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Prevalence and Molecular Characterisation of *Listeria* spp. in Retail and Mastitic Milk of Punjab, India

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A B S T R A C T

Listeriosis caused by *Listeria monocytogenes* is an important foodborne infectious disease and is associated with severe diseases in humans and animals, prevalent worldwide. In the present study, a total of 1018 retail milk samples and 250 mastitic milk samples were collected from different districts of Punjab for isolation and molecular characterization of *Listeria* spp. The isolates were phenotypically and genotypically characterised by biochemical tests, in-vitro pathogenicity assay followed by detection of genus specific gene and different virulence-associated genes *viz. hlyA, actA, iapA, plcA* and *prfA* using PCR along with multiplex PCR for geno-serotyping of *L. monocytogenes*. A total of seven samples were found positive for *Listeria* spp by biochemical and molecular tests thereby resulting in an overall *Listeria* spp. prevalence of (0.68%) in retail milk samples. These seven *Listeria* isolates belonged to *L. seeligeri* (2 isolates) and *L. grayi* (5 isolates). Further, analysis of the results based on the zones revealed prevalence of 1.30% and 0.82% from the central plain zone and sub-mountain zone of Punjab, respectively. Taking into consideration the districts, then Ludhiana, Patiala, Tarantaran and Pathankot yielded 3%, 1.66%, 3.33%, and 3.33% prevalence of *Listeria* spp respectively. From the total 7 isolates varying degree of hemolysis was exhibited by the 2 isolates on SBA. The two isolates of *L. seeligeri* were haemolytic in nature. The remaining five isolates of *L. grayi* were non-haemolytic. All the seven isolates were not pathogenic based on *in-vitro* pathogenicity assay. None of the mastitic milk sample was positive for *Listeria* spp. Retail milk samples in our study meet the food safety guidelines of zero tolerance of *L. monocytogenes*, but the presence of other non-pathogenic *Listeria* spp require further scaling of hygiene measures during production, processing and retailing.

Introduction

Listeriosis caused by *Listeria monocytogenes* is an important foodborne infectious disease of humans and animals, prevalent worldwide. In the past few years listeriosis has turn out to be one of the most dangerous foodborne diseases with a high mortality rate of 20-30% (Dmowska and Osek, 2010). It is the third main cause of death due to food-borne bacterial pathogens, with the fatality rates exceeding that of *Salmonella* and *Clostridium botulinum* (Ramaswamy *et al.*, 2007). There

are thirteen serotypes of *L. monocytogenes*. It is characterized by invasive and non-invasive illness and has a tendency to cause severe complications especially in pregnant women, neonates, elderly, and the immunosuppressed individuals, specifically in pregnant women it leads to abortion, septicaemia or infections of the central nervous system (Rebagliati *et al.*, 2009).

Farm animals and their environment act as an important source of food contamination and infections for humans (Jemmi and Stephen, 2006). Milk and other dairy products such as cheese and ice cream which are produced from unpasteurised milk (Brooks *et al.*, 2012) are often contaminated with this pathogen and have been reported as source of listeriosis in numerous widely publicized incidents. There are several documented studies which have found milk from infected animal i.e. raw milk (Rahimi *et al.*, 2010) or mastitic milk or milk available in the retail market contaminated with *L. monocytogenes* (Fretz *et al.*, 2010). Besides milk, meat and meat products have also been found to be contaminated with *L. monocytogenes* (Schwartz *et al.*, 1988).

However not phenomenal, there has been an upswing in the number of human Listeriosis cases in India, with the reports on sporadic cases and incidence in clinical samples which has been quoted as an emerging foodborne disease in by Chug, 2008. Similarly, Aurora *et al.*, (2006) also reported the incidence of *L. monocytogenes* in milk based foods from Agra region. Moreover a study conducted by Sawant *et al.*, (2016) in bovine raw milk samples from Punjab resulted in the isolation and recovery of four *L. monocytogenes* out of total 350 samples studied.

India ranks first among the world's milk producing nations since 1998 and has the largest bovine population in the world. Milk production in India during the period 1950-51 to 2017-18, has increased from 17 million tonnes to 176.4 million tonnes. During the year 2016–17, the estimates of milk production revealed the milk production in Punjab as 11.28 million tonnes. (Source: Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture Government of India).

Several molecular genotyping techniques such as DNA restriction endonuclease analysis, ribotyping, multilocus enzyme electrophoresis and PFGE have been established for molecular epidemiological studies (Borucki *et al* 2003). The expansion of PCR-based serotyping procedures has provided further benefits for the identification and grouping of *L. monocytogenes* (Doumith *et al.*, 2004). The present study, therefore is undertaken with a prime objective to assess the prevalence of *Listeria* species, with particular reference to *L. monocytogenes* in retail and mastitic milk in Punjab state of Northern India and molecular characterization of pathogenic *L. monocytogenes* isolates using virulence associated genes based PCR.

Materials and Methods

Bacterial strains

The standard strains of *L. monocytogenes* (ATCC 19115), *Staphylococcus aureus (*ATCC 11632) and *Rhodococcus equi* (ATCC 6939), used in this study were procured from Hi Media Labs Mumbai.

The standard strain of *Listeria monocytogenes* with serotype 1/2c was procured from Division of Veterinary Public Health, ICAR Research Complex, Goa. All the strains were stored in brain heart infusion (BHI) broth with 20% v/v glycerol at -20 $^{\circ}$C. The cultures were periodically sub cultured in BHI broth and agar.

Collection of milk samples

A total of 1018 retail milk samples were collected from retail outlets and shops of the local market of different districts of Punjab and 250 mastitic milk samples were collected from Mastitis laboratory, Department of Veterinary Medicine, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

The samples were collected aseptically in the sterilized 50 ml sampling tubes with proper labelling and were transported to the laboratory in an insulated ice box.

Processing and isolation of *Listeria* spp. from milk samples

Isolation of *Listeria* spp. from the milk samples collected from retail shops was attempted as per the study conducted by Sawant *et al.,* (2016).

Confirmation of the isolates

After a two-step selective enrichment of samples and selective plating on to polymixin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar, the typical morphological colonies of *Listeria* were picked and verified by

Gram's staining, catalase reaction, tumbling motility at 25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol, lactose, glucose and α-methyl- d-mannoside), followed by *in-vitro* pathogenicity tests such as phosphatidylinositol-specific phospholipase C (PI-PLC) activity on Agar Listeria according to Ottaviani and Agosti (ALOA), haemolysis on 7% sheep blood agar and CAMP test with *Staphylococcus aureus* and *Rhodococcus equi* (Seeliger and Jones, 1986).

Polymerase chain reaction

DNA extraction of *Listeria spp.* isolates was done by snap-chill method and used as template in multiplex PCR.

Molecular confirmation of *Listeria* isolates

After conventional biochemical and sugar fermentation tests, employed for the detection of *Listeria* spp., further confirmation was done by molecular technique especially, PCR.

The PCR was employed for the detection of *Listeria* genus targeting genus specific, putative phosphoribosyl pyrophosphate synthetase (*prs*) gene and for *L. monocytogenes*, specific haemolysin (*hly*A) gene. All the biochemically confirmed isolates were additionally confirmed by the molecular technique based PCR assay.

Initially the gradient PCR was standardized using ATCC standard strains with published set of primers.

Genus of the presumptive isolates was confirmed by standardizing PCR for genus specific *prs* gene whereas confirmation of the species *L. monocytogenes* was done by PCR targeting *hly* gene of *Listeria*.

PCR for genus *Listeria* and species *L. monocytogenes*

All DNA amplification reactions in conventional PCR for detection of genus *Listeria* and the species *L. monocytogenes* were performed in Mastercycler Gradient Thermocycler (Eppendorf, Germany) with a pre-heated lid as per protocol optimized in department by Sawant *et al.,* (2013). The amplified PCR products were analysed by using agarose gel electrophoresis and the bands in the gel were visualized by Gel Documentation System (Syngene, USA).

Multiplex PCR for the detection of virulence genes of *L. monocytogenes*

The multiplex PCR was standardized using *L. monocytogenes* ATCC (19115) culture strain for the detection of virulence associated genes namely, haemolysin (*hly*A), PI-PLC (*plcA*), actin (*actA*), p60 (*iap*A) and regulatory (*PrfA*) as per the method described by Rawool *et al.,* (2007). The annealing temperature varied from 53^{o}C to 56^{o}C depending on gene to be amplified. Amplification of *PrfA* was standardized at 56^{o}C, whereas *plcA* was standardized at 53^{o}C, *hlyA* at 53^{o}C or 56^{o}C, *actA* at 53^{o}C or 56^{o}C and *iapA* at 53^{o}C or 56^{o}C. Hence, depending on their annealing temperature two sets of multiplex reactions were carried out.

In one set virulent genes namely, *plcA*, *hlyA*, *actA* and *iapA* were amplified with annealing temperature of 53^{o}C, while in another set, virulent genes namely, *PrfA*, *hlyA*, *actA* and *iapA* were amplified with annealing temperature of 56^{o}C. Rest of the cycling conditions of PCR were same as described above for the genus specific PCR

Results and Discussion

In this study out of 1268 (both retail and mastitic) milk samples processed for the isolation, only 120 milk samples developed the typical greyish green colonies on PALCAM agar with visible esculin hydrolysis (Fig. 1), the colonies of 82 samples only were found to be Gram positive coco-bacillary rods. On biochemical examination only seven isolates showed typical reaction. For further characterization to species level they were subjected to sugar fermentation test and based on these test five isolates fermented α-methyl-D-mannoside, mannitol, glucose and lactose hence were designated as *L. grayi* (Table 1). The two isolates produced acid from xylose, lactose and dextrose and were designated as *L. seeligeri.* Summarizing the results on prevalence of *Listeria* as obtained by conventional culture and molecular based method, an overall positivity of 0.68%was reported in Punjab (Table 2). Among the five zones 1.3% and 0.8% prevalence were recorded in central and sub-mountain zone respectively. Taking into consideration the districts, then Ludhiana, Patiala, Tarantaran and Pathankot yielded 3%, 1.66%, 3.33%, and 3.33% prevalence of *Listeria* spp. respectively (Table 3). None of the retail milk samples from other three zones and mastitic milk samples were found positive. Therefore this study reported a very low prevalence of *Listeria* species in retail milk samples in Punjab.

The results of this study can be supported with the studies conducted in Mysore city, Karnataka wherein authors reported 0.76% prevalence of *Listeria* in milk and none of the sample was positive for *L. monocytogenes* (Shantha and Gopal, 2014). Whereas in another study from, Mohali Punjab documented zero prevalence of *Listeria* spp. in the milk samples (Agarwal *et al.,* 2013). Khan *et al.,* (2012) also specified the presence of *L. monocytogenes* from only two samples out of total 250 raw milk samples and milk products in Bareilly. In another study from Odissa by Sarangi *et al.,* (2009), three samples revealed the presence of *L.monocytogenes* (2.01%) out of the total 137 raw milk samples examined. Nayak *et al.,* (2015) screened a total of 200 milk samples and milk products, of these 18 (9%) were found positive for the *Listeria* spp. whereas *L.monocytogenes* was isolated from the three milk samples only with the prevalence 1.5%.

On the contrary some of the studies stated higher prevalence of *Listeria*. Kalorey *et al.,* (2008) conducted a large survey of central India and reported 5.1% prevalence of *L. monocytogenes* from 2060 raw milk samples.

Similarly, Soni *et al.*, (2013) reported 5.8% prevalence of *L. monocytogenes* in raw cow milk samples collected from Varanasi, Uttar Pradesh. Another study carried out in Tamil Nadu by Marry and Shrinithivihahshini (2017) reported 52.7% prevalence of *L. monocytogenes*. The results from these studies concluded that the high prevalence of *L. monocytogenes* in the milk was indication of the direct faecal contamination of milk, poor sanitary practices during collection and transportation of milk and further faulty handling process which leads to low standards of the milk and milk products sold at shops.

In the study, all seven isolates of *Listeria* (two *L. seelegiri* and five *L. grayi*) were subjected to haemolysin test on 7% sheep blood agar (SBA). Accordingly, the 2 isolates (*L. seeligeri*) turned out to be haemolytic in nature. The 5 isolates characterized as *L. grayi* were non-haemolytic. *Listeria* isolates were tested for their pathogenicity by plating them on ALOA, all the 7 isolates produced typical blue green colonies on ALOA but failed to produce a halo even after one week of incubation (Fig. 2).

Listeria spp. isolates when subjected to genus specific PCR for genus level confirmation were found to amplify the DNA fragments of 370 bp respectively. All the isolates were confirmed molecularly as *Listeria* spp (Fig. 3). The findings in the present study are in agreement with the work carried out by Shantha and Gopal (2014) who reported isolates recovered from raw cattle milk when subjected to the molecular identification by PCR for determining the genus *Listeria*, were found to be positive for the genus specific gene *prs*.

Another study carried out in Malaysia where raw milk was assessed for the presence of *Listeria* spp. (Chye *et al.*, 2004), reported 4.4% of the raw milk sample was positive for the *Listeria* spp. Among this, 1.9% were *L. monocytogenes*, 2.1% were *L. innocua* and 0.6% were *L. welshimeri*.

Table.1 Biochemical characterization and sugar fermentation test of *Listeria* spp. from raw retail milk

District	Isolates	Catalase	Nitrate reduction	Voges-Proskauer's test	Methyl red test	Xylose	Lactose	α-Methyl-D-Mannoside	Rhamnose	Dextrose	Mannitol	Designated spp.
Pathankot	PK5	+	-	+	+	-	+	+	V	+	+	*L. grayi*
Ludhiana	L28	+	-	+	+	-	+	+	V	+	+	*L. grayi*
	L10	+	-	+	+	+	+	-	-	+	-	*L. seeligeri*
	L24	+	-	+	+	+	+	-	-	+	-	*L. seeligeri*
Tarantaran	TT11	+	-	+	+	+	+	+	V	+	+	*L. grayi*
	TT35	+	-	+	+	-	+	+	-	+	+	*L. grayi*
Patiala	Pat14	+	-	+	+	-	+	+	-	+	+	*L. grayi*

Table.2 Prevalence of *Listeria* spp. in retail and mastitic milk samples

Sr. No.	Sample type	Sample size	Conventional methods		Molecular methods	
			Positive samples	Per cent positivity (%)	Positive samples	Per cent positivity (%)
1	Retail milk	1018	7	0.68	7	0.68
2	Mastitic milk	250	Nil	Nil	Nil	Nil

Table.3 Districts wise prevalence of *Listeria* spp. in retail milk samples in Punjab

Sr. No.	District	Sample	Positive sample	Per cent positivity
1	Ludhiana	100	3	3
2	Taran taran	60	2	3.33
3	Patiala	60	1	1.66
4	Pathankot	30	1	3.33

Fig.1 Typical greenish-yellow, glistening, lustrous and pointed colonies surrounded by a diffuse black zone of aesculin hydrolysis on PALCAM agar

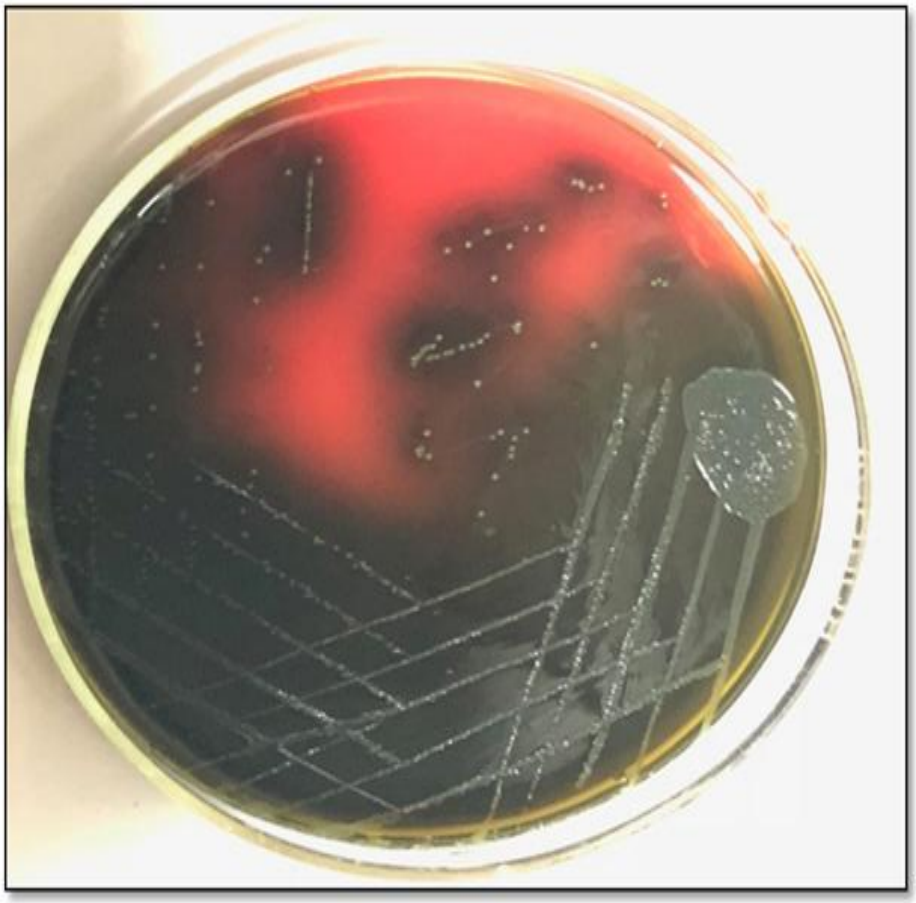
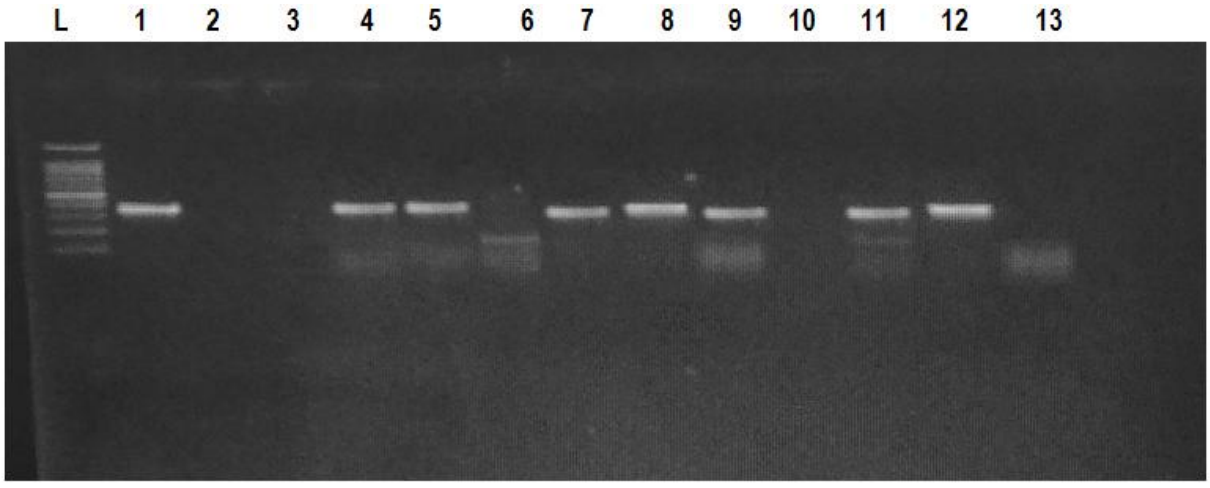


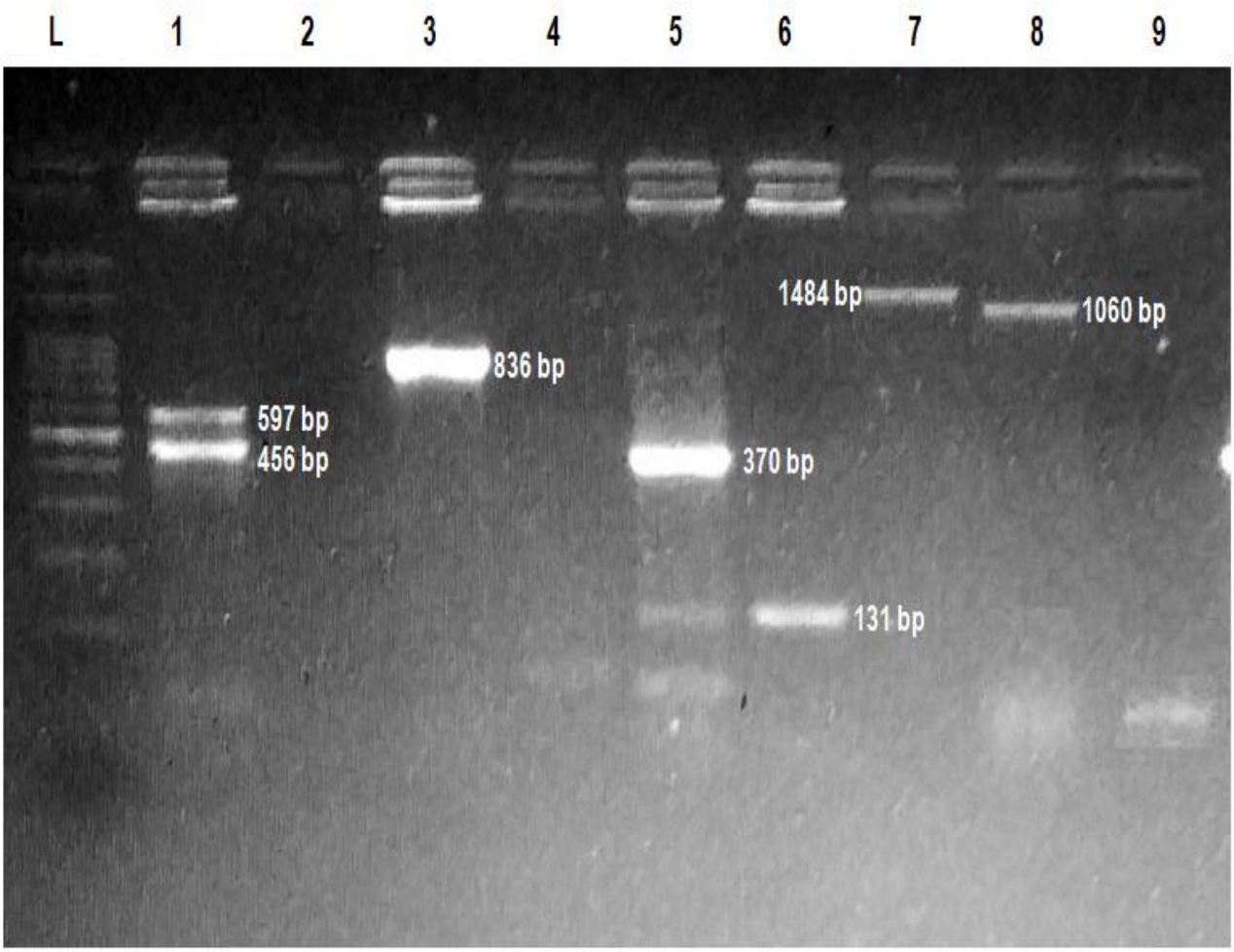
Fig.2 ALOA plate showing the typical green colour of *Listeria* isolates

Fig.3 *Genus specific PCR of Listeria*



Lane L: Marker (100 bp)
Lane 1: Standard
Lane 2, 3, 6, 10: Negative isolates
Lane 3: Negative isolate
Lane 4, 5, 7-9, 11, 12: *prs*
Lane 13: NTC (Negative control template)

Fig.4 Virulence marker genes of *Listeria monocytogenes*



Lane L: Marker (100 bp)
Lane 1: *HlyA*/ORF2110
Lane 2: Negative control
Lane 3: *act*A
Lane 4: Negative control
Lane 5: *prs*
Lane 6: *iap*
Lane 7: *plc*A
Lane 8: *prf*A
Lane 9: NTC (Negative control template)

The PCR for the *hlyA* gene employed in the study could not detect the gene in any of the seven isolates except the standard strains of *L. monocytogenes* which amplified the DNA fragment of 456 bp in the PCR (Fig. 4). As none of the isolates were found to be positive for the species *L. monocytogenes*, therefore multiplex PCR and PCR targeting the genes for *L. monocytogenes serovars* was not accomplished further.

Based on the documented scientific reports we can say that prevalence or contamination of *Listeria* spp, and especially *L. monocytogenes* varies in different studies done in different regions. *Listeria* spp,

including *L. monocytogenes* are ubiquitous microorganism that survive at different places in farm environment and serve as a source of contamination (Sunitha *et al.,* 2016). In some studies it has been found to be excreted intermittently in the faeces of apparently healthy animals at farm, more in animals that are kept indoor and in cooler parts of the year such as December (Husu, 1990). Excretion of these organisms in the faeces and their widespread presence in the farm environment make milk samples highly prone to contamination from these organisms. Contaminated dairy equipments and hands of milkers can also add to the contamination of raw milk with *Listeria* spp. (Tahoun *et al.,* 2017; Sunitha *et al.,* 2016)

Therefore strict hygiene of the farm and during milking is necessary to prevent contamination. Dairy farming in farm is either semi-organised or organised and farmers are aware of the importance of clean and hygienic milk production.

Although *Listeria* spp, especially *L. monocytogenes* have not been frequently associated with mastitis but some studies have documented their presence in the mastitic milk as well. Yadav *et al.,* (2010) reported *L. monocytogenes* from the mastitic milk samples of buffalo and cow. In another study, Rawool *et al.,* (2007) also detected *L. monocytogenes* from subclinical mastitic milk samples, indicating that even if the farm hygiene is good direct excretion of these organisms in milk samples also act as another source of milk contamination. None of our milk samples from the mastitis cases were positive for *Listeria* spp.

The other milk samples (raw retail milk samples) of our study which reported presence of *Listeria* spp, were however not examined for their mastitis status.

Milk samples collected in the study were from retail milk shops and from the milk sellers, which usually collect milk from different household and pool them all. This pooling effect results in dilution of the organisms and become difficult to detect.

There are also different isolation methods available for the isolation of *Listeria* spp. along with different amount of sample processed for *Listeria* isolation by different research workers. This difference in processing also adds to the variation in prevalence level reported from different studies. Absence of *L. monocytogenes* in retail milk samples in this study meets the zero tolerance criteria set by food safety agency. However, the detection of *Listeria* spp. in the milk samples raises the food safety and public health concerns in Punjab and highlights the need to further strengthen production, processing, packaging, storage and distribution system into the retail market to safeguard consumer health.

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