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Pregnancy - associated human listeriosis: Virulence and genotypic analysis of *Listeria monocytogenes* from clinical samples

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Listeria monocytogenes, **a life-threatening pathogen, poses severe risk during pregnancy, may cause abortion, fetal death or neonatal morbidity in terms of septicemia and meningitis. The present study aimed at characterizing *L. monocytogenes* isolated from pregnant women based on serotyping, antibiotic susceptibility, virulence genes, *in vivo* pathogenicity test and ERIC- and REP-PCR fingerprint analyses. The results revealed that out of 3700 human clinical samples, a total of 30 (0.81%) isolates [12 (0.80%) from placental bit (1500), 18 (0.81%) from vaginal swab (2200)] were positive for *L. monocytogenes*. All the isolates belonged to serogroup 4b, and were + ve for virulence genes tested i.e. *inlA*, *inlC*, *inlJ*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap*. Based on the mice inoculation tests, 20 isolates showed 100% and 4 isolates 60% relative virulence while 6 isolates were non-pathogenic. Moreover, 2 and 10 isolates were resistant to ciprofloxacin and cefoxitin, respectively, while the rest susceptible to other antibiotics used in this study. ERIC- and REP-PCR collectively depicted that the isolates from placental bit and vaginal swab had distinct PCR fingerprints except a few isolates with identical patterns. This study demonstrates prevalence of pathogenic strains mostly resistant to cefoxitin and/or ciprofloxacin. The results indicate the importance of isolating and characterizing the pathogen from human clinical samples as the pre-requisite for accurate epidemiological investigations.**

Keywords: antibiotic susceptibility, serotype identification, virulence genes, mice virulence assay, ERIC- and REP-PCR

Introduction

The genus *Listeria* includes following species, namely, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi*, *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, and those recently added (*L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, *L. grandensis*, *L. booriae*, and *L. newyorkensis*) (den Bakker *et al*., 2014; Weller *et al*., 2015). Among these, *L. monocytogenes* is primarily pathogenic to humans in causing listeriosis. However, very rarely, *L. ivanovii* and *L. seeligeri* have also been associated with human infection (Guillet *et al*., 2010; Poulsen and Czuprynski, 2013). *L. monocytogenes* is ubiquitous and survives a wide range of pH, salt, and temperature. Its commonly contaminates raw products and also other food items through cross-contamination thus targeting the human beings. Human listeriosis is a major public health concern due to high mortality rates (20–30% of cases), severity of the disease (abortion, stillbirth, septicemia, meningitis, and meningoencephalitis), and the predilection for pregnant women, neonates, elderly or immunocompromised people (Mateus *et al*., 2013; Kashish *et al*., 2015; Soni *et al*., 2015).

Listeriosis ranks among the most frequent causes of death and attributes to about 1,600 cases and 255 deaths in United States annually (Scallan *et al*., 2011; Datta *et al*., 2013). Pregnant women are 18-times more prone to listeriosis than non-pregnant counterpart which is apparently due to decrease in cell-mediated immune function wherein placenta provides a protective niche for *L. monocytogenes* (Jackson *et al*., 2010; Lamont *et al*., 2011; Awofisayo *et al*., 2015). In a survey by WHO (2004), 20 countries with 782 listeriosis cases revealed that 43% of the infections were related to pregnancy, 29% to septicemia, 24% to central nervous system infection, and 4% to atypical forms of the infection. Recently, the percentage of pregnancy-associated listeriosis from *Listeria* infection is reported as 11%, 12%, 16.9%, and 17.7% from Italy, United Kingdom, United States, and France, respectively (Jackson *et al*., 2010; Mook *et al*., 2010; Goulet *et al*., 2012; Mammina *et al*., 2013). From India, no data are available and also a mandatory notification of listeriosis is lacking. However, only a few reports are available on prevalence of *L. monocytogenes* in human clinical samples, pregnant women with a bad obstetric history (Dhanashree *et al*., 2003; Kaur *et al*., 2007). These studies are limited to the virulence attributes, antibiotic sensitivity and subtyping of isolates, and do not provide enough evidence for the extent of infection by this pathogen in human beings.

In view of above, it seemed the prerequisite to have estimates of the disease load among the Indian population. A combination of species identification with molecular subtyping would certainly abridge isolate characterization, tracking and prevention of *L. monocytogenes*, and also help in the establishment of efficient surveillance strategy. The present study was undertaken to gain more insight into the prevalence, virulence genes and antibiotics susceptibility of *L. monocytogenes* in clinical samples of pregnant women. Mice virulence assay was also performed for more accurate as-

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sessment of the virulence potential. Further, serotyping and ERIC- and REP-PCR were used to assess the molecular clustering of *L. monocytogenes* isolates and to know about the clonal relationships among the human isolates.

Materials and Methods

Study site and sample collection

During June 2010 - November 2013, a total of 3700 human clinical samples (pregnant women with bad obstetric history like repeated abortions, still births and pre-term labour) including placental bits (n=1500) and vaginal swabs (n=2200) were collected from 4 private and 2 government hospitals, Varanasi region of the province (Uttar Pradesh), India. The selection of hospitals for the purpose of sample collection was based on the large number of outdoor patients attending for routine health check-up and gynaecological related issues. All the samples were collected aseptically, quickly transported to the laboratory under chilled condition and processed within 24 h of collection.

Identification of *L. monocytogenes*

Human clinical samples (HPb1-12 and HVS1-18) used in this study from our laboratory stock collection were examined following the standard double enrichment method of ISO 11290:1 with slight modifications (Anonymous, 1997; Soni *et al.*, 2013). Further, identification of the pathogen was confirmed by biochemical and molecular characterizations (Soni and Dubey, 2014). The 16S rRNA and *hlyA* gene sequences have been deposited to NCBI GenBank under accession number KJ 765663-KJ 765692 and KJ 883238-KJ 883267, respectively. *L. monocytogenes* strain MTCC1143, *L. monocytogenes* strain ATCC19115, *Staphylococcus aureus* strain MTCC1144 and *Rhodococcus equi* strain MTCC1135 were used as controls. All the *L. monocytogenes* isolates and control strains were preserved in tryptic soy agar slants at room temperature for use in the subsequent analyses.

Antibiotics susceptibility test

All *L. monocytogenes* isolates were tested for their susceptibility to 10 antibiotics, commonly used in veterinary and human therapy, using the disc diffusion method of Bauer *et al.* (1966). Antibiotics discs (Oxoid) with the following concentrations were used: ampicillin (A, 10 µg), chloramphenicol (C, 30 µg), ciprofloxacin (Cf, 5 µg), cefoxitin (Fox, 30 µg), co-trimoxazole (SXT, 25 µg), gentamicin (G, 10 µg), oflaxacin (Of, 5 µg), rifampicin (R, 5 µg), streptomycin (S, 10 µg), and tetracycline (T, 30 µg). The diameter of the clearance zones was recorded and interpreted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006) for Gram + ve bacteria.

DNA isolation

Chromosomal DNA was extracted from *L. monocytogenes* isolates grown overnight (37°C) with shaking (200 oscillations/min) in brain heart infusion broth (BHIB, Difco) following the protocol of QIAGEN DNeasy® Blood and Tissue kit. Harvested biomass (maximum 2×10^9 cells) was centrifuged in a microcentrifuge tube (7,500 rpm, 10 min), re-suspended in 180 µl lysis buffer [20 mM Tris-Cl; pH 8.0, 2 mM sodium EDTA, 1.2% Triton® X-100, 20 mg lysozyme (Sigma) per ml], and incubated for 30 min (37°C). Proteinase K (25 µl) and 200 µl Buffer AL (without ethanol) were added, mixed by vortexing and the mixture re-incubated at 56°C (30 min). Thereafter, 4 µl RNase A (100 mg/ml) was added and incubated (2 min) at room temperature. Pure ethanol (200 µl) was added to the sample, and mixed by vortexing. The DNA was eluted in Buffer AE and the concentration and purity determined with the help of Nano Drop spectrophotometer (ND 1000, Nano Drop Technologies, Inc).

Species- and virulence-specific genes and serogroup identification

The presence of internalin genes (*inlA*, *inlC*, and *inlJ)*, virulence-associated genes (*plcA*, *actA*, *hlyA*, *iap*, and *prfA)* and serogroup (1/2a, 1/2b, 1/2c, and 4b) were determined by multiplex PCR with slight modifications as described by Liu *et al.* (2007a), Notermans *et al.* (1991) and Doumith *et al.* (2004), respectively. The PCR products were analyzed on agarose gel (1.5%), stained with ethidium bromide, and visualized under UV transilluminator (AlphaImager EC). The details of oligonucleotide sequences (Sigma) and PCR cyclic conditions used in this study are given elsewhere (Soni *et al.*, 2013, 2014).

Mice-virulence assay

In the present investigation, 5-weeks-old female laboratory mice belonging to Parkes (P) strain, weighing 20–22 gm were used. Mice were maintained under hygienic conditions in well-ventilated rooms (23 ± 2°C) with 12 h photoperiod (8 a.m. to 8 p.m. light) and relative humidity of 50 ± 20%. Animals were provided with pellet food (Amrut Laboratory Animal Feeds) and drinking water *ad libitum*. Each group (n=5) of experimental animals were housed separately in polypropylene cages (450 × 270 × 150 mm) with dry rice husk as the bedding material. Mice virulence assay was performed with modification of the method described by Menudier *et al.* (1991). All thirty isolates tested positive for *L. monocytogenes* and were grown along with control strains (ATCC19115 and MTCC1143) for 24 h at 37°C on Brain Heart Infusion Agar (BHIA; Difco) slants and harvested in 5 ml sterile normal saline solution (NSS). Each suspension was washed by agitation with sterile pipette, and standardised turbidometrically, adjusted to McFarland nephelometric tube number 1 (approximately 3×10^8 CFU/ml) by adding either NSS or bacterial suspension. A 0.4 ml of the inoculum (~10^7 CFU/ml) was administered through intraperitoneal injection. Sets of five mice received each isolate. In each experiment, control mice were dosed with the known pathogenic bacteria (ATCC19115 and MTCC1143) with saline alone. Mice were then observed every 6 h for 72 h, and mortality recorded (Aurora *et al.*, 2008; Kaur *et al.*, 2010). General health conditions were investigated from time to time throughout the experiment. Mice were sacrificed under chloroform anaesthesia by decapitation after death and the rest living mice

were sacrificed after 72 h. Animals were maintained according to guidelines of Institutional Ethical Committee. The results are expressed as relative virulence (%) by dividing the number of dead mice by the total number of mice tested for a particular isolate.

ERIC- and REP-PCR based genomic fingerprinting

ERIC- and REP-PCR were performed as described by Rivera *et al.* (1995) and Versalovic *et al.* (1991), respectively. The amplicons were electrophoresed in 1.8% agarose gel at 60 V (6 h), stained with ethidium bromide and analyzed as described earlier (Soni *et al.*, 2013, 2014). The fingerprint pattern was measured in a Fluoro-S-Imager (Bio-Rad) and analyzed using Bionumerics fingerprint analyst (Applied Maths) software with a simple-matching similarity matrix, and the data clustered by the un-weighted pair group method with arithmetic means (UPGMA). The clustering analysis of the ERIC- and REP-PCR patterns could be affected by factors like position bias in gels, band assignment, and different settings in the BioNumerics software. Therefore, the similarity of the ERIC- and REP-PCR fingerprint profiles was calculated using the average simple-match similarity matrix and the default cluster settings of 0% optimization and 1% band position tolerance.

Results

Prevalence and antibiotics susceptibility of *L. monocytogenes*

During the three years and four months period of the present study, a total 30 (0.81%) isolates out of the 3700 human clinical samples, tested positive for *L. monocytogenes.* Out of which, 12 (0.80%) were from placental bit (n=1500), 18 (0.81%) from vaginal swab (n=2200). Further, all the isolates were tested for their antibiotic susceptibility wherein 2 isolates (from placental bit and vaginal swab each) were resistant to ciprofloxacin, and 10 isolates (4 from placental bit and 6 from vaginal swab) were resistant to cefoxitin. All the isolates, however, were susceptible to other antibiotics tested (Table 1).

Species- and virulence-specific genes and serogroup identification

Twelve isolates of *L. monocytogenes* from human placental

Table 1. Source of isolation, serogroup, resistance pattern, % virulence, ERIC- and REP- fingerprints and virulence profiles of *L. monocytogenes* used in this study

Sl. No.	Strains	Source of isolation	Date of isolation	Serogroup	Resistance Pattern	% virulence	ERIC type	REP type	Presence of following genes determined by PCR							
									inlA	*inlC*	*inlJ*	*plcA*	*prfA*	*actA*	*hlyA*	*iap*
1	HPb1	Human placental bit	15.06.2010	4b, 4d, 4e	Fox	0	XXIb	XIXa	+	+	+	+	+	+	+	+
2	HPb2	Human placental bit	02.10.2010	4b, 4d, 4e		100	XXIb	XIXa	+	+	+	+	+	+	+	+
3	HPb3	Human placental bit	08.01.2011	4b, 4d, 4e		60	XXIb	XIXb	+	+	+	+	+	+	+	+
4	HPb4	Human placental bit	18.04.2011	4b, 4d, 4e	Cf	100	XXII	XX	+	+	+	+	+	+	+	+
5	HPb5	Human placental bit	03.02.2012	4b, 4d, 4e		60	XXIa	XXIV	+	+	+	+	+	+	+	+
6	HPb6	Human placental bit	05.08.2012	4b, 4d, 4e		100	XXb	XXII	+	+	+	+	+	+	+	+
7	HPb7	Human placental bit	12.09.2012	4b, 4d, 4e		100	XXb	XXIIIa	+	+	+	+	+	+	+	+
8	HPb8	Human placental bit	16.12.2012	4b, 4d, 4e	Fox	100	ND	ND	+	+	+	+	+	+	+	+
9	HPb9	Human placental bit	19.04.2013	4b, 4d, 4e	Fox	100	XXIa	XXIIIa	+	+	+	+	+	+	+	+
10	HPb10	Human placental bit	28.05.2013	4b, 4d, 4e		0	ND	ND	+	+	+	+	+	+	+	+
11	HPb11	Human placental bit	20.07.2013	4b, 4d, 4e	Fox	0	XXc	XXIIIb	+	+	+	+	+	+	+	+
12	HPb12	Human placental bit	10.11.2013	4b, 4d, 4e		100	XXIV	XXI	+	+	+	+	+	+	+	+
13	HVS1	Human vaginal swab	13.07.2010	4b, 4d, 4e		0	XXa	XXI	+	+	+	+	+	+	+	+
14	HVS2	Human vaginal swab	06.09.2010	4b, 4d, 4e		100	XXa	XXI	+	+	+	+	+	+	+	+
15	HVS3	Human vaginal swab	09.12.2010	4b, 4d, 4e	Fox	60	XXa	XXVIII	+	+	+	+	+	+	+	+
16	HVS4	Human vaginal swab	12.02.2011	4b, 4d, 4e	Cf	100	XXV	XXVIII	+	+	+	+	+	+	+	+
17	HVS5	Human vaginal swab	18.04.2011	4b, 4d, 4e		60	ND	ND	+	+	+	+	+	+	+	+
18	HVS6	Human vaginal swab	24.01.2012	4b, 4d, 4e		100	ND	ND	+	+	+	+	+	+	+	+
19	HVS7	Human vaginal swab	03.02.2012	4b, 4d, 4e		100	ND	ND	+	+	+	+	+	+	+	+
20	HVS8	Human vaginal swab	07.03.2012	4b, 4d, 4e	Fox	100	XXIIIIa	XXV	+	+	+	+	+	+	+	+
21	HVS9	Human vaginal swab	11.05.2012	4b, 4d, 4e	Fox	100	XXIV	XXV	+	+	+	+	+	+	+	+
22	HVS10	Human vaginal swab	22.06.2012	4b, 4d, 4e		0	XXIV	XXVII	+	+	+	+	+	+	+	+
23	HVS11	Human vaginal swab	12.09.2012	4b, 4d, 4e		100	XXIIIb	XXVI	+	+	+	+	+	+	+	+
24	HVS12	Human vaginal swab	16.12.2012	4b, 4d, 4e		100	XXIV	XXV	+	+	+	+	+	+	+	+
25	HVS13	Human vaginal swab	25.03.2013	4b, 4d, 4e	Fox	100	ND	ND	+	+	+	+	+	+	+	+
26	HVS14	Human vaginal swab	28.05.2013	4b, 4d, 4e	Fox	100	ND	ND	+	+	+	+	+	+	+	+
27	HVS15	Human vaginal swab	20.07.2013	4b, 4d, 4e		0	XXV	XXVII	+	+	+	+	+	+	+	+
28	HVS16	Human vaginal swab	14.09.2013	4b, 4d, 4e		100	XXV	XXV	+	+	+	+	+	+	+	+
29	HVS17	Human vaginal swab	17.10.2013	4b, 4d, 4e		100	ND	ND	+	+	+	+	+	+	+	+
30	HVS18	Human vaginal swab	10.11.2013	4b, 4d, 4e	Fox	100	ND	ND	+	+	+	+	+	+	+	+

Cf, ciprofloxacin; Fox, cefoxitin; ND, not determined

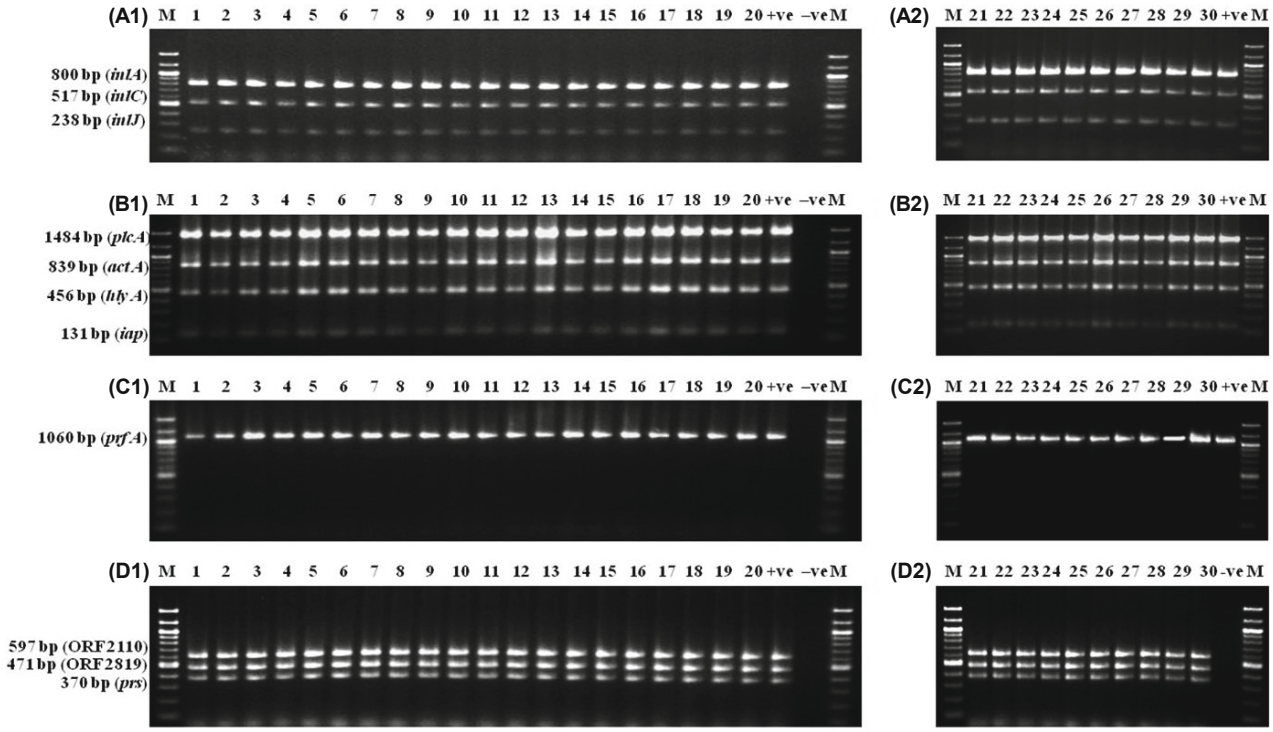

Fig. 1. (A1 and 2) Multiplex PCR amplification of species-specific (*inlA*) and virulence-specific (*inlC* and *inlJ*) genes; (B1 and 2) virulence-associated genes *plcA*, *actA*, *hlyA*, and *iap*; (C1 and 2) gene *prfA*; (D1 and 2) serogroups specific genes ORF2819, ORF2110 and *prs* from human placental bit (HPb1-12; lanes 1–12) and vaginal swab (HVS1-18; lanes 13–30) isolates of *L. monocytogenes*. +ve control MTCC 1143 and –ve control. Lane M contains 100 bp molecular size DNA ladder.

bits and 18 from human vaginal swabs were screened for the presence of virulence genes. All the 12 isolates from human placental bits tested were positive for *inlA*, *inlC*, *inlJ*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap* genes. Similarly, all the 18 isolates from human vaginal swabs were positive for *inlA*, *inlC*, *inlJ*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap* genes (Fig. 1). In serotype specific multiplex PCR, all the isolates were positive for ORF2110 and ORF2819 genes indicating that these belonged to 4b, 4d or 4e serogroups (Fig. 1).

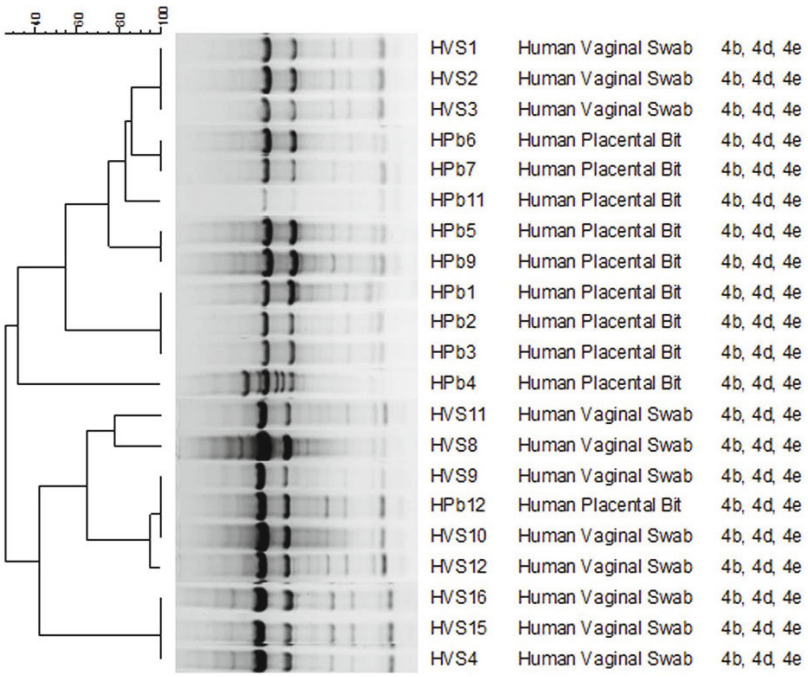

Fig. 2. DNA fingerprints generated by ERIC-PCR amplification from human placental bit and vaginal swab isolates of *L. monocytogenes*. The dendrogram was generated using the Bionumerics Fingerprint Analyst Software (Applied Maths), and data clustered by the unweighted pair group method with arithmetic means. Similarity of the ERIC-PCR fingerprint profiles was calculated using the average simple-match similarity matrix and the default cluster settings of 0.00% optimization and 1.00% band position tolerance were used.

Mice virulence assay

Thirty isolates from human clinical samples assessed by mice inoculation test. Among these isolates 20 and 4 showed 100% and 60% relative virulence within 72 h, respectively. The remaining 6 isolates were non-pathogenic to mice. The relative virulence for individual isolates from placental bit and vaginal swab human samples is shown in Table 1.

ERIC- and REP-PCR fingerprint analysis

ERIC-PCR of genomic DNA from *L. monocytogenes* isolates from human placental bit (10 isolates), and vaginal swab (11 isolates) showed a total of seven fingerprint profiles (profiles XX through XXV), not described earlier. The number of bands consists of 5 to 11 ranging from 500 and 5200 bases (Fig. 2). Four fingerprint patterns were characteristics of 10 isolates from the placental bit, while one had a distinct fingerprint pattern. Although 9 isolates from the vaginal swab yielded three fingerprint profiles ranging from identical to similar and closely related patterns, 3 isolates had a separate pattern analogous to that of the placental bit isolates. A correlation in fingerprint patterns could be seen among a few isolates of placental bit and vaginal swab.

Similarly, REP-PCR of genomic DNA from *L. monocytogenes* from human placental bit and vaginal swab showed amplification of multiple fragments of DNA consisting of 6 to 13 bands ranging from 500 to 5800 bases. Like the ERIC-profile, two major clusters comprising isolates from placental bit and vaginal swab were obtained, of which, five fingerprint profiles were shown by 10 isolates from placental bit (Fig. 3). Whereas four fingerprint patterns were shown by isolates from vaginal origin, 2 isolates had distinct fingerprint profile but identical to placental bit isolates indicating a close relationship between them. At least 2 isolates showed correlation in the fingerprint pattern between the placental bit and vaginal swab isolates.

Discussion

Pregnancy-associated listeriosis is a challenging issue not only for researchers but also for clinicians because of the asymptomatic or nonspecific clinical symptoms linked with the disease such as mild flu-like symptoms, fever, muscle ache, backache, headache, nausea or diarrhea (Lamont *et al.*, 2011; Awofisayo *et al.*, 2015). In India, the most common clinical symptom reported is genital listeriosis. It has been reported that *L. monocytogenes* is one of the major etiological factors in the causation of abortions and premature births. Our results indicate that 30 isolates from human clinical samples tested positive for internalin A (*inlA*) gene, and all of them identified as *L. monocytogenes*. The results are in agreement with our previous report on the identification of *L. monocytogenes* based on the 16S rRNA and *hlyA* gene sequence similarity (Soni and Dubey, 2014). There are very scarce reports on listeriosis from Indian human population. However, *L. monocytogenes* was isolated (0.3%) of 633, (1.31%) of 305 and (1.7%) of 300 samples from patients with poor obstetric history of abortions, miscarriages, stillbirths or neonatal deaths as reported by Dhanashree *et al.* (2003); Kaur *et al.* (2007); Soni *et al.* (2013), respectively. The findings of the present study on the prevalence of *L. monocytogenes* in human clinical samples are similar to the earlier reports 0.3% to 14% range in pregnant women. These reports highlight the importance of the pathogen as a causative agent for spontaneous human abortions in this ecozone of the world.

Under *in vitro*, *L. monocytogenes* are usually susceptible to wide range of antibiotics, however, some studies also reported increased resistance to one or several clinically relevant antibiotics (Conter *et al.*, 2009; Allerberger and Wanger, 2010; Morvan *et al.*, 2010; Lamont *et al.*, 2011). In India, Dhanashree *et al.* (2003) reported sensitivity of *L. monocytogenes* to ampicillin, ciprofloxacin, cotrimoxazole, erythro-

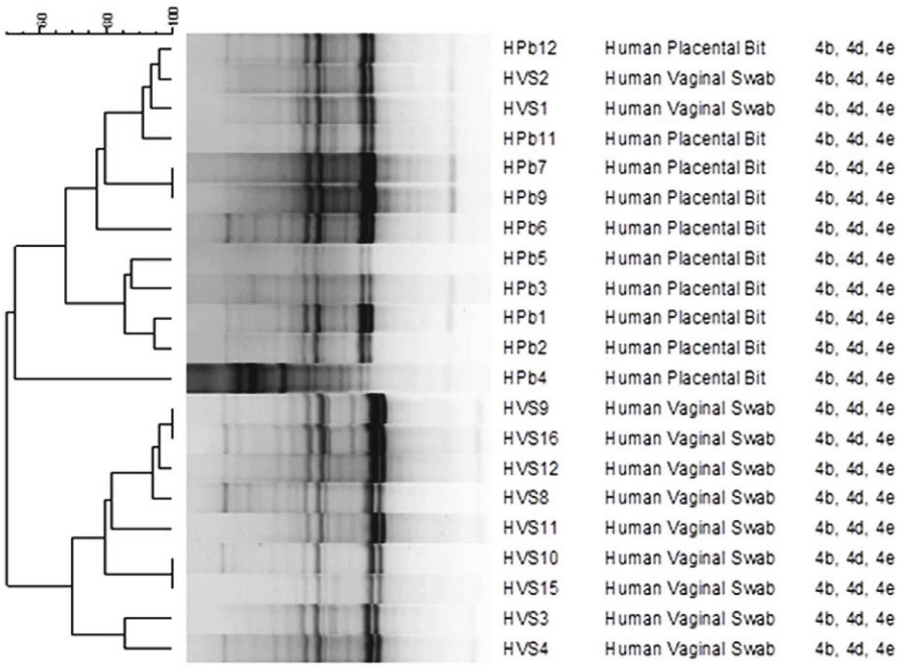

Fig. 3. DNA fingerprints generated by REP-PCR amplification from human placental bit and vaginal swab isolates of *L. monocytogenes*. The dendrogram was constructed using the Bionumerics Fingerprint Analyst Software (Applied Maths) as described in the legend of Fig. 2.

mycin, penicillin, and chloramphenicol. Sharma *et al.* (2012) and Soni *et al.* (2013) reported multidrug resistant isolates of *L. monocytogenes* from water, milk, and human clinical samples. The possible source of human exposure to multidrug-resistant bacteria is the intake of uncooked food grown in agricultural land contaminated by the disposal of untreated effluents, application of faeces or dung slurries of infected (or carrier) animals (Nightingale *et al.*, 2005; Rodas-Suarez *et al.*, 2006; Nwachukwu *et al.*, 2010). The resistance of majority of the isolates from human clinical samples to ciprofloxacin and/or cefoxitin as observed in this study, indicates the emergence of antibiotic resistance in *L. monocytogenes*. This finding is significant in context of the incidence of spatio-temporal changes in the antibiotics resistance (Lyon *et al.*, 2008; Yan *et al.*, 2010). Therefore, such studies underline the necessity for the continuous surveillance of the emerging antimicrobial resistance of the pathogen to ensure the effective treatment of human listeriosis. This study can be used to improve the background data on antibiotic resistance of strains from human clinical samples and for epidemiological and public health studies related to *L. monocytogenes*.

L. monocytogenes with several putative virulence genes associated with the infection cycle may be potentially pathogenic. The presence of genes for the expression of internalins (encoded by *inlA*, *inlC*, *inlJ*), listeriolysin O (LLO encoded by *hlyA*), actin (*actA*), phosphatidyl-inositol-phospholipase C (PI-PLC encoded by *plcA*), iap (invasion associated protein encoded by *iap*) and virulence regulator (encoded by *prfA*) have been reported as the most important virulence factors for *L. monocytogenes* (Camejo *et al.*, 2011; Poulsen and Czuprynski, 2013). The multiplex-PCR results of this study showed that all the *L. monocytogenes* isolates possessed all the virulence associated genes, including *inlA*, *inlC*, *inlJ*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap* (Table 1). The observations indicate that all of the isolates had the requisite properties of a virulent strain. The presence of either or both *inlC* and *inlJ* genes together with the other virulence genes (*plcA*, *prfA*, *actA*, *hlyA*, and *iap*) indicates its virulence potential and to cause mouse mortality via intraperitoneal route and also to cause the infections in humans.

The presence of virulence-related genes in *L. monocytogenes* isolate does not always imply the certainty of the strains to cause disease (Mammina *et al.*, 2009; Van Stelten *et al.*, 2010; Chen *et al.*, 2014). Therefore, to improve the discrimination between pathogenic and non-pathogenic isolates, mice virulence assay was performed, as it is regarded as the gold standard for pathogenicity test for bacterial isolates including *L. monocytogenes* virulence, due to its capability to provide an *in vivo* measurement of all the virulent determinants (Takeuchi *et al.*, 2006; Liu *et al.*, 2007b). In this study, relative virulence (%) based on the number of dead and total mice and independent from CFU estimation has been used. It is much more direct, precise and appears to offer the more accurate assessment of *L. monocytogenes* virulence (Takeuchi *et al.*, 2006; Liu *et al.*, 2007b). All the isolates having the virulent genes were tested, belonged to 4b, 4d or 4e serovars, though the pathogenicity rate varied among different isolates (Table 1). However, most of the isolates i.e. 24 showed pathogenicity while the remaining isolates were non-pathogenic. The results are in accordance with the previous reports indicating that only the presence of virulence associated genes does not bear correlation with the virulence of *L. monocytogenes* (Kaur *et al.*, 2007, 2010; Aurora *et al.*, 2008). Further, it is also reported that some naturally virulence attenuated *L. monocytogenes* strains (particularly those from human carriers) often contain mutations in their *prfA*, *hlyA*, *actA*, and *inlA* genes and possibly because of this reason, they express truncated or non-functional PrfA, LLO, ActA, and InlA proteins (Roche *et al.*, 2005; Orsi *et al.*, 2007; Shen *et al.*, 2013). Hence, in order to determine the virulence of *L. monocytogenes*, only PCR detection of these genes does not provide the accurate information. Therefore, an optional strategy of *L. monocytogenes* virulence testing remains the detection of virulence-specific genes with the *in-vivo* bioassays and *in-vitro* cell assays.

Serotyping along with molecular typing methods has proven their scientific utility in characterization of *L. monocytogenes* over several decades (Huang *et al.*, 2011; Chenal-Francisque *et al.*, 2013). On the basis of information available, thirteen serotypes of *L. monocytogenes* have been described so far. However, only three serotypes (1/2a, 1/2b, and 4b) cause the vast majority of human clinical cases, in which serotype 1/2a is the most frequent one from food while serotype 4b causes the majority of human epidemics (Almeida *et al.*, 2010; Mateus *et al.*, 2013). Therefore, serotype might be valuable in describing the virulence potential of *L. monocytogenes*. This study shows the prevalence of 4b serotype in human clinical samples wherein serotype 4d and 4e are relatively rare.

ERIC- and REP-PCR were used in this study to record the genetic relatedness of *L. monocytogenes* isolates from placental bit and vaginal swab samples along with their serotypes. Placental bit isolates showed identical to similar and closely related ERIC- and REP fingerprints but distinct from the vaginal swab isolates indicating the distinct association of strains in placental and vaginal swab. The fingerprints as shown by these isolates, are distinct from those reported earlier by Soni *et al.* (2013). The results of the present study corroborate with the report on 47 REP types among the 57 isolates from the human clinical samples (Chou and Wang, 2006). In the overall, there was a close correlation between the PCR methods applied and the pathogens isolated from placental bit that yielded identical to closely related fingerprint but quite distinct from vaginal isolates. The observed difference between the banding pattern of isolates from different sources suggests divergence in genomic organization that could have arisen from the genetic reassortment as applicable to different ecological niche. These findings are similar to those on the diversity of fingerprint patterns among food isolates using REP-PCR (Soni *et al.*, 2013, 2014; Chen *et al.*, 2014; Shi *et al.*, 2015) and PFGE (Fugett *et al.*, 2007; Jamali and Thong, 2014). There was no correlation observed between fingerprint profiles and the pathogenicty of bacteria, although these had similar virulence genes coding for cell invasiveness, suggesting the possibility of their uneven expression in different *L. monocytogenes* isolates. In addition, isolates with the same fingerprint profile responded differently in their antibiotic susceptibility. Furthermore, there was no correlation between serogroup and the PCR fingerprint profiles of the pathogens. These findings corroborate with

the earlier report that the same serotype could also be assigned to different molecular subtypes (Chou and Wang, 2006). Therefore, REP- and ERIC-PCR based approach seems useful in epidemiology to trace the origin of isolates and/or screening of the laboratory isolates, and to establish relationships among them, if any.

The results in the present study provide information for the presence of pathogenic as well as non-pathogenic *L. monocytogenes* in pregnant women. In general, no relationships was observed among placental bit and vaginal swab, except for a few isolates that exhibited identical patterns with distinct ERIC- and REP-PCR PCR fingerprints profiles. All the isolates of 4b serogroup, exhibited multiple antibiotic resistance and tested + ve for all the virulence genes (*inlA*, *inlC*, *inlJ*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap*) used in this study. This highlights the action needed to protect vulnerable populations specially the pregnant women. The routine screening for the prevalence of *L. monocytogenes* in environmental, food and human clinical samples will be helpful in the better understanding of the pathogen epidemiology and may prevent the recurrence of still more cases in India. Further, governmental surveillance programme is warranted to better estimate the disease burden and to the control strategies.

Competing interests

The authors have no conflict of interest.

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