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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

LISTERIA 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 0.3 cases per 100,000 in Europe, Canada, Australia, and the United States, while slightly higher rates (0.6–1.3) have been observed 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

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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, meningoencephalitis, 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 (Kalekar *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 (Jersek *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 (MVLST) (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 MVLST 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, MVLST 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 ECVII are in serotype 1/2a, and ECVI is 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 (Murhekar *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 (Mehendale *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 MVLST 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).

MVLST

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 MVLST database at https://sites.google.com/site/mvlstdatabase/. A cluster diagram was constructed

TABLE 1. SOURCE OF *LISTERIA MONOCYTOGENES* ISOLATES OF SEROTYPE 4B OF INDIAN ORIGIN

ID	*Source*	*Location*	*VT (EC)*	*Year of isolation*
India01	Vegetables	Central India	VT20 (ECI)	2005
India02	Human	West Coast region	VT20 (ECI)	2010
India03	Vegetables	Central India	VT20 (ECI)	2005
India04	Human	Western India	VT20 (ECI)	2009
India05	Human	West Coast region	VT20 (ECI)	2008
India06	Human	West Coast region	VT20 (ECI)	2008
India07	Milk	Central India	VT20 (ECI)	2001
India08	Milk	Central India	VT20 (ECI)	2002
India09	Poultry	Western India	VT20 (ECI)	2005
India10	Meat	Northern India	VT20 (ECI)	2001
India11	Animal	Western India	VT20 (ECI)	2003
India12	Freshwater fish	Central India	VT20 (ECI)	2006
India13	Wildlife	Central India	VT98 (novel VT)	2005
India14	Poultry	Western India	VT98 (novel VT)	2005
India15	Animal	Western India	VT98 (novel VT)	2003
India16	Milk	Central India	VT20 (ECI)	2002
India17	Poultry	Central India	VT20 (ECI)	2005
India18	Milk	Central India	VT20 (ECI)	2002
India19	Animal	Northern India	VT20 (ECI)	2003
India20	Animal	Northern India	VT20 (ECI)	2003
India21	Wildlife	Central India	VT20 (ECI)	2005
India22	Animal	Northern India	VT20 (ECI)	2010
India23	Animal	Western India	VT20 (ECI)	2003
India24	Animal	Western India	VT99 (novel VT)	2003
India25	Milk	Central India	VT20 (ECI)	2003

VT, virulence type; ECI, Epidemic Clone I.

with the Neighbour-Joining method in MEGA. Progressive new virulence types (VTs) were assigned to isolates showing at least a one-nucleotide difference with all other previously typed *L. monocytogenes* strains. Gene sequences were deposited into GenBank under accession number KC839823-KC839972.

Results and Discussion

Recent advances in the development of molecular subtyping methods have provided tools that allow rapid and highly accurate determination of microbial sources and have 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.

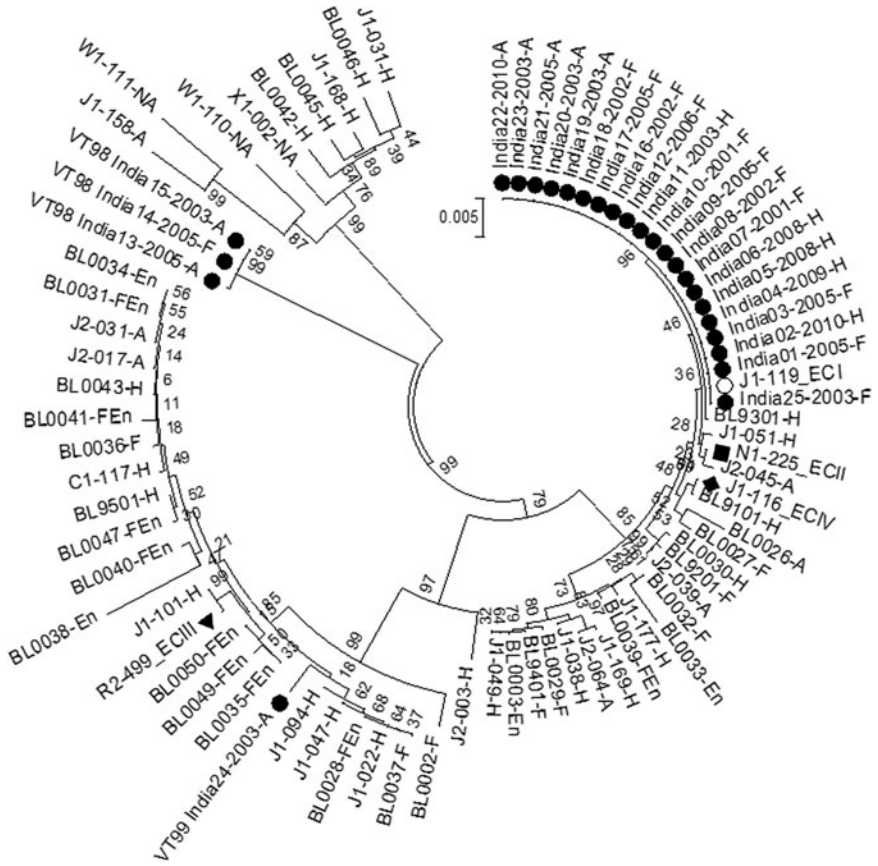

FIG. 1. Unrooted neighbor-joining tree computed in MEGA 5.1 for multi-virulence locus sequence typing data based on sequencing of six virulence genes*: prfA, inlB, inlC, dal, clpP*, and *LisR*. Sequence data from randomly selected Indian isolates (●) were compared with previously known multi-virulence-locus 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.

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.*

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No competing financial interests exist.

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