chevon, knife, choppingboard, and butcher's hand swabs to be positive for all the five virulence genes *viz*., *hly*A, *prf*A, *plc*A, *act*A, and *iap*. Similarly, Darshan, (2019) in their study found *L.monocytogenes* from pork to be carrying all the five virulence genes examined in the present study. However,some *L.monocytogenes* isolates carried not all the five virulence genes but were positive for *hly*A, *iap*, and *act*A only.

Genoserotyping

The method for detecting different serotypes of *L.monocytogenes* was standardised using *L.monocytogenes* standard strain (ATCC 19115) serotype 4b. As none of the isolates in the study were *L.monocytogenes*, therefore genoserotyping of the *L.innocua* isolates was not attempted. *L.monocytogenes* with different serotypes exist in the environment. However serotype, 4b,1/2a, 1/2b, 1/2c are associated with food of animal origin (Doumith *et al.*,2004). Alka *et al.,* (2019) found *L.monocytogenes* serotype 4b predominant in their study. The presence of other serovars 1/2a, 1/2b, and 1/2c were also recorded by them.

Sequencing of the *prs* gene and phylogenetic analysis

The amplicons of the *prs* gene (370bp) were purified using a PCR product purification kit (Qiagen) and sequenced in both the directions from Eurofins Genomics India Pvt Ltd. The obtained nucleotide sequences were blasted with the available sequences in the NCBI database using online blast software. Matched sequences were aligned in Clustal2.1. The sequences were submitted to the NCBI database and Accession numbers MT936890 and MT876628 were obtained for RCB-216 and RCB-212 isolates, respectively. The percent identity for our isolates was compared with the already submitted *prs* gene sequences of *L. innocua* isolates in the NCBI database. Both the *Listeria innocua* isolates, RCB-216 (Accession No. MT936890), and RCB-212 (Accession No. MT876628) of the current study showed 99.72 % similarity among themselves. *L. innocua* isolates RCB-216 (Accession No. MT936890) and RCB-212 (Accession No. MT876628) were 98.66% and 100%, respectively similar to *L. innocua* (Accession No. MT797215) isolated from chicken samples in another study (unpublished) already conducted in the department. Sequence analysis of *L. innocua* isolated from beef in Washington (Accession No. EF071912) revealed a similarity of 98.67% and 99.45 % with *L. innocua* isolates RCB-212 and RCB-216 respectively obtained from carabeef.

Phylogenetic analysis

The evolutionary history was inferred using the Maximum Likelihood phylogenetic tree method using Mega-X software based on the Poisson's model. The bootstrap consensus tree inferred from 500 replicates represented the evolutionary history of the taxa analyzed. The phylogenetic tree based on the nucleotide sequences of *L. innocuaprs* gene fragment indicated that both the positive samples belonged to *L. innocua*. Both the *L.innocua* isolates from carabeef clustered with other *L. innocua* isolates recovered from the chicken samples in the department (Accession No. MT797214 and MT797215) as shown in Fig. 2. The findings of the current study indicated that the same clonal lineages of *Listeria* species are circulating in this region. The study also points out the possible transmission of *Listeria innocua* in the varied food animals of Punjab.

Based on the results of the present study, we can conclude that carabeef samples from Punjab were negative for *L.monocytogenes*, however presence of *L.innocua* pointed to the potential presence of *L.monocytogenes* which may be present in low number as both share the same ecological niche. The slaughterhouse environment also did not support persistence of *Listeria* spp. as none of the samples were found positive. Further, the present study recommend regular awareness of slaughterhouse workers and consumers on foodborne pathogens and their public health importance to reduce the burden of occupational and food borne diseases.

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