Genotypic characterization of *Listeria monocytogenes* isolated from humans in India

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Listeria monocytogenes is a foodborne pathogen associated with severe diseases in humans and animals. The genotypic analysis of 17 *L. monocytogenes* isolates recovered from humans in India during 2006–2009 using multiplex serotyping PCR allowing serovar predictions, conventional serology and by pulsed field gel electrophoresis (PFGE) is presented. The isolates were recovered from patients exhibiting various clinical conditions. A multiplex-PCR based serotyping assay revealed 88.24% (15/17) of the strains belonging to the serovar group 4b, 4d, 4e and 11.76% (2/17) to the serovar group 1/2b, 3b. Conventional serology indicated that 13 (76.47%) *L. monocytogenes* isolates to be of serotype 4b, 2 (11.76%) serotype 4d, and 2 (11.76%) serotype 1/2b. Ten *ApaI* and nine *AscI* pulsotypes were recognized among the 17 human isolates. PFGE analysis allowed discrimination among isolates of the same serotype and among isolates from the same sampling areas or those isolated from different areas. Thus, PFGE together with multiplex-PCR serotyping allows rapid discrimination of *L. monocytogenes* strains. In addition, the predominance of *L. monocytogenes* serotype 4b is of concern, as this serotype has been most frequently associated with human listeriosis outbreaks.

Listeria monocytogenes, a cause of listeriosis, is a Gram-positive, facultative intracellular pathogen capable of causing severe invasive disease in animals and humans. The genus *Listeria* consists of six species namely, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. grayi* and *L. welshimeri*. Recently, two new species of *Listeria*, *L. marthii* (Graves *et al.*, 2010) and *L. rocourtiae* (Leclercq *et al.*, 2010) have been described. *L. monocytogenes* and *L. ivanovii* are pathogenic species of public health and veterinary significance (Guillet *et al.*, 2010). *L. monocytogenes* can cause serious diseases,

such as septicemia, meningitis, gastroenteritis, meningoencephalitis in immunocompromised individuals, newborns and the elderly; and abortion and stillbirth in pregnant women (Vazquez-Boland *et al.*, 2001; Barbuddhe and Chakraborty, 2009).

L. monocytogenes strains are serotyped according to variation in the somatic (O) and flagellar (H) antigens. *L. monocytogenes* isolates have been assigned to their lineages using a range of genotypic and phenotypic approaches, including ribotyping, pulse-field gel electrophoresis (PFGE), and multi-locus sequence typing. *L. monocytogenes* can be classified into four lineages: lineage I encompasses serotypes 1/2b, 3b, 4b and 3c; lineage II includes serotypes 1/2a, 1/2c, 3a, lineage III comprises serotypes 4a, 4b and

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4c and lineage IV comprises 4a, 4b, 4c (Orsi *et al.*, 2011). Several molecular genotyping methods such as DNA restriction endonuclease analysis, ribotyping, multilocus enzyme electrophoresis and PFGE have been developed for *L. monocytogenes* (Borucki and Call, 2003; Liu, 2006). However, most of these methods are not always convenient for routine use in clinical laboratories and require special instruments and skilled personnel. Serotyping of *Listeria* is difficult and expensive due to non-availability of commercial antisera (Dhanashree *et al.*, 2003; Liu, 2006). The multiplex PCR used for serotyping separates the four major serovars (1/2a, 1/2b, 1/2c and 4b) by using the marker genes as well as the species specific gene. This assay is highly specific as well as reproducible (Doumith *et al.*, 2004). PFGE has provided the most sensitive strain discrimination but it is labor-intensive and time consuming and thus for practical purposes is often preceded by serotyping. PFGE has become the standard subtyping method to detect listeriosis outbreaks (Graves and Swaminathan, 2001; Barbuddhe *et al.*, 2008).

In India, *L. monocytogenes* have been isolated from humans (Kaur *et al.*, 2007), animal clinical cases (Shakuntala *et al.*, 2006; Rawool *et al.*, 2007) and foods (Barbuddhe *et al.*, 2002; Jallewar *et al.*, 2007; Parihar *et al.*, 2008a). However, no significant work has been carried out on molecular/genomic level typing of listerial isolates. The objective of this study was to characterize the clinical isolates of *L. monocytogenes* by subtyping both phenotypically and genotypically and to further discriminate strain relatedness by PFGE. Data gathered from the above study provide valuable information about the genotypes on *Listeria* and the distribution of serotypes and pulsotypes in the Indian subcontinent.

MATERIALS AND METHODS

Bacteria

The standard strains of *Listeria monocytogenes* 4b (MTCC 1143) was procured from

IMTECH, Chandigarh. The other reference strains of *Listeria*, *L. ivanovii* (NCTC 11846), *L. seeligeri* (NCTC 11856), *L. innocua* (NCTC 11288), *L. monocytogenes* 4b (NCTC 11994), *L. monocytogenes* 1/2a (NCTC 7973) and *L. monocytogenes* 1/2b (NCTC 10887) were available at Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar, India. These strains were used for standardization of multiplex PCR assay.

L. monocytogenes strains isolated from human clinical cases during 2006–2009 were included in the study (Table). The isolates originated from samples collected from private and government hospitals in Goa, Maharashtra (Western India), Punjab and Uttar Pradesh (Northern India). Species designation of all isolates was confirmed by biochemical characterization (Gorski, 2008) and 16S rRNA sequencing (Czajka *et al.*, 1993).

PFGE

PFGE was performed according to the PulseNet standardized protocol (Graves and Swaminathan, 2001). Sample plugs were digested with 25 U of *AscI* (New England Biolabs, Beverly, MA, USA) at 37°C for 3 hours or 160 to 200 U of *ApaI* (New England Biolabs) at 30°C for 5 hours. Plugs were electrophoresed on a CHEF-DR II apparatus (Bio-Rad, Hercules, CA, USA) using the following parameters: initial switch time, 4 seconds; final switch time, 40 seconds; run time, 22 hours; angle, 120°; gradient, 6 V/cm; temperature, 14°C; and ramping factor, linear. The photographs were analyzed visually, and the DNA restriction fragments were sized against Lambda ladder PFG Marker NO 340 S (New England Biolabs). The TIFF images were compared using the Applied Maths BioNumerics software package (version 2.0, Applied Maths, Saint-Martins-Latem, Belgium) and visual inspection (Tenover *et al.*, 1995). Pattern clustering was performed using the unweighted pair group method using arithmetic averages and the Dice

TABLE. Sources and serotypes of *Listeria monocytogenes* isolates included in the study

Isolate	Syndrome	Sample type	Species	Serotyping	Serogrouping by PCR
1	Kidney disease	Urine	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
2	Septicaemia	Blood	<i>L. monocytogenes</i>	4d	4b, 4d, 4e
3	Septicaemia	Blood	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
4	Abortion	Placental tissue	<i>L. monocytogenes</i>	1/2b	1/2b, 3b
5	Septicaemia	Blood	<i>L. monocytogenes</i>	1/2b	1/2b, 3b
6	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
7	Septicaemia	Blood	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
8	Kidney disease	Urine	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
9	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
10	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
11	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
12	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
13	Abortion	Vaginal swab	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
14	Abortion	Placental tissue	<i>L. monocytogenes</i>	4d	4b, 4d, 4e
15	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
16	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e
17	Abortion	Placental tissue	<i>L. monocytogenes</i>	4b	4b, 4d, 4e

correlation coefficient with a position tolerance of 1.0%.

Serotyping

The isolates were serotyped using commercial *Listeria* antisera (Denka Seiken, Japan), in accordance with the manufacturer's instructions.

Multiplex-PCR Serotyping

The multiplex-PCR serotyping was performed as per the methodology described by Doumith *et al.* (2004). The primers used for multiplex serotyping PCR were from Sigma Aldrich, USA.

RESULTS

We present the analysis of 17 *L. monocytogenes* isolates recovered from humans in India by multiplex serotyping PCR allowing serovar predictions, conventional serology and by PFGE. The isolates were recovered from clinical samples from patients with various clinical conditions. A majority of isolates included were from human cases experiencing abortion, but included cases of

septicemia and kidney disease (Table). Multiplex-PCR based serotyping assay was used for serogrouping the isolates. Multiplex-PCR serotyping assay revealed 88.24% (15/17) of the strains belonging to serovar group 4b, 4d, 4e, 11.76% (2/17) to serovar group 1/2b, 3b. By serology, 13 (76.47%) *L. monocytogenes* isolates were assigned to serotype 4b, 2 (11.76%) each to serotype 4d and serotype 1/2b. PCR based and traditional serotyping gave congruent results.

Ten *ApaI* pulsotypes were recognized among the 17 human isolates. Eight isolates showed unique *AscI* pulsotypes, and the remaining *AscI* pulsotypes were assigned to four clusters. Each cluster contained one to three isolates (Figs. 1 and 2). The *ApaI* profiles of the strains with indistinguishable *AscI* profiles showed no difference in the number or position of bands in all clusters.

DISCUSSION

In India, infections caused by *L. monocytogenes* have been considered to be sporadic. It is not a notifiable disease in India. Consequently, no systematic data are available on the occurrence of listeric infection. In order

FIG. 1. Dendrogram derived from a PFGE profile of *ApaI* macrorestriction showing restriction patterns among the *L. monocytogenes* isolated from humans. Isolate identification numbers correspond to isolate numbers in Table.

to gain new information on infection clusters and on the distributions of subtypes of *L. monocytogenes*, 17 clinical isolates from a 4 year period in India were analyzed by PFGE and serotyping. Typing of *L. monocytogenes* plays an important role in epidemiological studies for investigation of foodborne outbreaks and in the food-processing environment, to identify the source of contamination and routes of dispersal. To accomplish this, rapid and reliable molecular methods are currently in use. A rapid multiplex-PCR serotyping assay has been developed which separated the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c and 4b) into distinct groups (Doumith *et al.*, 2004; Doumith *et al.*, 2005). This assay was employed in the present study to study the

FIG. 2. Dendrogram derived from a PFGE profile of *AscI* macrorestriction showing restriction patterns among the *L. monocytogenes* isolated from humans. Isolate identification numbers correspond to isolate numbers in Table.

serotype profile of *L. monocytogenes* isolates recovered from human clinical cases. Our data showed that most of the isolates belonged to serotype 4b, a serotype that is strongly associated with human listeriosis (Liu, 2006). Earlier studies (Parihar *et al.*, 2008b) reported the predominance of serotype 1/2a isolates among human clinical cases in Sweden; however, predominantly serotype 4b was detected from human listeriosis cases diagnosed in Hungary (Kiss *et al.*, 2006). The strains included were collected from four different geographical locations in India isolated at different time points, thus were representative of sporadic cases of listeriosis in India. In India, commonly reported clinical form is genital listeriosis. *L. monocytogenes* was isolated from the cervix of 14% of 150 patients of bad obstetric history (Krishna *et al.*, 1966), from 3% (Bhujwala *et al.*, 1973) and 10% women with a history of abortions (Stephen *et al.*,

1978). Pathogenic *L. monocytogenes* were isolated from 3.3% cases of spontaneous abortions (Kaur *et al.*, 2007). *L. monocytogenes* has been reported as a cause of meningitis and hydrocephalus in children born of infected mothers (Gogate and Deodhar, 1981). A perinephric abscess caused by *L. monocytogenes* in a 5-year-old malnourished child has also been reported (Gomber *et al.*, 1998). A case of meningitis caused by *L. monocytogenes* in a 17-year-old immunocompetent patient was reported (Kalyani *et al.*, 2006).

In India, spontaneous abortions, subclinical mastitis, meningoencephalitis and endometritis were the commonest forms reported among animal populations. The prevalence of *L. monocytogenes* was found to be 4.4% from buffaloes with a history of reproductive disorders (Shakuntala *et al.*, 2006). During three outbreaks of encephalitis, overall morbidity, mortality and case fatality rates of 7.89, 7.08 and 89.85%, respectively were reported in a population of 875 sheep (Kumar *et al.*, 2007).

Listeria has been isolated from a variety of foods in India. *Listeria* spp. were isolated from 139 (6.75%) of 2060 milk samples; 105 (5.1%) samples were positive for *L. monocytogenes* (Kalorey *et al.*, 2008). The microbiological analysis of 1098 samples comprising of milk and ready-to-eat indigenous milk products revealed a prevalence of *L. monocytogenes* as 1.64% (Aurora *et al.*, 2008). The prevalence of *L. monocytogenes* was reported as 6.66 to 7.08% in goat meat (Barbuddhe *et al.*, 2000; Rekha *et al.*, 2006), 7.4% in sheep meat (Barbuddhe *et al.*, 2000), and as 3.07 to 6% in beef samples (Brahmbhatt and Anjaria, 1993; Barbuddhe *et al.*, 2002). Isolation of *L. monocytogenes* was reported from 1.3% of fresh raw fish samples from Mangalore, India (Dhanashree *et al.*, 2003) and 8.69% from seafood of markets in Goa, India (Parihar *et al.*, 2008a).

In our study, PFGE analysis allowed discrimination among isolates of the same serogroup and also among isolates recovered

from the same sampling areas or from those isolated from different areas. Listeriosis outbreaks similar to the massive outbreaks observed in Europe and North America have probably not occurred in India yet. According to earlier data, in the period between 1966 and 2005, a number of bacteriologically confirmed human listeriosis cases have been reported (Malik *et al.*, 2002; Dhanashree *et al.*, 2003; Gupta *et al.*, 2003). The isolates from these studies were not available for analysis. Earlier, Dhanashree *et al.* (2003) reported serotypes 1 and 4 from *L. monocytogenes* isolated from fish. The PFGE types were compared with pulsotypes for isolates from other countries (Yde and Genicot, 2004; Mammina *et al.*, 2009). The banding pattern was compared as described by Tenover *et al.* (1995). The isolates reported in this study have different PFGE patterns when compared with the isolates described earlier (Yde and Genicot, 2004; Mammina *et al.*, 2009).

Most industrialized countries have an annual listeriosis incidence of 2 to 10 reported cases per million people per year (Goulet *et al.*, 2008). However, listeriosis has a high case fatality rate that can exceed 30% (Swaminathan and Gerner-Smith, 2007; Drevets and Bronze, 2008). Rapid and discriminatory subtyping methods, such as various DNA-based methods, e.g. PFGE, PCR and restriction fragment length polymorphism analysis, or sequencing of some specific sequences, are essential for the epidemiological investigation of *L. monocytogenes* and the tracking of specific clones along food-processing chains (Wiedmann, 2002; Sauders *et al.*, 2006). More recently, we have reported on the development of a 10-minute assay based on matrix assisted laser desorption/ionization-time of flight spectroscopy directly from colonies on agar plates. The method allows not only discrimination between pathogenic and non-pathogenic *Listeria* spp., but also permits resolution up to the level of the PCR serotype analysis described above (Barbuddhe *et al.*, 2008). Nevertheless, the high

cost of the capital equipment involved, despite being offset by cheap running costs of the assay, puts this method beyond the means of smaller diagnostic laboratories.

The results obtained in this study may be helpful in reinterpreting the epidemiology of *L. monocytogenes* in India. Investigations of food-borne events and surveillance by complementing conventional with molecular epidemiology are essential for the prevention and control of human listeriosis. The communication of results of typing of human and food isolates between health and food authorities should improve the efficacy of control measures.

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