

Review Article

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Incidence of *Listeria monocytogenes* in Bovine Environment with Respect to Phenotypic and Genotypic Characterization: A Review

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ABSTRACT

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Listeria is an ubiquitous organism and can be isolated from a variety of sources from different parts of the world. Various methods are used to sanitize the bovine and food processing environment and to control the organism from bovine environment. Proper surveillance, rapid detection of *Listeria* is important to ensure the safety. The article reviews major *Listeria monocytogenes* incidences in bovine and their environment.

Introduction

The genus *Listeria* is currently grouped into 17 species identified as *Listeria monocytogenes*, *L. ivanovii*, *L. marthi*, *L. innocua*, *L. fleischmanni*, *L. aquatica*, *L. newyorkensis*, *L. rocurtia*, *L. weihenstephanensis*, *L. grandensis*, *L. booriae*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. floridensis*, *L. comellensis* and *L. riparia*. *Listeria* are small rod shaped and Gram positive bacteria. Out of 17 species, two species viz. *L. monocytogenes* and *L. ivanovii* have been recognized as pathogenic. *L. monocytogenes* has now recognized as a facultative intracellular pathogen, accountable for a number of foodborne human infections

that lead to invasive disease of high fatality rate (25 to 30 %). Listeriosis is highly prevalent in human as well as in farm ruminants (sheep, cattle, goats). In USA, listeriosis ranks third among foodborne infections associated with mortality (Scallan *et al.*, 2011).

L. monocytogenes is prevalent in products such as water, sludge, vegetation, feed, food, plants, soil, and milk products in a number of geographical regions (Leite *et al.*, 2006). It also transmits from infected humans and animals (Liu, 2008; Dhama *et al.*, 2013). About 11-52% of infected animals remain as healthy fecal carriers of the pathogens (Rocourt and Cossart, 1997).

L. monocytogenes becomes potent pathogen as it can survive in the bio-film and can sustain temperatures as low as -1.5 °C to as high as 50°C and tolerates a pH between 4.3 and 9.6. Once an area gets contaminated by this organism, its eradication becomes difficult (Rodriguez *et al.*, 2008). *Listeria* is also prevalent in dairy environment (Nightlingale *et al.*, 2004). Spread of *Listeria* between animals or human generally starts from cattle farm which acts as a major source of transmission (Pritchard and Donnelly, 1999). Farm ruminants are regularly exposed to *Listeria* sp. through a continuous fecal-oral cycle (Vazquez-Boland *et al.*, 2001). Silage feeding has been reported to increase the *Listeria* exposure in ruminants (Donnelly, 2002).

L.monocytogenes has been reported as a milk contaminant from mastitic animals. It is responsible for mastitis in cattle along with evident abortion, infertility, repeat breeding, encephalitis and septicemia (Barbuddhe *et al.*, 2008; Deb *et al.*, 2013). In a cattle farm, prevalence of *Listeria* is reported to be on higher side especially in feed bunks, feedings and water trough. Moreover, cattle feces and silage are found to be the major sources of *L. monocytogenes* infection (Yoshida *et al.*, 1998). Sepsis, nervous and listerial abortion are three common forms of listeriosis based on symptoms (Carare, 2006). In ruminants, *L.monocytogenes* has also been responsible for encephalitis and uterine infections

In early years, it was found to be tough to correlate the epidemiology of *L. monocytogenes* in cow as no such method was available and hence reports were mostly on lower side (Fenlon, 1986b). In recent time, PCR based detection of *hlyA*, *actA*, *plcA* and *inlB* gene assist in easy detection of *L. monocytogenes* (Winters *et al.*, 1999 and Yong *et al.*, 2003). In addition, serotyping further adds the details to characterize *L.*

monocytogenes with respect to virulence and pathogenicity (Douminth *et al.*, 2004). Molecular typing of *L.monocytogenes* serotypes assist in quick detection of isolates involved in disease outbreaks and thus adds to the efficiency of diagnosis (Hofer *et al.*, 2000). At present pulse field gel electrophoresis scores a high rank in detection and phylogenetic analysis of *L. monocytogenes*. The method is featured by high discriminatory power, being reproducible and easily gets standardized and proved specific at genetic level (Swaminathan *et al.*, 2001 and Connor *et al.*, 2010).

Being a pathogen *L. monocytogenes* featured with biofilm formation for its survive bility in the environment (Jeong and Frank, 1994). In a surviving instinct, *L. monocytogenes* has found to be resistant to commonly used disinfectants which are generally used in dairy and food plants. Despite of using rigorous protocol of disinfection, *L.monocytogenes* is found to be a milk contaminant especially in farm conditions.

Recently due to the awareness towards food safety, it is important to develop suitable methods which could be specific, reliable and rapid since many traditional methods lack sensitivity, specificity and rapidity.

Furthermore Real time PCR (qPCR) technique is advantageous by ruling out the many limitations of conventional methods and also lowers the risk of cross contamination. qPCR method is surely a fast and the results show increasing response once positive sample recorded and also post PCR processing is not required which makes it an easy tool for detection.

A number of studies reported the occurrence of *Listeria* species in India from various sources (Dhanashree *et al.*, 2003; Manoj *et al.*, 1991; Moharem *et al.*, 2007; Kalorey *et al.*,

2008; Jallewar *et al.*, 2007; Yadav *et al.*, 2011). Poor understanding and lack of awareness on Listeriosis in India, the *L. monocytogenes* infection goes unnoticed especially in human and animals. In India, the data do not represent the clear picture of its epidemiological scenario. Not many reports are available for the source of *L. monocytogenes* contamination.

Listeria

Species in Listeria are very well known as non spore forming, facultative anaerobic rods, non capsulated organism. They measure about 0.4 by 1 to 1.5 μ in size and motile at 10 to 25°C as reported by Sallen *et al.*, (1996) and Rocourt, (1999). *Listeria* sp. are sometimes found to be positive for the mucopolysaccharides capsule along with catalase positive nature as reported by Smith and Metzger (1962). This large group of Listeria genus is recognized by seventeen well known species, *L. monocytogenes*, *L. welshimeri*, *L. marthii*, *L. innocua*, *L. grayi*, *L. seeligeri*, *L. ivanovii*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, *L. cornellensis*, *L. rocourtia*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, and *L. booriae*. Among them only two are genetically pathogenic, *L. monocytogenes* and *L. ivanovii* as reported by Henke *et al.*, (2015). A very first report of the pathogenicity with *L. monocytogenes* has been recorded way back in 1926 (Murray *et al.*, 1926) and recently *L. monocytogenes* has been listed in the food borne pathogen category by World Health Organization (WHO, 2002) making it more featured organism to investigate.

Listeria monocytogenes

Among *Listeria* species, *L. monocytogenes* dominates in pathogenicity and recognized with food borne bacterial zoonotic disease (Ben Embarek, 1994). It is found to be infecting wider range of hosts like cattle,

chicken, goat, and sheep and comes out with disease, listeriosis in high risk group hosts, pregnant women, neonates and even immunocompromised adults which puts up a serious concern about its spread and prevalence in different ecology (Schuchat *et al.*, 1992). With about 0.2 to 0.8 cases per 100,000 persons reported with infection (Gellin *et al.*, 1991, McLauchlin 1996, Kela and Holmstrom, 2001, Lukinmaa *et al.*, 2003); according to research of Dalton *et al.*, (1997) among infected population progression of sepsis, meningitis, abortion, and gastroenteritis is very common and in total, mortality rate reaches close to 20 to 30%, hence making it a serious pathogen (Goulet *et al.*, 1998). Keeping in view, origin of pathogen always remain the concern as sometimes it may lead to sporadic or even epidemic disease situation as reported in United states. This may sometimes put up the situation of food product recall and hence creates the public health concern (Borucki *et al.*, 2004).

Not only are the animals, human also is the host for *L. monocytogenes* causing human listeriosis, a potentially fatal food borne infection especially in European countries (Goulet *et al.*, 2008; Smith and Osborn, 2009). In India, incidences of Listeriosis has extensively been reported by Malik *et al.*, (2002) and undoubtedly responsible for many abortions and premature birth in human (Bhujwala, *et al.*, 1974). In recent time, India has accounted prevalence of *L. monocytogenes* in women with poor obstetrics history also (Gupta *et al.*, 2003; Dhanashree *et al.*, 2003; Kaur *et al.*, 2007; Sonegaokar, 2009; Dixit, 2013).

L. monocytogenes is not only remain restricted to food items like raw and ready to eat produces but also recovered from fecal samples of healthy population (Gilbert *et al.*, 1989; Sheridan *et al.*, 1994; Wesley, 1999). With a apparent presence in fish, squid and

crustaceans as detailed by Miettinen and Wirtanen (2005), incidence of *L. monocytogenes* also been recorded in animals with mastitis, reproductive disorders and septicaemia (Shakuntala *et al.*, 2006; Rawool *et al.*, 2007). *L. monocytogenes* additionally putting up a serious question in front of us as they are showcasing now recurrent presence in the regularly consumed meat and milk of goats, sheep and buffaloes, and from seafood especially in India as reported by Barbuddhe *et al.*, 2000; Barbuddhe *et al.*, 2002; Karunasagar and Karunasagar, 2000; Parihar *et al.*, 2007).

Prevalence of *Listeria monocytogenes*

Prevalence of *L.monocytogenes* from bovine environment

Like any other pathogen, *L. monocytogenes* certainly spans several niches throughout the world such as forest soil, mud, feed, feeding grounds, birds, wildlife faeces and other as reported by Weis and Seeliger, (1975). According to Ueno *et al.*, (1996) and Borucki *et al.*, (2004) farm animals and farm environment also carries *L. monocytogenes* and act as potential source of human listeriosis. Allerberger and Wagner, (2010) proposed that bacterium can easily transmit through consumption of contaminated food, water and feed and also related this incidences with type of food production technology used which makes food "Listeria-risk foods". Cattle with 9.4% detection of *L. monocytogenes* puts up a serious question about health profile of cattle used for milk (Atil *et al.*, 2011). Gelbíčova and Karpiskova (2012) reported 11 % sample prevalence in faeces, feed, water and environment of cattles for *L. monocytogenes*. Esteban *et.al.*,(2009)reported occurrence of *L.monocytogenes*in dairy farm environment, 46.3% prevalence of *L. monocytogenes* was reported in dairy cattle. In one report Santorum *et al.*, (2012) evidenced that *L. monocytogenes* easily getting

transmitted *via.*, farm effluents, along with feed and food also to the nearby animals and plant materials.

In India, Sarangi and Panda (2012) corroborated the *L. monocytogenes* presence in environmental and clinical samples of cattle, sheep, goat, poultry and pig and mostly cattle found to be positive for the species. Soil containing *L. monocytogenes* also been reported in Kerala by Kumar *et al.*, (2014).Raorane *et al.*, (2014) recorded 12% prevalence in clinic and farm environment and Soni *et al.*, (2014) observed *L. monocytogenes* was also dominating close to the soil.

Prevalence of *L. monocytogenes* from animal clinical cases

Nightingale *et al.*, (2004) put us a concern about dispersal of *L. monocytogenes* from farm environment and Vidic *et al.*, (2007) confirmed the 1.2% positivity for the listeriosis.Silva *et al.*, (2009) also confirm the presence of *L. monocytogenes* in cattle aborted fetus with 2.39%. Barkallah, *et al.*, (2014) confirmed the *L. monocytogenes* (4.66%) in bovine abortion of Tunisian dairy cattle.

Srivastava *et al.*, (1985) and Shakuntala *et al.*, (2006) confirmed the presence of *L. monocytogenes* in farm animals suffering from various reproductive disorders when tested for the fecal, nasal, vaginal swabs and blood samples with 4.4 and 7.4% prevalence of *L. monocytogenes* and other *Listeria* sp, respectively. Presence of *L. monocytogenes* in milk furthermore been confirmed by the PCR by targeting the *hlyA* gene of *L. monocytogenes* with about 17.9% positive samples (Parihar *et al.*, 2007). Animal tissue samples from cases of abortion, repeat breeding, retention of placenta also been used to confirm the presence of *L. monocytogenes* and about 16samples was recorded positive out of 359 sampled (Chopra *et al.*, 2012). Not only *L. monocytogenes* 12(5.11%), remain

present in animal samples but also the other species such as *L. innocua* 11(5.11%), *L. seeligeri* 2(0.93%), *L. ivanovii* 2(0.93%), and *L. welshimeri* 1(0.46%) was recorded (Raorane, *et al.*, (2014). The prevalence of *L.monocytogenes* in cattle is higher in contrast to other animals reported by Dixit (2013).

Prevalence of *L. monocytogenes* from milk

Presence of *L. monocytogenes* found to be variable in percent incidences across the world. As per Schlegelova *et al.*, (2002) only two samples found to be positive out of 111 milk samples tested which accounts for 1.8% contamination for *L. monocytogenes*. According to Kessel (2004) in U.S dairy total 861 bulk tank sample when tested, they recorded 21 samples found to be positive. Centinkaya *et al.*, (2014) confirmed the presence of *L. monocytogenes* in milk and dairy products when tested 512 food samples overall.

Presence of *Listeria* species has been detected in number of milk and milk products in India such as ice cream (Pednekar *et al.*, 1997), raw milk (Bhilegaonkar *et al.*, 1997). Kalorey *et al.*, (2008) reported that about 5.1% of milk samples from dairy having presence of *L. monocytogenes* along with other *Listeria* species. Not only the milk and its products, but also the appliances used in dairy was reported to contaminated with *L. monocytogenes* such as raw milk collector, milk silos, butter milk miner and other as reported by Doijad *et al.*, (2011). Many authors published the similar incidences of prevalence of *L. monocytogenes* in milk (Soni *et al.*, (2013), Nayak *et al.*, (2015), Yadav *et al.*, (2010), Najand *et al.*, (2015) and Sawantet *et al.*, (2016).

Molecular characterization of *L. monocytogenes*

With the implicit severity of *L.*

monocytogenes as it is prevailing in many food products that human consumes, early or easy detection of contamination certainly is a need of an hour and in requirement number of protocols or methods have been put forward. Since the food industry demands early detection of contaminant, so that food could be either dispatched or discarded at early time, techniques like PCR making its presence which is not only sensitive but also remained to be specific to its target.

In the present scenario, detection of *L. monocytogenes* by PCR could be achieved by targeting the number of virulent genes (e.g., *hlyA*, *prfA* and *actA*) which are one kind of marker genes for easy detection of *Listeria monocytogenes* in intramammary origin (Warke *et al.*, 2007). In India and abroad number of stories has been written with these genes for achievement in PCR amplification for contaminated samples like fish, poultry, and wild life (Gunjal, 2006; Kalorey *et al.*, 2006; Jellewar *et al.*, 2007).

Virulence factors of *L. monocytogenes plc's* (Phospholipase)

L. monocytogenes posses two phospholipase 1) phosphatidyl inositol- specific PLC (PI-PLC or PLC-A; encoded by *plc A*) and a broad range phosphatidyl choline- specific PLC (PC-PLC or PLC-B; encoded by *plc B*). Protein PI-PLC is a 33kDa protein encoded by *plcA* gene which is one of the six genes located in small chromosomal virulence cluster and positively been regulated by *prfA* gene (Portnoy *et al.*, 1992; Bannam and Goldfine, 1999). It functions to escape pathogen from primary defense of phagocytic vacuole (Kiasrsfel *et al.*, 1994, Marquis *et al.*, 1997), as well as from double membrane secondary vacuole (Smith *et al.*, 1995), and assist in cell to cell spread (Kiasrsfel *et al.*, 1994) and hence noted as essential virulence factor (Moser *et al.*, 1997).

***hly* (previously called *hly A* and *lis A*)**

hlyA gene is known for encoding Listeriolysin O (LLO). LLO act by pore forming toxin, for lysing the vacuolar membrane in the host cell, resultant evading *L. monocytogenes* escapes from vacuole (Lhopital *et al.*, 1993). Probably for the first time, Paziak – Domanska *et al.*, (1999) standardized the PCR protocol for *hlyA* gene of *L. monocytogenes* having total amplicon of 731 base pairs. Further it has been proven by nested PCR and *Hind I* digestion to strongly support the primers and its specificity.

***actA*(previously called *prt B*)**

In a recent research titles, sure attention has been put forward towards *actA* gene as it is essential for *L. monocytogenes* transmission from one cell to another by formation of listeriopods. This gene function in a way that pathogens avoid direct contact with antibodies or other immuno agents and reaches safety to neighboring cells (Kocks, 1992).

***iap*(Invasion associated protein)**

Invasion associated protein gene (*iap*) found to be involved in cell division of *L. monocytogenes* and also been the important protein in phagocytosis of *L. monocytogenes* (Kuhn and Goebel, 1989). Any mutation in 'iap gene' may lead to the decrease in invasiveness of *L. monocytogenes* which makes it important in survival of this pathogen in host (Sun *et al.*, 1990). As gene *iap* expression is not dependent on *prf A* (Bubert *et al.*, 1997) but certainly controlled by post transcriptional level (Kohler *et al.*, 1990). It is important to note when designing PCR primer for *Listeria* sp with *iap* gene is that, gene showcase sequence variation at species level (Bubert *et al.*, 1992).

***prfA* (Positive regulatory factor A)**

Presence of *prfA* protein has been related with gene expression regulation of several virulence gene expression, such as LIPI-1 gene, *inlA*. Not only is that, *prf A* found to be negatively regulated some of the *L. monocytogenes* genes like *clpC* who acts in stress response mediator gene and motility associated genes *motA* and *flaA*.

Multiplex PCR targeting virulence marker genes

In an order to understand pathogenicity and identification of *L. monocytogenes* number of virulent genes such as *hly A* encoding Listeriolysin O (LLO), invasion associated p60 protein (*iap*) gene (Kohler *et al.*, 1990), listeriolysin O (*hlyA*) (Bessesen *et al.*, 1990; Border *et al.*, 1990; Deneer and Boychuk, 1991; Rossenet *al.*, 1991), the *iap* gene (Jatonet *al.*, 1992 and Kohler *et al.*, 1990) and the *plc A* gene has been targeted.

In number of food samples, *L. monocytogenes* *prf A* and *hly A* gene has been distinctly marked by PCR to detect contamination (Niederhauser *et al.*, 1992, Wernars *et al.*, 1992) and LLO gene (*hly A*) was successfully used in case of low copy number of bacteria in sample (Wiedmann *et al.*, 1994). Another gene *actA* found to be important to detect by PCR especially in *L. monocytogenes* which many times showcase sequence variability (Moriishi *et al.*, 1998).

For the first time, Lawrence and Gilmour (1994) provided the success of multiplex PCR which is targeting virulence genes of *L. monocytogenes* in a single reaction. Along with that, genes like *iap*, *prf A* and *hlyA* found to be superior in multiplex PCR (Almeida and Almeida, 2000). Wesley *et al.*, (2002) sensibly used multiplex PCR to target 16s rRNA region of 938 bp along with 174 bp of listeriolysin

(*hlyA*) of *L. monocytogenes*. In a set of PCR, this two amplicon acted as successful model to recognize *Listeria* species and their serotypes successfully.

Multiplex PCR targeting virulence marker genes in bovine environment, Animal clinical cases and milk

For the first time, Cooray *et al.*, (1994) reported the multiplex PCR for *L. monocytogenes* for three genes *prfA*, *hlyA* and *plcA* successfully and then in 1995 Rasmussen *et al.*, reported that *L. monocytogenes* could be divided into three lineages amplification and subsequent determination of sequence variation of gene *hly*. Use of RFLP-PCR in the virulence gene *hlyA* detection and by also ribotyping found to be successful as reported by Wiedmann *et al.*, (1997).

Multiplex PCR also assists in understanding maximum likelihood phylogenies of several genes especially to classify *L. monocytogenes* into two deeply separated evolutionary lineages by targeting genes *gap*, *prs*, *purM*, *ribC*, *sigB*, *actA* and *inlA* (Nightingale *et al.*, 2005).

Presence of *L. monocytogenes* in milk, bulk tank, swabs, cheese, feed, water, environment and faeces was confirmed by PCR techniques along with many species such as *L. innocua*, *L. grayi*, *L. welshimeri* and others (Atil *et al.*, 2011). Gelbicova and Karpiskova, (2012) successfully PCR amplified 11.2% virulent *L. monocytogenes* from environmental samples for several virulence genes *prfA*, *hlyA*, *actA*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ*. Similar to that Meloni *et al.*, (2012) carried out multiplex PCR to target *prfA*, *hlyA*, *rrn*, *inlA*, *inlB*, *actA*, *mpl*, *iap*, *plcA* and *plcB*. Park *et al.*, (2012) identified 35 *L. monocytogenes* present in animals, foods, environmental samples by carrying out multiplex PCR, and

detected six virulence associated genes (*inlA*, *inlB*, *plcA*, *plcB*, *hlyA* and *actA*) along with 16S rRNA targeting. Usman *et al.*, (2016) identify virulence associated genes such as *prfA*, *inlA*, *hlyA*, *actA* and *iap* from milk and milk products in Nigeria.

In India, recently Kaur *et al.*, (2006) targeted *L. monocytogenes* genes like *iap*, *actA*, *hlyA*, *plcA* and *prfA* by multiplex PCR with samples of abortions in human and found to be 5 genes positive in two samples out of 305 tested. Shakuntala *et al.*, (2006) also found to be successful in targeting virulence associated genes *prfA*, *plcA*, *hlyA*, *actA* and *iap* in variable source samples containing *L. monocytogenes* and other species and most of them found to be positive for five virulence associated genes. In a similar attempt number of worker successfully demonstrated this approach can detect *L. monocytogenes* (Parihar *et al.*, 2007; Rawool *et al.*, 2007; Yadav *et al.*, 2010; Shoukat *et al.*, (2014); Nayak *et al.*, (2015); and Sawant *et al.*, 2016).

Serotyping PCR

It is noted that no direct link could be made among particular forms of listeriosis and certain serotypes, till the Gellin *et al.*, (1991) put forward some relation between perinatal listeriosis and serovars 1/2b, 3b and 4b.

By multiplex PCR, Doumith *et al.*, (2004) first time separated *L. monocytogenes* serovars from food and patients into distinct groups (1/2a, 1/2b, 1/2c and 4b), Yde and Genicot (2004) studied 48 strains of *L. monocytogenes*, out of them 26 belonged to serovar 4b, another 18 to serovar 1/2a and remaining four to serovar 1/2b.

According to Kessel *et al.*, (2004) tank milk samples from farms in United States found to be positive for *L. monocytogenes* (6.5%) of total tested 861 samples and by serotyping,

five serotypes found to be dominating 1/2a, 1/2b, 3b, 4b and 4c. Esteban *et al.*, (2009) reported that fecal samples of animal herd found to be dominating in serotype 4b (84.2%) followed by 1/2a (13.2%). Gebicova and Karpiskova (2012) confirmed the serotype 1/2a as dominating in *L. monocytogenes* recovered from wild, farm environment and vegetation. Centinkaya *et al.*, (2014) when tested raw milk, dairy products and food products like chicken meat, sea food and raw vegetables presence of *L. monocytogenes* was confirmed with dominating serotype 1/2a, 1/2b, 1/2c and 4b in descending order when serotyped by multiplex PCR.

Arora *et al.*, (2009) reported by multiplex PCR assay that milk and milk product isolated *L. monocytogenes*, found to be dominating (72%) with serovar group 4b, 4d, 4e 22% (4/18) to serovar group 1/2b, 3b and 5.5% (1/18) to serovar group 1/2a, 3a. Similar report published by Sonegaonkar (2009) recorded that food of animal origin *L. monocytogenes* majorly belongs to serovar 4b group. In several samples, animal origin, raw poultry meat found to be mainly positive with serovar 4b.

Mastitic milk samples also found to be positive for *L. monocytogenes* and when serotyped by multiplex PCR, all of them serotyped for 4b (Yadav *et al.*, 2010). Dixit (2013) tested 150 clinical samples and successfully detected *Listeria* species with majority of them serotyping positive for 4b. Raorane *et al.*, (2014) when tested 215 samples clinical (n=182) and farm environment (n=33) most of them found to contain *L. monocytogenes* dominating with serotype 4b and 1/2b. Negii *et al.*, (2015) when tested food, human and animal clinical cases, three serotypes found to be dominating in *L. monocytogenes* which are 4b, 4d, 4e followed by 1/2a, 3a and last by 1/2b and 3b serogroups.

Pulse field gel electrophoresis

In a diagnostic epidemiological approach detection of *L. monocytogenes* is possible by number of molecular techniques like Random Amplification of polymorphic DNA (RAPD), Microarray analysis, Amplified Intergenic Locus Polymorphism (AILP), Multilocus Enzyme Electrophoresis (OIE, 2008). Along with that, Genomic macro restriction endonuclease digestion followed by pulse field gel electrophoresis is becoming popular assay to detect number of food pathogens in several applications as suggested by Graves and Swaminathan (2001); Fugett *et al.*, (2007). This technique certainly making its presence owing to higher discriminatory power, accuracy and proven to be useful in surveillance and control of listeriosis (Graves and Swaminathan, 2001; Jeffers *et al.*, 2001).

As it is known *L. monocytogenes* represents number of clonal group which can have different pathogenic potential (Jeffer *et al.*, 2001). In requirement, power PFGE certainly been useful to study epidemiological aspect of *L. monocytogenes* (He *et al.*, 2008; Felix *et al.*, 2012; Fox *et al.*, 2012). It has also been successful to track the *L. monocytogenes* contamination in food source (Fugett *et al.*, 2007; Conter, 2008). Zhang *et al.*, (2007) performed the PFGE analysis of *L. monocytogenes* isolated from retail ready to eat meat fresh produce and raw chicken along with techniques like PCR.

Wojciech *et al.*, (2004) put forward the success of PFGE with detection of *L. monocytogenes* in food stuff and animal clinical cases. They recorded that ERIC-PCR and PFGE has different genotypes of *L. monocytogenes* from food derived samples than the infected animal samples. Vela *et al.*, (2001) studied *L. monocytogenes* with PFGE when they were isolated from sheep, cattle,

feed stuffs and from human. They recorded 84 animals, 51 human and total 18 food strains showcasing 31, 29 and 7 different pulsotype, respectively, which has indicated a great genetic diversity among Spanish *L. monocytogenes*.

Borucki *et al.*, (2004) demonstrated that PFGE could be useful in comparative studies of *L. monocytogenes* originating from different sources like human and animals dairy associated strains such as milk, environment and bovine they recorded that 23% of human sporadic strain had PFGE pattern common to that of farm isolates which makes PFGE important diagnostic or surveillance tool. Borucki *et al.*, (2005) put forward the use of PFGE in testing transmission of listeriosis from one farm to another and also reported that fecal samples found to be positive for most of samples of cow and serotype 1/2a reported most of the time. Okwumabua *et al.*, (2005) put forward that isolated *L. monocytogenes* from animal clinical samples of mastitis, abortion, meningitis and from food could be analyzed by PFGE. They reported that a caprine, two bovine and an ovine brain isolate resulted in identical PFGE banding pattern which gives a theory that available strains of *L. monocytogenes* are not host specific. Further by dendrogram of PFGE pattern isolates clustered majorly with respect to serotype.

Negi *et al.*, (2015) profiled PFGE of human and animal clinical samples collected from different geographical locations for *L. monocytogenes*. They recorded 17 pulsotypes among 36 *L. monocytogenes* recovered from animal, human and clinical cases and foods with 6 major clusters having similar finger print profile and within them 11 unique cluster finger print profile was recorded. Hence, they recommended PFGE analysis in food of animal origin for infection source detection. According to Barbudhhe *et al.*, (2016)

majority of the serotype 4b strains from India were clonal, and altogether 68.80% serotype 4b strains exhibited identical pulsotype for the samples isolated from animals, humans, food and environment.

Biofilm production of *L. monocytogenes*

Biofilm production is considered as one of the major weapons of pathogen defense as evidenced in following reports. Norwood and Gilmour, (2001) reported that biofilms are surface associated, sessile bacterial communities. This film confers protection against physical and chemical stresses. In human many chronic illness are reported with biofilm formation and hence major focus has been put forward for the genes involved in such activity (Parsek and Singh, 2003). Vatanyoopaisarn *et al.*, (2000) firstly related flagella of *L. monocytogenes* on initial attachment to stainless steel. They recorded this mechanism assist in early stage of attachment. Lemon *et al.*, (2007) also reported that flagellum mediated motility of *L. monocytogenes* is responsible for the both initial surface attachment and thereafter biofilm formation.

As per reports, it is important to detect ability to form biofilm and Djordjevic *et al.*, (2002) designed a polyvinyl chloride (PVC) microfilter plate assay which can test biofilm formation by *L. monocytogenes*. They successfully demonstrated quantitative biofilm formation by epifluorescence microscopy. Borucki *et al.*, (2003) further related *L. monocytogenes* in relation to biofilm formation, phylogenic division and persistence in the environment. They have observed that serotypes 1/2a and 1/2c showcase increased biofilm formation and persistent strains majorly showing increased biofilm formation. Stepanovic *et al.*, (2004) estimated biofilm formation on plastic with *L. monocytogenes* and observed that nutrient content of the

medium governs the quantity of biofilm production. Di Bonaventura *et al.*, (2008) related *L. monocytogenes* biofilm with material and temperature. They recorded that biofilm level is on higher side in glass material with incubation at 4, 12 and 22°C as compared to polystyrene and stainless steel. Similarly when culture allowed to grow at 37°C, *L. monocytogenes* produced higher level of biofilm on glass and stainless steel, as compared to polystyrene material.

Dubravk *et al.*, (2007) put up a relation between temperature and medium used for biofilm production. They tested *L. monocytogenes* strains growing on 1/20 diluted tryptophan- soy broth with yeast extract (TSB-YE), Brain-heart infusion (BHI) and tryptophan- soy broth with yeast extract at temperature 4 °C, 25 °C and 37 °C. As per report no significant change occurs with biofilm production even by changing medium and temperature which highlighted independence of this medium or temperature for biofilm formation. Harvey *et al.*, (2007) used microtiter plate assay to quantify biofilm production of 138 strains of *L. monocytogenes* isolated from environment animal food and clinical were classified (92.0%) as weak, (6.5%) as moderate and (1.5%) as strong biofilm farmers.

Looking at these features, microbial biofilms put up serious questions in clinical and industrial settings and majorly to food processing environments which may lead to food contamination and spoilage and that certainly will assist in transmission of pathogens (VanHoudt and Michielis, 2010). In a view to monitor the food contamination by biofilm, Latorre *et al.*, (2010) demonstrated the use of scanning electron microscopy (SEM) by collecting small pieces like milk meter and rubber liners of tankers and exposing it in SEM. This approach is successful in milk industry for regular

monitoring (Latorre *et al.*, 2010).

Borges *et al.*, (2011) simulated vaginal fluid maintained at pH 4.2, 5.5, and 6.5 and observed that *L. monocytogenes* can produce biofilm but highest was produced in nutrient rich medium. Slama *et al.*, (2012) related activity of biofilm formed by *L. monocytogenes* when isolates were given before and after cold stress (-20°C). They observed that cold stressed strains were more adhesive to material like polyethylene; polyvinyl chloride, glass and even stainless steel. Barbosa *et al.*, (2013) mentioned that clinical isolates of *L. monocytogenes* are strong biofilm producer than food isolates when tested in laboratory condition at 37°C for 24 hours using polystyrene tissue culture plates.

With microtiter assay, *L. monocytogenes* isolated from minced meat/chicken showed stronger biofilm producing ability as compared to isolates from sausages when tested in laboratory conditions for 24 h at 20°C on polystyrene surface (Fouladynezhadet *al.*, 2013).

Sonegaonkar (2009) confirmed the *L. monocytogenes* isolated from human, raw poultry, fish and milk samples are majorly biofilm producers. Gurjer (2010) also confirmed by multiplex PCR and by CRA method that virulence and biofilm production is positive in poultry isolates, human isolates and milk isolates.

Doijad *et al.*, (2015) related serotypes with biofilm formation and with micro titer plate assay. Majority of clinical and food source *L. monocytogenes* are weak, biofilm producer and hence no firm correlation was recorded with serotype and respective biofilm formation capability.

Biofilm associated genes (*lux S* and *fla A*) of *L. monocytogenes*

Inactivation of flagellar gene *flaA* has been related with impairment of biofilm formation and initial attachment in *L. monocytogenes* (Kumar and Anand, 1998). In response Gray and Kroll (1995) firstly reported the standardized PCR method which can distinctly amplify internal fragment of *fla A* gene of genus *Listeria* which allows specific genus discrimination between other Gram-positive and Gram-negative bacteria and other *Listeria* species.

By using PCR technique, Strachan and Gray (1995) amplified a 200 mer fragment of the *flaA* gene by incorporating biotin and fluorescein amidite (FAM) - labelled primers as one of the success to diagnose *L. monocytogenes*.

Another gene *luxS* has been found to be involved in repression of components that take part in attachment of biofilm formation and this is confirmed when *L. monocytogenes luxS* strain produce denser biofilm than normal to a glass surface as recorded by Sela (2006). Many studies confirm that *lux S* gene is present virulent *L. monocytogenes* along with *flaA* gene (Gurjer, 2010; Kumar *et al.*, 2009).

Disinfectant resistance of *L. monocytogenes*

Presence of *L. monocytogenes* in animal farm environment and animal clinics with disinfectant resistance is not so well studied. It is also not so clear about the exact mechanism of resistance present in *L. monocytogenes*.

To tackle the power of sanitizers, probably *L. monocytogenes* uses efflux pump which can transport a range of structurally dissimilar compounds. As per Toet *et al.*, (2002) very little is known about regulation of these transporters at a level of gene expression in *L. monocytogenes*.

In number of resistance assay of disinfectant successor *L. monocytogenes* does found to be winning the race. According to Best *et al.*, (1990) out of 14 disinfectants, only 3 disinfectants (providone-iodine, chlorhexidine, gluconate and glutaraldehyde) were found to be effective against *L. monocytogenes* in a mass screening. Aase *et al.*, (2000) recorded that out of 200 *L. monocytogenes*, only 10% were reported resistant to benzalkonium chloride (BC). This resistant has been linked with *apmf*- driven efflux pump.

Efflux pump and disinfectant resistance gene

Mata *et al.*, (2000) probably for the first time reported chromosomal gene (*mdr L*) in *L. monocytogenes* L028, resembling with multidrug efflux transporters of the major facilitator super family. In the same year, Mereghetti, (2000) detected *mdrL* gene in *L. monocytogenes* which codes multidrug efflux pump and another gene *orf A* which act as transcriptional repressor of *mdr L*. Thereafter, Romanova *et al.*, (2006) further confirmed *mdrL* gene with sanitizer and antibiotic resistance as it encodes efflux pump which has been related to quaternary ammonium compounds. These genes are encoded by both chromosomal as well as plasmid. This *mdrL* efflux pump has been confirmed by the Mullapudiet *et al.*, (2008) also which can act in extrusion of toxic ions such as BC, ethidium bromide and heavy metals.

Another gene *Sig B*, encoding major transcriptional regulator of stress response gene found to be associated with resistance to disinfectant via continuous formation of biofilm as reported by Van der veen and Abee (2010). According to Rakic-Martinez *et al.*, (2011) efflux pump once gets expose to BC or certain antibiotics or heavy metals, it gets sensitize and starts removing the ions that sensed as a toxic.

Real time PCR assay (q PCR)

It is always been suggested to follow traditional microbial methods to detect *Listeria spp.* which involves pre enrichment followed by isolation on solid/liquid medium and confirmation by biochemical methods (Jasson *et al.*, 2010, Mandal *et al.*, 2011). However, as the technology advances have been experienced towards more specific and sensitive PCR technology to identify organisms at molecular level.

Number of researchers successfully demonstrated the use of real PCR assay to detect *L. monocytogenes* in number of samples. They have also successfully discriminated the close *Listeria* species by targeting *hlyA*, *iap* gene for *L. monocytogenes* (Lazaro *et al.*, 2004; Guilbaud, 2005). Burbano, (2006) developed a PCR technique which can detect as low as 10 CFU/ml of *L. monocytogenes* in raw milk DNA with the amplicon of 938 bp and 750 bp and it certainly remained genes and species specific. Rossmanith *et al.*, (2006) utilizes combine/real time PCR method for the detection of *L. monocytogenes* by targeting universal marker gene *prfA* for *L. monocytogenes*. They detected as low as 7.5 CFU/25 ml in raw milk, minimum 1CFU/15g of cheese and its relative accuracy stands up to 96% with relative specificity 100% and relative sensitivity, 76.9%. In another approach, Yanget *al.*, (2007) adapted a method of combining nanoparticles based immunomagnetic separation (IMS) with the use of real time PCR to detect *L. monocytogenes*. The method successfully detected milk sample with *L. monocytogenes* as low as $\geq 10^2$ CFU/0.5 ml and CT value found to be recording 1.5 to 7 times higher value than those derived from plate count suggested its success.

O'Grady (2008) detected the *ssrA* gene by using real time PCR by amplifying its 162 bp

fragment available in *L. monocytogenes*. The success lies in the fact that only 1-5 CFU of *L. monocytogenes* per 25 gram/ml of food sample in 30 hour could be detected especially in soft cheese, meat, milk, vegetables and fish. Omiccioli *et al.*, (2009) developed the multiple PCR based platform which can detect along with *L. monocytogenes*, pathogens like *Salmonella sp.*, *E. coli*. This developed multiplex real time PCR utilizes either dual labelled probes (m RT-PCR) or melting curve analysis (m HRH) with the detection limit of 1 CFU for each pathogen in a total of five with 25 ml aliquots of raw milk.

Vanegas *et al.*, (2009) successfully used real time PCR to detect *L. monocytogenes* in raw milk by targeting 149 bp fragment of metalloprotease (*mpl*). They reported the success of real time as out of 81 samples, real time PCR confirmed 21 positive while, only 13 reported positive by conventional method.

In number of reports, success of real time has been highlighted use of qPCR found to be useful in food and clinical sample was demonstrated by Alessandria *et al.*, 2010; Piednoir *et al.*, 2013; Wang *et al.*, 2014. Real time PCR method for detection of *L. monocytogenes* in chilled pork was successfully demonstrated which need no prior enrichment. Amagliani *et al.*, (2012) showcase the success of multiplex real time PCR assay as compared to culture based approach to detect *L. monocytogenes* infarm quality assurance program. Claytonet *al.*, (2011) developed real time PCR assay which can detect LLS-positive *L. monocytogenes* by targeting *lls X* gene. The test is highly specific with detection limit of 1 CFU of an LLS-positive strain 25 g/ml of spiked foods in less than 30 h. when this assay was coupled with culture enrichment. Wang *et al.*, (2011) and Liu *et al.*, (2012) used real time PCR to detect *L. monocytogenes* in simulated milk samples by targeting *hlyA* gene involving pair of

primers and Taq-Man probe for qualitative detection. The test is highly specific to *L. monocytogenes* with sensitivity of assay lies with 9 copies per PCR reaction. Amagliani *et al.*, (2012) confirmed the higher sensitivity of real time PCR as compared to culture approach especially to detect *L. monocytogenes*, *E. coli* and *Salmonella* sp in bulk tank milk, in-line milk filters, manure and faeces. Success of real time PCR once again evidenced, when Dadkhah *et al.*, (2012) demonstrated real time PCR superiority compared to plating method. Where plate count can detect minimum 1.58 to 1.58x10⁷ CFU/ ml in milk sample and by PCR it can detect as low as four copies of *iap*-gene which delivers the sensitivity and specificity of real time PCR. Dehkardi *et al.*, (2013) demonstrated series of success in detection of *L. monocytogenes* in milk, faeces, vaginal swab and urine samples when tested for conventional cultural method, conventional PCR and light cyclers real time PCR. They demonstrated 8.53% positive results for cultural, 9.19% for conventional PCR and 11.96% positive results with same samples for real time PCR which put forward the defined success of real time PCR.

Singh *et al.*, (2011) utilized the power of duplex real time PCR assay which could simultaneously detect *L. monocytogenes* by targeting *hly* gene, respectively. The protocol could detect as low as 3 and 4 CFU per ml of *L. monocytogenes* and *Salmonella* sp without any pre-enrichment required. However, with pre-enrichment this limit improves upto 1 CFU per ml of pathogens.

Overall it has been evidenced that by targeting the virulence associated genes in real time PCR fast, accurate, sensitive and specific detection of *L. monocytogenes* is possible and may be extended for diagnosis of *L. monocytogenes* in milk and clinical samples.

The study of incidence of *Listeria monocytogenes* in bovines and their environment provide information about contamination status of milk, animal clinical cases and bovine environment. The presence of *L. monocytogenes* in bovines and their environment could be a potential risk for animals and human beings. Further strategies to reduce the occurrence of the organism in bovine environment are required to overcome the contamination.

Table.1 Serovar grouping of the isolates

Sr. No	Serogroup	Milk	Animal Clinical Cases	Environment	Total
1	4b,4d,4e	2(M8,M14)	-----	1(E3)	3
2	1/2a,1/2c,3a,3c	----	-----	1(E16)	1
3	1/2b,3b,4b,4d,4e	10(M1,M2,M5,M6,M7,M9,M11,M13,M16,M17)	14 (ACC-2,4,5,6,8,9,10,11,12,13,14,15,16,17)	10(E5,E9,E11,E12,E13,E14,E15,E18,E24,E25)	34
	Total	12	14	12	38

Plate.1 Agarose gel showing Multiplex PCR serotyping for determination of the serovargroups of isolates obtained from bovine environment, animal clinical cases and milk . Lane1: isolates E-16 *L. monocytogenes* serogroup1/2a ;Lane 2: *E coli*- Negative Control Lane3: 100 bp DNA ladder; Lane 4 : isolates M-5 *L. monocytogenes* serogroup1/2b and 4b ; Lane5: isolatesACC-3 *L. monocytogenes* serogroup 1/2band 4b ; Lane6 : isolate-E-3 *L. monocytogenes* serogroup 4b ; Lane7: *L.monocytogenes*EGDe as a standard for serotypes 1/2a; Lane8 : isolate M-14 *L. monocytogenes* serogroup 4b.

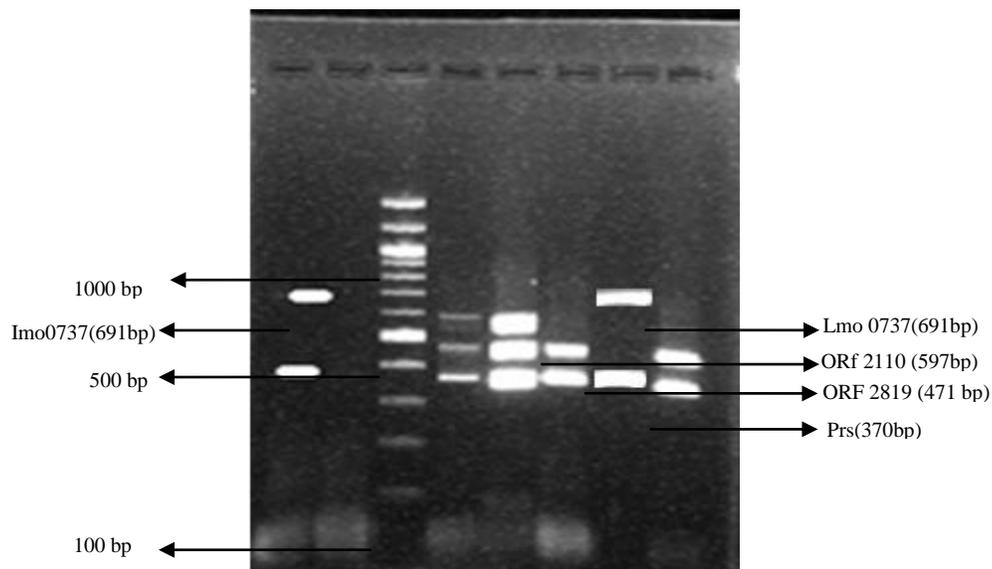
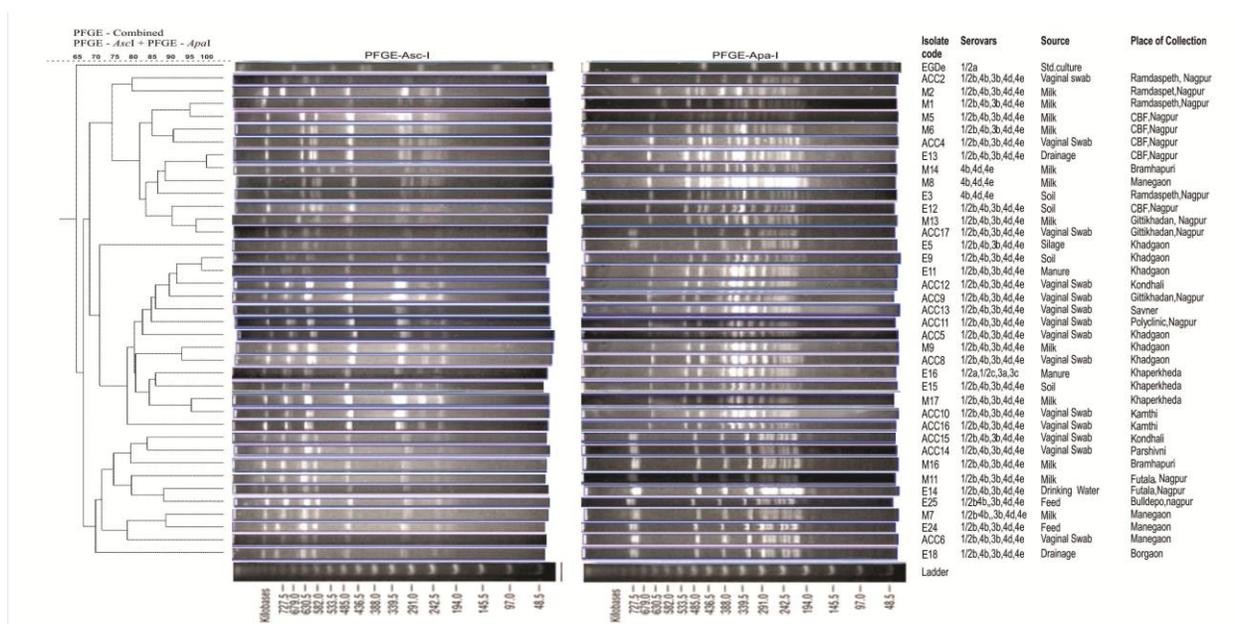


Plate.2 Dendrogram derived from PFGE Profile of *AscI* and *ApaI* macrorstriction showing restriction pattern similarity among 38 *L. monocytogenes* bovine environment, animal clinical and milk isolates with strain *EGDe*.



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