Multi-Virulence-Locus Sequence Typing of 4b *Listeria monocytogenes* Isolates Obtained from Different Sources in India over a 10-Year Period

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**Abstract**

*Listeria monocytogenes* is an emerging foodborne pathogen responsible for listeriosis. The incidence of listeriosis has increased during the last 2 decades due to the increase in consumption of ready-to-eat foods and change in food consumption habits. Outbreaks and sporadic cases of listeriosis have been reported in developed countries. These reports have helped determine the safety practices needed to control listeriosis. Although *L. monocytogenes* has been reported from humans, animals, and a variety of foods in India, limited data exist with respect to prevalence and distribution of *L. monocytogenes* in the Indian subcontinent. The Indian *Listeria* Culture Collection Centre in Goa maintains all of the isolates received for subtyping and molecular characterization. Of the listerial isolate collection maintained by this center, three fourths of the isolates are of 4b serotype, while the number of other serotypes is very low. Therefore, we screened *L. monocytogenes* serotype 4b isolates to determine their relevance to previously defined epidemics and/or outbreaks using multi-virulence-locus sequence typing (MVLST). A total of 25 isolates in serogroup 4b of *L. monocytogenes* were randomly selected from a repository of 156 *L. monocytogenes* 4b isolates obtained from different sources in India over a period of 10 years. MVLST sequence types (virulence types, VTs) were compared to known epidemic clones and other known isolates in the *L. monocytogenes* MVLST database. The 25 isolates were grouped into three clusters. Cluster I comprised 21 isolates including animal (n=9), human (n=4), and food (n=8), which matched Epidemic Clone I (ECI, VT20). Three isolates—two from animal and one from food—formed a cluster while a single animal isolate was placed into two novel VTs (VT98 and VT99), respectively. Based on these findings, it can be inferred that ECI has been isolated from a variety of sources and places and has persisted in India for at least 10 years.

**Introduction**

*L. monocytogenes* is a facultative intracellular pathogen and is widely distributed in the environment. Foodborne illness caused by *L. monocytogenes* is a serious public health concern because of high mortality in susceptible populations, such as newborn children, the elderly, and immune-compromised persons (Farber et al., 1991; Ramaswamy et al., 2007; Swaminathan et al., 2007). According to FoodNet US, listeriosis accounted for 30% of foodborne deaths from 1996 to 2005 with a high case fatality rate of 16.9% (Barton Behravesh et al., 2011). In developed countries, listeriosis accounts for 0.6–1.3 cases per 100,000 in New Zealand and some European countries (Todd and Notermans, 2011). *Listeria* is more likely to cause death than other bacteria that cause food poisoning (Ramaswamy et al., 2007). Since the first reported foodborne outbreak of *L. monocytogenes* in 1981 in Canada, cases of foodborne listeriosis have become increasingly frequent throughout the world (Lungu et al., 2011). *L. monocytogenes* can colonize food-processing facilities and can persist there for long periods of time (Carpentier et al., 2011). The presence and persistence of *L. monocytogenes* in food-processing facilities can lead to postprocessing contamination and subsequent growth in food. Currently, many different kinds of foods have been associated with *L. monocytogenes* outbreaks (Swaminathan et al., 2007).
et al., 2007). Unlike in developed countries, the prevalence, distribution, and outbreaks caused by L. monocytogenes in India are poorly understood (Barbuddhe et al., 2012). In India, listeriosis associated with the reproductive system is the most common clinical form reported in humans. In animals, spontaneous abortions, subclinical mastitis, meningocoeplithitis, and endometritis have been frequently reported (Barbuddhe et al., 2012). Listerial outbreaks tend to be reported as sporadic cases, due to lack of an established surveillance system for monitoring outbreaks in India (Gupta et al., 2003; Mokta et al., 2010; Adhikari and Joshi, 2011). In India, listeriosis has also been detected in immune-compromised individuals (Peer et al., 2010; Mukherjee et al., 2011). Recently, a case of listerial meningitis with disseminated tuberculosis in a human immunodeficiency-positive individual was reported (Joel et al., 2013).

Listeria monocytogenes has been differentiated into 13 serotypes, of which 4b, 1/2a, and 1/2b are associated with 98% of listeriosis outbreaks and sporadic cases (Nelson et al., 2004). While serotype 4b is less common in food than other serotypes, it is responsible for the majority of outbreaks and human clinical cases (Pinner et al., 1992; Aureli et al., 2000; Donnelly 2001; Shen et al., 2006), and has a high case-fatality rate (McLauchlin 1990; Gerner-Smidt et al., 2005). In addition, the clinical manifestation of the L. monocytogenes serotype 4b is severe compared to other serotypes (Czuprynski et al., 2002). While the incidence of outbreaks and cases due to serotype 1/2a have risen recently in developed countries, the incidence due to serotype 4b remains high in India (Kale et al., 2011).

Tracking and control of L. monocytogenes has been accomplished by the conventional microbiologic techniques, traditional epidemiologic and molecular subtyping methods. Subtyping methods such as serotyping (McLauchlin et al., 1998), polymerase chain reaction (PCR) serotyping (Doumith et al., 2004), phage typing (Capita et al., 2002), plasmid typing (Lebrun et al., 1992), multilocus enzyme electrophoresis (Piffaretti et al., 1989), RAPD (Williams et al., 1990), pulsed-field gel electrophoresis (Brosch et al., 1991), repetitive-element sequence-based–PCR (Jerse et al., 1999), hybridization-based typing (Liu et al., 2006), DNA array (Rudi et al., 2003), multilocus sequence typing (Salcedo et al., 2003), multi-virulence-locus sequence typing (MLVST) (Zhang et al., 2004) and single nucleotide polymorphisms (Ducey et al., 2007) have been employed to distinguish L. monocytogenes strains isolated from clinical, food, and environmental sources. Molecular subtyping of L. monocytogenes has been valuable for discriminating strains that are clinically significant (Ramaswamy et al., 2007; Cheng et al., 2008). However, the MLVST scheme developed by Zhang et al. (2004) has been shown to have high discriminatory power (D = 0.99), excellent epidemiological concordance (E = 1.0), stability, and typeability (Zhang et al., 2004; Chen et al., 2005; Chen et al., 2007). Also, MLVST has been successfully used to detect epidemic clones and outbreak clones (Chen et al., 2005; Chen et al., 2007; Lomonaco et al., 2013; Rocha et al., 2013). Epidemic clones (EC) are genetically related isolates implicated in geographically and temporally unrelated outbreaks that are presumably from a common ancestor (Cheng et al., 2008). Since the introduction of this concept in 2004, seven ECs have been recognized, of which ECI, ECII, and ECIV are serotype 4b, ECIII, ECV, and ECVI in serotype 1/2a, and ECVI in serotype 1/2b (Zhang et al., 2004; Chen et al., 2007; Knabel et al., 2012; Lomonaco et al., 2013).

Listeriosis has been reported in sporadic, outbreak, and epidemic forms across many countries; however, data are lacking from developing countries such as India (Dandona et al., 2004; Reddy, 2006; Dandona et al., 2009). The absence of such data impedes epidemiological studies, which could otherwise be employed to control the spread of L. monocytogenes in developing countries. In addition to lack of funds for studying epidemiology (Murheker et al., 2010), the presence of major traditional diseases such as malaria, cholera, leprosy, tuberculosis, and so on in India divert resources away from the study of other emerging diseases such as listeriosis. Also, database reliability, and accessibility constitute additional major challenges in India (Mehande et al., 2013). Many studies from India have shown the presence of Listeria in a variety of foods such as milk and milk products, meat and meat products, seafood, and vegetables; however, it is unknown whether the presence of L. monocytogenes in food has led to sporadic cases or outbreaks to lack of epidemiologic surveillance. On the other hand, the isolation rates of L. monocytogenes from foods in India appears to be comparable to rates in other countries worldwide (Barbuddhe et al., 2012). Therefore, in an attempt to determine the epidemiological relevance of L. monocytogenes in India, we used MLVST to analyze isolates of serotype 4b that were collected from different sources over a period of 10 years.

Materials and Methods

Bacterial isolates

A total of 25 L. monocytogenes 4b isolates were randomly selected (based on geographic location, source, and year of the isolation) out of 156 L. monocytogenes 4b isolates in the repository of the Indian Listeria Culture Collection Centre. Only 4b serotypes were chosen due to the higher proportion (3:1) of the 4b than other serotypes. These isolates were recovered from human and animal clinical cases, and different food sources over a 10-year period (2000–2010) (Table 1) (Barbuddhe et al., 2012). Isolates were obtained from different laboratories across India and were confirmed as 4b serogroup by PCR serotyping (Doumith et al., 2004) and Listeria antiserum Seiken kit (Denka Seiken Co., Tokyo, Japan).

MLVST

Overnight-grown cultures in Brain Heart Infusion broth were subjected to DNA isolation using PureLink Genomic DNA Kits (Invitrogen) following the manufacturer’s instruction. For PCR, primer and PCR conditions were used as described by Zhang et al. (2004). PCR products were then run on 1.5% agarose gel and purified by using a gel extraction kit (Promega). The purified products were sequenced in both forward and reverse directions by Davis Sequencing (California). The 6 virulence gene sequences for the 25 isolates were concatenated (2608 bp) using Molecular Evolutionary Genetic Analysis software ver. 5.1 (MEGA5.1) software and then compared to the MLVST database at https://sites.google.com/site/mlvlstdatabase/. A cluster diagram was constructed
animal clinical (subtyped 25 a novel epidemic clone (Chen et al., 2007). In this study, we epidemic clones (epidemic clones I, II, and III) and redefined and also has accurately identified three previously known method has proven its significance in epidemiological studies and by different personnel. Twenty-five over a period of 10 years from different sources, locations, breaches clone sequence types. These isolates were collected and matched with previously described epidemic clone or out-

Cluster-I comprised 21 isolates from human clinical (serotype 4b isolates belonged to three clusters (Fig. 1). and refined epidemiologic studies focused on tracking the spread of pathogens with much greater effectiveness than ever before (Cheng et al., 2008; Moorman et al., 2010). The MVLST method has proven its significance in epidemiological studies and also has accurately identified three previously known epidemic clones (epidemic clones I, II, and III) and redefined a novel epidemic clone (Chen et al., 2007). In this study, we subtyped 25 L. monocytogenes serotype 4b isolates using MVLST to determine whether their virulence sequence types matched with previously described epidemic clone or outbreak clone sequence types. These isolates were collected over a period of 10 years from different sources, locations, and by different personnel. Twenty-five L. monocytogenes serotype 4b isolates belonged to three clusters (Fig. 1). Cluster-I comprised 21 isolates from human clinical (n = 4), animal clinical (n = 9), and food (n = 8) sources. The second cluster comprised three isolates from clinical (n = 2) and food sources (n = 1), while a single isolate from an animal clinical case was placed separately from all other typed isolates. The MVLST data generated were compared with the previously known MVLST data (Zhang et al., 2004; Chen et al., 2005; Chen et al., 2007) to determine the MVLST VTs. A major cluster with 21 isolates grouped with VT20, which is the virulence type for ECI. While the second and third clusters grouped separately from currently known VTs, these two types were then considered as novel virulence types and allotted VT98 and VT99, respectively.

It is significant to note the high prevalence of ECI (80%) from different sources and regions throughout India over the 10-year period (Table 1). As stated earlier, there is a lack of documented surveillance data on listeriosis in India, while the available data preclude the ability to compare and contrast molecular epidemiologic data with that of developed countries. At the serotype level, our findings concur with the findings of Kalekar et al. (2011). They observed that 17 of 149 human clinical samples screened from 2006 to 2009 were positive for L. monocytogenes, of which 15 (88%) isolates were positive for L. monocytogenes serotype 4b. In a recent study, Soni et al. (2013) examined L. monocytogenes isolated from the Ganges River, and clinical human specimens and milk samples from Varanasi, India. They observed that isolates from humans and water belonged to 4b, 4d, 4e or 1/2c, 3c serogroups, while milkborne isolates belonged to serogroups 1/2b, 3b or 1/2a, 3a. Other studies documenting human listeriosis in India have been reported in the last 10 years (Dhanashree et al., 2003; Gupta et al., 2003; Mokta et al., 2010; Adhikary et al., 2011; Peer et al., 2010; Kaur et al., 2010); however, some of these studies did not report serotypes of L. monocytogenes, and the strains from these studies could not be obtained for further characterization. Based on the findings of our study, it is speculated that ECI is broadly distributed and could be responsible for outbreaks in India.

### Table 1. Source of Listeria monocytogenes Isolates of Serotype 4b of Indian Origin

<table>
<thead>
<tr>
<th>ID</th>
<th>Source</th>
<th>Location</th>
<th>VT (EC)</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>India01</td>
<td>Vegetables</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2005</td>
</tr>
<tr>
<td>India02</td>
<td>Human</td>
<td>West Coast region</td>
<td>VT20 (ECI)</td>
<td>2010</td>
</tr>
<tr>
<td>India03</td>
<td>Vegetables</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2005</td>
</tr>
<tr>
<td>India04</td>
<td>Human</td>
<td>Western India</td>
<td>VT20 (ECI)</td>
<td>2009</td>
</tr>
<tr>
<td>India05</td>
<td>Human</td>
<td>West Coast region</td>
<td>VT20 (ECI)</td>
<td>2008</td>
</tr>
<tr>
<td>India06</td>
<td>Human</td>
<td>West Coast region</td>
<td>VT20 (ECI)</td>
<td>2008</td>
</tr>
<tr>
<td>India07</td>
<td>Milk</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2001</td>
</tr>
<tr>
<td>India08</td>
<td>Milk</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2002</td>
</tr>
<tr>
<td>India09</td>
<td>Poultry</td>
<td>Western India</td>
<td>VT20 (ECI)</td>
<td>2005</td>
</tr>
<tr>
<td>India10</td>
<td>Meat</td>
<td>Northern India</td>
<td>VT20 (ECI)</td>
<td>2001</td>
</tr>
<tr>
<td>India11</td>
<td>Animal</td>
<td>Western India</td>
<td>VT20 (ECI)</td>
<td>2003</td>
</tr>
<tr>
<td>India12</td>
<td>Freshwater fish</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2006</td>
</tr>
<tr>
<td>India13</td>
<td>Wildlife</td>
<td>Central India</td>
<td>VT98 (novel VT)</td>
<td>2005</td>
</tr>
<tr>
<td>India14</td>
<td>Poultry</td>
<td>Western India</td>
<td>VT98 (novel VT)</td>
<td>2005</td>
</tr>
<tr>
<td>India15</td>
<td>Animal</td>
<td>Western India</td>
<td>VT98 (novel VT)</td>
<td>2003</td>
</tr>
<tr>
<td>India16</td>
<td>Milk</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2002</td>
</tr>
<tr>
<td>India17</td>
<td>Poultry</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2005</td>
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<td>India18</td>
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<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2002</td>
</tr>
<tr>
<td>India19</td>
<td>Animal</td>
<td>Northern India</td>
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<tr>
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<td>Animal</td>
<td>Northern India</td>
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<td>2005</td>
</tr>
<tr>
<td>India22</td>
<td>Animal</td>
<td>Northern India</td>
<td>VT20 (ECI)</td>
<td>2010</td>
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<tr>
<td>India23</td>
<td>Animal</td>
<td>Western India</td>
<td>VT20 (ECI)</td>
<td>2003</td>
</tr>
<tr>
<td>India24</td>
<td>Animal</td>
<td>Western India</td>
<td>VT99 (novel VT)</td>
<td>2003</td>
</tr>
<tr>
<td>India25</td>
<td>Milk</td>
<td>Central India</td>
<td>VT20 (ECI)</td>
<td>2003</td>
</tr>
</tbody>
</table>

VT, virulence type; ECI, Epidemic Clone I.
ECI has been characterized as having a unique and stable genome (Jersek et al., 1999), which could partly explain the circulation of ECI over a 10-year period of time (Cheng et al., 2008). In addition, ECI has been considered a cosmopolitan clone that has been linked to several foodborne outbreaks of listeriosis worldwide (Herd et al., 2001; Ying et al., 2008). Such high prevalence of ECI was observed in Italy, where Rocha et al. (2013) found that 12 of 20 isolates associated with ruminant rhombencephalitis over 13 years were ECI. Outbreaks related to animal-adapted ECs (such as ECI) have been associated with cross-contamination (Kathariou, 2003; Chen et al., 2007; Cheng et al., 2008) (e.g., use of sheep manure as fertilizer in the 1981 Canada outbreak); the use of raw materials (outbreaks in Boston 1979, France 1992, and California 1985), improper food handling (outbreak in Italy, 1997), or postpasteurization contamination (outbreak in Boston 1973 and Switzerland 1983–1987) (Norton et al., 2007).

With the increase in urbanization in India, and as acceptance and consumption of ready-to-eat food has steadily risen, many of the ready-to-eat foods require refrigeration (ASA, 2013; Prakash, 2013). Failure to properly refrigerate foods could favor the growth of *L. monocytogenes* in refrigerated foods, especially when power outages occur for extended periods of time in populated towns and cities of India. Furthermore, diverse ecological niches along with varied environmental and climatic conditions add considerable challenge to determining the prevalence and distribution of *L. monocytogenes* in humans, animals, and food. It is recommended that a region-based approach be adopted to undertake surveillance and monitoring of foodborne pathogens including *L. monocytogenes* in human populations, raw and retail foods, and food-processing plants.

In conclusion, ECI appears to be prevalent and persistent in India over a period of at least 10 years. The dissemination of ECI in animals, food, and humans in India could perhaps be due to complex transmission routes of *L. monocytogenes* from animals to environment to humans; or through animal-origin raw products, to ready-to-eat foods, to humans. Understanding these key transmission pathways with the assistance of MVLST will greatly assist in developing prevention and control strategies for *L. monocytogenes*. The prevalence and persistence of ECI in India is a matter of concern. Therefore, systematic and coordinated study is needed to understand the epidemiologic nature of *L. monocytogenes* ECI and other possible epidemic clones in India. In addition, strong control measures and regulations should be implemented in India to minimize the spread of *L. monocytogenes*.

### FIG. 1. Unrooted neighbor-joining tree computed in MEGA 5.1 for multi-virulence locus sequence typing data based on sequencing of six virulence genes: *prfA*, *inlB*, *inlC*, *dal*, *clpP*, and *LisR*. Sequence data from randomly selected Indian isolates (●) were compared with previously known multi-virulence-locus sequence typing database (Chen et al., 2005; Chen et al., 2007; Knabel et al., 2012), and major epidemic clones (ECI-●, ECII-●, ECIII-●, ECIV-●). Twenty-one of the 25 selected isolates grouped with ECI. Three of the 25 isolates grouped with a novel VT (VT98) and one grouped with a novel VT (VT99). Keys for source of isolation: A, animal; F, food; Fen, food environment; H, human; En, environmental; NA, not available.
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Disclosure Statement

No competing financial interests exist.

References


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