

## Original Research Article

### Isolation and genotypic characterization of *Listeria monocytogenes* from pork and pork products

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#### A B S T R A C T

#### Keywords

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*Listeria monocytogenes* is an important pathogen of public health significance. Isolation and characterization of *L. monocytogenes* was attempted from the different components of pork production system in the west coast region of India. A total of 501 samples were collected from pig rearing environment (n=146), sick and healthy pigs (n=199), slaughter house environment (n=62), raw pork (n=37) and pork products (n=57). *Listeria* spp. were isolated from 31 (6.18%) samples. The isolates were further subjected to biochemical and genotypic characterization. Out of 31 isolates, 11(2.19%) isolates were confirmed as *L. monocytogenes*, 8 (1.59%) as *L. innocua*, 6 (1.19%) as *L. seeligeri*, 3 (0.59%) as *L. ivanovii* and 3 (0.59%) as *L. welshimeri*. Multiplex PCR based serotyping revealed 10 *L. monocytogenes* isolates to be of serovar group 4b, 4d, 4c, while, one isolate was of serovar group 1/2b, 3b, 7. PFGE analysis revealed clonality of the strains prevalent in pork production chain and were similar with strains prevalent in India.

## Introduction

*Listeria monocytogenes*, an important foodborne pathogen, has a significant impact on public economy worldwide. Though human infections are rare, *L. monocytogenes* has the potential to cause serious and life-threatening disease and the majority of cases of human listeriosis are associated with contaminated foods (Andritsos *et al.*, 2013). *L. monocytogenes* has been described as an opportunistic pathogen affecting primarily neonates, pregnant women, the aged and immune-challenged individuals, patients with a history of transplantation and under

the treatment of immune suppressive drugs (Liu, 2006; Schlech *et al.*, 2000). Ingestion of *L. monocytogenes* contaminated foods is associated with central nervous system disease, sepsis, endocarditis, focal infections and gastroenteritis, and still births and abortions in pregnant women (Zhou and Jiao, 2004).

It has been hypothesized that the live animals like healthy carrier pigs may be the major sources of contamination at abattoirs and of final food products (Autio *et al.*,

2000; Giovannacci *et al.*, 1999; Hellström *et al.*, 2010). Besides this, the occurrence of *L. monocytogenes* has been reported in abattoirs, raw pork and pork products (Hellström *et al.*, 2010; Kanuganti *et al.*, 2002; Thevenot *et al.*, 2005). Food-borne listeriosis outbreaks have been reported worldwide implicating pork and pork products such as deli meats (Goulet *et al.*, 1998).

Different reports are available describing the characterization of *L. monocytogenes* isolates from swine slaughterhouses, pork and pork products by PCR-based serotyping, presence of virulence-associated genes, and pulsed-field gel electrophoresis analysis (Meloni *et al.*, 2013). *L. monocytogenes* serotypes 1/2a, 1/2c, 4b, and 4d have been reported from pork and pork products (Bērziņš *et al.*, 2010; Boscher *et al.*, 2012). Isolation of *L. monocytogenes* strains with similar serotypes and pulsotypes have been reported from sows and fattening pigs from the same farms, suggesting common sources of contamination (Boscher *et al.*, 2012).

In India, *L. monocytogenes* has been reported from a variety of foods including meat, milk and seafood (Barbuddhe *et al.*, 2012; Kalorey *et al.*, 2008; Parihar *et al.*, 2007). However, no reports are available which illustrate the risk factors for *Listeria* contamination on farm, abattoir, raw pork and pork products in India. The objective of the present study was to isolate and characterize *L. monocytogenes* from different components of the Indian pork production system.

## Materials and methods

### Sample collection

A total of 501 samples were collected from the piggeries (n=17) and its associated

environment, samples from slaughter house, and raw pork and pork products during August 2012 to December 2013 from the West Coast region of India. Samples comprised of clinical (n=199) and environmental (n=146) sources. Deep vaginal, nasal, oral, skin swabs, feces, autopsied tissues were collected as clinical samples. Soil, feed, and water along with floor swabbings were collected from piggery associated environment. Furthermore, tonsils, intestinal contents from slaughtered pigs (n=62), and swabs from floor, hands of workers, and carcasses were also collected. Raw pork (n=37) and pork products (n=57) samples mainly sausages (cooked and uncooked) were also collected. All the samples were collected aseptically in the containers (Himedia, India) and brought to the laboratory in chilled conditions and processed for isolation of *Listeria*.

### Isolation and identification

Isolation of *Listeria* from collected samples was attempted as per the US Department of Agriculture (USDA) method as described by Donnelly and Baigent (1986) after making necessary modifications. Briefly, the tonsil tissues, intestinal contents, faeces and autopsied tissue samples (approx. 5 g) were triturated under aseptic conditions and inoculated into 45 ml of University of Vermont medium I (UVM-I) and incubated at 30°C for 18- 24 h. Water (25 ml), feed (5 g), soil, raw pork samples and sausages were enriched in UVM I. Enriched inocula (0.1 ml) from UVM-I were then transferred to 10 ml of UVM II and incubated again for 24 - 36 h at 30°C.

Enriched broths were streaked on PALCAM agar (Himedia) and incubated at 30°C for 48 h. Typical greenish gray colored colonies with black sunken centers surrounded with black halo on PALCAM agar were selected

as presumptive *Listeria* spp. and streaked on 5% sheep blood agar (SBA). Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and  $\alpha$ -methyl D-mannopyranoside). All biochemically characterized *Listeria* isolates were further subjected to haemolysis on SBA, Christie, Atkins, Munch Petersen (CAMP) test, phosphatidylinositol phospholipase C (PI-PLC) assay as per Gorski (2008). Standard strains of *L. monocytogenes* MTCC 1143, *Staphylococcus aureus* MTCC 1144, and *Rhodococcus equi* MTCC 1135 were used as controls or standards for all reactions.

### **Multiplex PCR for serotyping and virulence determination**

The genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India) and was subjected to PCR amplification for detection of the *actA*, *hlyA* and *plcA* genes (Rawool *et al.*, 2007). The multiplex PCR serotyping assay was standardized as per the protocol described by Doumith *et al* (2004). Details of oligonucleotide primers are given in Table 1. The PCR amplification of the virulence associated genes was set for 25 $\mu$ l reaction volume. The reaction mixture was subjected to an initial denaturation at 95°C for 2 min followed by 35 cycles each of 15 s denaturation at 95°C, 30 s annealing at 60°C and 1 min 30 s extension at 72°C. It was followed by final extension of 10 min at 72°C and held at 4°C.

For multiplex PCR based serotyping, 50  $\mu$ l reaction mixtures were prepared each containing 2 U Taq DNA polymerase, 10X PCR Buffer (50mM Tris-HCl, 10mM KCl,

50mM ammonium sulphate, 2mM MgCl<sub>2</sub>), 300 $\mu$ M dNTP mix, 2 mM MgCl<sub>2</sub>, 2  $\mu$ M each of primer *lmo0737*, *ORF2819*, *ORF2110* and *prs* and 5 $\mu$ l of DNA template. The reactions were performed in Mastercycler epGradient (Eppendorf, Germany) with a preheated lid with an initial denaturation step at 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 1 min 15 s, and 72°C for 1 min 15 s; and one final cycle of 72°C for 10 min in thermocycler. The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining under UV illumination.

### **Pulsed field gel electrophoresis (PFGE)**

PFGE was performed according to the PulseNet standardized protocol (Graves and Swaminathan, 2001). Bacterial cultures were embedded in PFGE grade agarose (Bio-Rad). Sample plugs were digested with 25 U of *AscI* (New England Biolabs, Beverly, MA) at 37°C for 3 h or 160 to 200 U of *ApaI* (New England Biolabs) at 30°C for 5 h. Generated DNA fragments were separated on the 1.2% PFGE grade agarose gel in 0.5x TBE buffer at 14°C using 6 V/cm with time ramped for 4-40 s over 22 h using a CHEF-DR II module (BioRad). Gels were stained with ethidium bromide and visualized by a UV transilluminator. Gels were analysed with Phoretix 1D-pro software (Total Lab, UK). The obtained pulsotypes were compared with PFGE profile database of *L. monocytogenes* isolates from food and clinical cases in India.

### **Result and Discussion**

#### **Isolation and identification of *Listeria***

In the present study, a total of 501 samples from pig rearing environment, sick and healthy pigs, slaughter house environment,

raw pork and pork products were subjected for isolation of *Listeria* species (Table 2). Overall, a total of 31 (6.18%) strains of *Listeria* were isolated. Of these, 11(2.19%) isolates were confirmed as *L. monocytogenes*, 8 (1.59%) as *L. innocua*, 6 (1.19%) as *L. seeligeri*, 3 (0.59%) as *L. ivanovii* and 3 (0.59%) as *L. welshimeri*. Eight *L. monocytogenes* strains were isolated from clinical and environmental samples, while, two isolates were recovered from slaughter house environment and one from sausage. *L. ivanovii* was isolated from vaginal swabs (2) and nasal secretions (1) of pigs. All 11 *L. monocytogenes* isolates were haemolytic, CAMP positive and PI-PLC positive.

#### **Multiplex based serotyping and virulence determination**

Ten *L. monocytogenes* isolates were serogrouped as 4b, 4d, 4c by multiplex PCR serotyping, while one isolate belonged to serovar group 1/2b, 3b, 7 (Fig. 1). All the *L. monocytogenes* isolates tested positive for the virulence genes, *hlyA*, *plcA* and *actA*.

#### **Genetic diversity and its comparison with food, animals and humans strains**

PFGE analysis of the *L. monocytogenes* isolates using *ApaI* and *AscI* restriction digestions revealed three different pulsotypes (PTs). Nine isolates clustered together, while other two isolates showed different banding patterns. *L. monocytogenes* serovar group 4b, 4d, 4c isolates showed clonality with the dominant pulsotypes observed from India (Fig. 2).

*Listeria* spp. are widespread in the environment including soil, water, sewage, vegetation, wild animal faeces, animal farms and in food processing facilities (Sauders and Wiedmann, 2007; Todd and Notermans, 2011). *Listeria* carriage by pigs at the farm

level could be a primary source for carcass contamination. The type of feed (wet/dry) and water could be major responsible factors associated with the occurrence of *Listeria* (Beloeil *et al.*, 2003). *L. monocytogenes* occurs frequently in raw pork (Norrung *et al.*, 1999), although the origin of the contamination is unclear (Thevenot *et al.*, 2006).

In present study, prevalence of *L. monocytogenes* in piggery, associated environment, slaughterhouse, raw pork and pork products was studied. *Listeria* spp. were isolated from 31 (6.18%) samples. Earlier studies reported the occurrence of *Listeria* in piggeries and related environment as 0.8% (Iida *et al.*, 1998), 1.7% (Skovgaard and Norrung, 1989), 2.4% (Kanuganti *et al.*, 2002), 3% (Buncic *et al.*, 1991) and 5.9% (Weber *et al.*, 1995). Few studies reported higher occurrence of *Listeria* in slaughterhouses, and raw pork and pork products than farm animals (Autio *et al.*, 2000; Meloni *et al.*, 2013; Ortiz *et al.*, 2010; Skovgaard and Norrung, 1989). Studies also illustrated the contamination from farm to final products (Hellström *et al.*, 2010; Thevenot *et al.*, 2006). In India, the occurrence of *Listeria* from pork and port products has been reported (Doijad *et al.*, 2010; Shrinithiviahshini *et al.*, 2011). Also an outbreak of listerial meningoencephalitis affecting 75 indigenous pigs was observed in a pig farm (Rahman *et al.*, 1985). In another outbreak, circling movements with lateroventral deviation of the head, sudden ataxia and epileptic seizures among the affected pigs was reported (Dash *et al.*, 1998). *L. monocytogenes* was isolated from pig blood and pig rearing environment, besides this, occurrence of *L. ivanovii* was found in placental material of aborted sows (Raorane *et al.*, 2013). In case of pork and pork products, limited data has been reported from India.

**Table.1** Oligonucleotide primers used for determination of serotyping and virulence genes

Target		Sequence (5'-3')	Amplicon size (bp)	Reference
lmo0737	F	AGGGCTTCAAGGACTTACCC	691	Doumith et al., 2004
	R	ACGATTTCTGCTTGCCATTC		
lmo1118	F	AGGGGTCTTAAATCCTGGAA	906	Doumith et al., 2004
	R	CGGCTTGTTTCGGCATACTTA		
ORF2819	F	AGCAAAATGCCAAACTCGT	471	Doumith et al., 2004
	R	CATCACTAAAGCCTCCCATTG		
ORF2110	F	AGTGGACAATTGATTGGTGAA	597	Doumith et al., 2004
	R	CATCCATCCCTTACTTTGGAC		
<i>Prs</i>	F	GCTGAAGAGATTGCGAAAGAAG	370	Doumith et al., 2004
	R	CAAAGAAACCTTGGATTTGCGG		
<i>actA</i>	F	CAGCGACAGATAGCGAAGATT	965	Present work
	R	TGTTTCCCGGATGATTTCTAGTT		
<i>plcA</i>	F	GGAAGTCCATGATTAGTATGCCT	803	Present work
	R	CTGGAATAAGCCAATAAAGAACTCTG		
<i>hlyA</i>	F	GCAGTTGCAAGCGCTTGGAGTGAA	456	Rawool et al., 2007
	R	GCAACGTATCCTCCAGAGTGATCG		

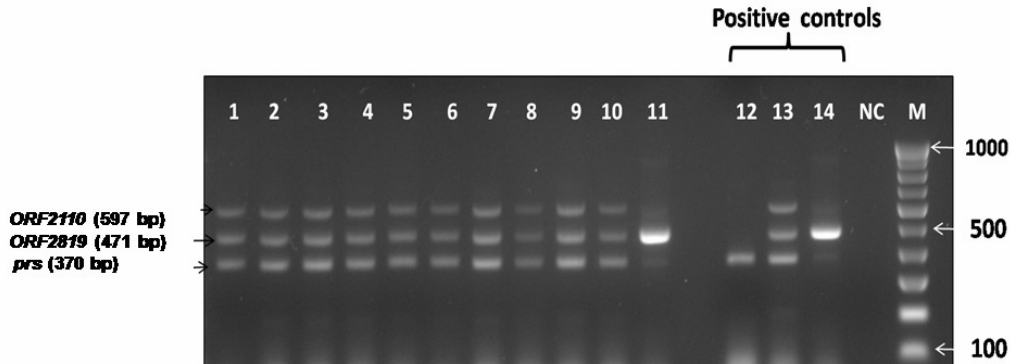
**Table.2** Incidence of *Listeria* in piggery, abattoir, pork and pork products

Source	Sample size	L <sub>m</sub> (%)	L <sub>iv</sub> (%)	L <sub>se</sub> (%)	L <sub>in</sub> (%)	L <sub>we</sub> (%)
Clinical	199	4 (2.01)	3 (1.5)	2 (1)	5 (2.5)	1 (0.5)
Environmental	146	4 (2.7)	-	4 (2.7)	2 (1.4)	2 (1.4)
Abattoir	62	2(3.3)	-	-	-	-
Raw pork	37	-	-	-	-	-
Pork product	57	1 (1.7)	-	-	1 (1.7)	-
Total	501	11 (2.2)	3 (0.6)	6 (1.2)	8 (1.6)	3 (0.6)

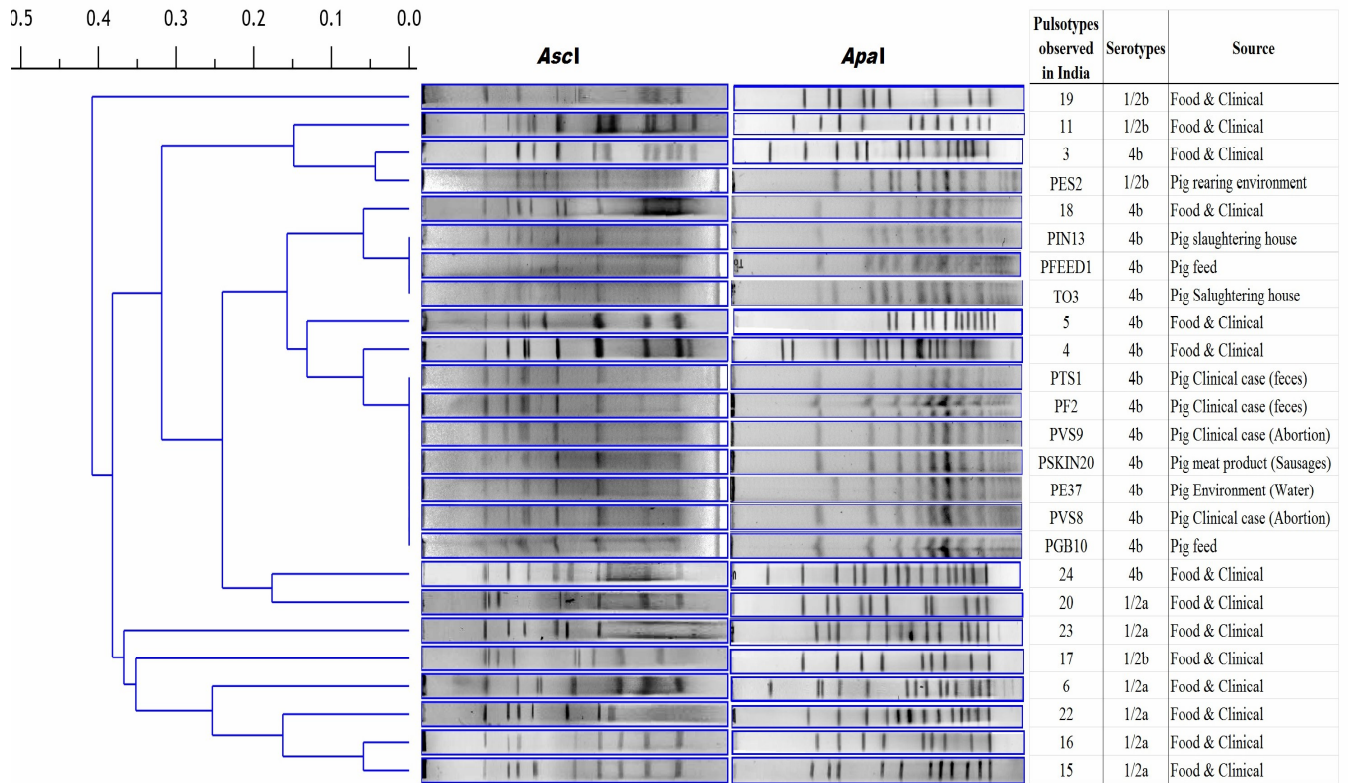
L<sub>m</sub>: *L. monocytogenes*, L<sub>iv</sub>: *L. ivanovii*, L<sub>se</sub>: *L. seeligeri*, L<sub>in</sub>: *L. innocua* and L<sub>we</sub>: *L. welshimeri*.



Fig 1



**Fig. 1.** Lane 1: Pgb10 (*L. monocytogenes* ), Lane 2: PF2 (*L. monocytogenes* ), Lane 3: PTS1 (*L. monocytogenes* ), Lane 4: PVS8 (*L. monocytogenes* ), Lane 5: PVS9 (*L. monocytogenes* ), Lane 6: 37(*L. monocytogenes*), Lane 7: Skin20 (*L. monocytogenes* ), Lane 8: Cfeed 1(*L. monocytogenes* ), Lane 9: X-In 13 (*L. monocytogenes* ), Lane 10: Tongue 3 (*L. monocytogenes* ), Lane 11: PES 2 (*L. monocytogenes*) Lanes 12, 13 and 14: Positive control: ILCC 523 ( *L. innocua*), (*L. monocytogenes* MTCC1143) and ILCC 27 (*L. monocytogenes* serogroup 1/2b, 3b and 7), respectively, Negative control (*Staphylococcus aureus* MTCC1144), Lane M: 100bp DNA ladder



**Fig. 2.** Pulsotypes compared with PFGE profile database of *L. monocytogenes* from India.

In the present study, *L. monocytogenes* was isolated from deep vaginal swabs of two sows having a history of abortions. Spontaneous abortions as attributed to *L. monocytogenes* have been frequently reported in sheep, goats and cattle but rare in pigs (Kaur *et al.*, 2010; Kumar *et al.*, 2007). One each *L. monocytogenes* strain was isolated from faeces and tongue swab of sow and fatteners. The occurrence of *L. monocytogenes* in faeces, tongue, and tonsil scrapings from live hogs has been reported earlier (Autio *et al.*, 2000; Felon *et al.*, 1996; Kanuganti *et al.*, 2002). All four *L. monocytogenes* strains linked with clinical cases were isolated from the same farm. Pig rearing environment plays a significant role in spread and/or persistence of infection in herds.

In the present study, *L. monocytogenes* strains were isolated from pig feed, drinking water and soil. Earlier workers (Beloeil *et al.*, 2003; Skovgaard and Norrung, 1989) demonstrated that the wet feeding was responsible for the development of listeriosis. In the present study, samples were collected from pig farms where feeding of hotel waste and poultry waste like feathers and viscera was being practiced. This could facilitate ease of entry of *L. monocytogenes* in pig habitat. Along with feeding, general hygiene of pig farm workers is mandatory factor, as farm worker may be the source of cross contamination (Beloeil *et al.*, 2003). Other factor like very short duration of the “empty and clean” period prior to stocking the fattening room has also been found as a risk factor for *L. monocytogenes* contamination at the end of the fattening period. Apart from this, *L. monocytogenes* has been isolated from drinking water and soil from piggery environment. Nightingale *et al.* (2004) reported the occurrence of *L. monocytogenes* in soil. Two isolates were recovered from

abattoir environment. Autio *et al.* (2000) hypothesized that *L. monocytogenes* could spread through contact between the tonsils and tongue and the other viscera and carcass during the evisceration process. Isolation of *L. monocytogenes* has been reported from 8.3% intestinal samples (Kanuganti *et al.*, 2002).

Processed pork products may be contaminated by *L. monocytogenes* at several stages. Either the raw ingredients may be contaminated or contact with unprocessed raw materials, unclean surface may lead to contamination (Chasseignaux *et al.*, 2001). *L. monocytogenes* was isolated from a sausage sample. Earlier, *L. monocytogenes* was isolated from 10.6 % of raw sausages (Thevenot *et al.*, 2005). *L. ivanovii* was isolated from placental tissues and nasal secretions of sows that were aborted. Outbreaks attributed to *L. ivanovii* have been reported in sheep (Sahin and Beytut, 2006; Santagada *et al.*, 2004). Reports of abortions due to *L. ivanovii* in sows are lacking. Besides other species, *L. innocua* was also isolated. Presence of *L. innocua* could be one of the indications of presence of *L. monocytogenes* in the samples.

Detection of multiple virulence associated genes in combination with *in vitro* pathogenicity tests may be required for confirming the pathogenic potential of *Listeria*. In the present study, all 11 *L. monocytogenes* isolates showed the amplification of the *hly*, *plcA* and *actA* genes and considered as pathogenic strains of *L. monocytogenes*. *L. monocytogenes* strains were serogrouped as 4b, 4d, 4c and 1/2b, 3b, 7. Meloni *et al.* (2013) detected serotypes 1/2c and 1/2a from pig slaughterhouses in Italy. Serotypes 1/2a, 1/2b, 4b, and 1/2c were detected from *L. monocytogenes* isolates recovered from

sows and fattening pigs in farrow-to-finish pig farms in France (Boscher *et al.*, 2012).

Among the several methods used to type *L. monocytogenes*, pulsed field gel electrophoresis (PFGE) is considered as the gold standard method due to high discriminating power. Based on the excellent resolving power for epidemiological typing, PFGE has been used to track isolates of *L. monocytogenes* and other pathogens responsible for food-borne illnesses (Aarnisalo *et al.*, 2003; Nakamura *et al.*, 2004; Wagner *et al.*, 2003). Various studies analyzed the prevalence in pig farm, slaughterhouse and raw pork by using PFGE (Giovannacci *et al.*, 1999; Hellstrom *et al.*, 2010). In this study, we determined the PFGE profiles of *L. monocytogenes* isolates and demonstrated the three different pulsotypes. Five clinical isolates, two isolates from pig feed, one from pig rearing environment and one isolate from sausage clustered together. The other two isolates clustered separately. The presence of single clone at pig rearing environment and clinical cases suggest probable cross contamination. Such contamination may get carried forward in pork processing chain and final products may remain contaminated. Various workers highlighted the occurrence of *L. monocytogenes* in herd animals could be responsible for final products contamination (Beloeil *et al.*, 2003; Hellstrom *et al.*, 2010; Thevenot *et al.*, 2006).

Further the profiles of isolates were compared with database of PFGE profiles of isolates from India (unpublished). The pulsotypes reported in this study were found to be identical with the dominant pulsotype observed in India. This pulsotype has been reported across India from different regions and wide sources and has been identical with strains isolated from outbreaks.

In conclusion, *L. monocytogenes* was isolated from piggery environment and could be a primary source for carcass contamination. Besides this abattoir environment could add to contamination of final products. Pig feeding practices could be a major source of *L. monocytogenes* in herd animals. Control measures should be designed to reduce the *L. monocytogenes* load at the pre-harvest stage. PFGE analysis demonstrated that the *L. monocytogenes* isolated from different components of food chain were clonal.

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