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Characterization of *Listeria monocytogenes* isolated from Ganges water, human clinical and milk samples at Varanasi, India

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ABSTRACT

Listeria monocytogenes isolated from Ganges water, human clinical and milk samples were characterized by antibiotic susceptibility, serotype identification, detection of virulence genes and ERIC- and REP-PCR fingerprint analyses. All isolates were uniformly resistant to ampicillin, except two isolates, and showed variable resistance to gentamicin, cotrimoxazole, ofloxacin, rifampicin and tetracycline. Of the 20 isolates found positive for pathogens, seven (four human and three water isolates) belong to serogroups 4b, 4d and 4e; six (one human and five water isolates) belong to serogroups 1/2c and 3c; four milk isolates belong to serogroups 1/2b and 3b; and three milk isolates belong to serogroups 1/2a and 3a. Two water isolates, all human isolates, except one (Pb1) lacking *inlJ* gene, and three milk isolates possess *inlA*, *inlC*, *plcA*, *prfA*, *actA*, *hlyA* and *iap* genes. The remaining water and milk isolates showed variable presence of *inlJ*, *plcA*, *prfA*, and *iap* genes. ERIC- and REP-PCR based analyses collectively indicated that isolates of human clinical samples belong to identical or similar clone and isolates of water and milk samples belong to different clones. Overall study demonstrates the prevalence of pathogenic *L. monocytogenes* species in the environmental and clinical samples. Most of the isolates were resistant to commonly used antibiotics.

1. Introduction

Human listeriosis, a public health problem, has been reported in the industrialized nations (Poulsen et al., 2011). Pregnant women, neonates, elderly, or immunocompromised people are particularly susceptible to *Listeria* which manifests as abortion, stillbirth, septicemia, meningitis and meningoencephalitis (WHO, 2004). The genus *Listeria*, a Gram-positive bacteria, comprised of eight species *Listeria monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria grayi*, *Listeria marthii* and *Listeria rocourtiae* (Graves et al., 2010; Leclercq et al., 2010) of which *L. monocytogenes* and *L. ivanovii*, are considered virulent (Guillet et al., 2010). *L. ivanovii* is rarely associated with human infection (Cummins et al., 1994).

L. monocytogenes are usually susceptible to wide range of antibiotics, the resistance to several antibiotics has been reported by several workers (Carpentier and Courvalin, 1999; Nwachukwu et al., 2010). Drug-sensitive strains of *L. monocytogenes* were isolated from clinical and food samples by Dhanashree et al. (2003). Sharma et al. (2012) reported multidrug-resistance strains from milk samples. A number of virulence factors such as 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 for their important role in the virulence and pathogenicity of *L. monocytogenes* (Vazquez-Boland et al., 2001; Liu et al., 2007).

Serotyping has been used to characterize *L. monocytogenes* that differs in their virulence and pathogenicity (Doumith et al., 2004; Liu, 2006). Although 13 serotypes of *L. monocytogenes* have been reported, only three serotypes (1/2a, 1/2b and 4b) are frequently isolated from the clinical cases of which 1/2a is mostly isolated from food and 4b from human epidemics (Liu, 2006). All strains isolated from human clinical, food and environment belong to a small number of serotypes (Farber and Peterkin, 1991; Chen et al., 2010). Therefore, highly discriminatory typing methods that correlate with serotyping are necessary. Among various approaches for molecular typing of *L. monocytogenes*, PFGE (pulsed field gel electrophoresis) has been considered to be the "gold standard" due to its high reproducibility and discriminatory ability (Swaminathan et al., 2001; Gerner-Smidt et al., 2006). However, it is labor intensive and time consuming method. REP (repetitive element sequence) and ERIC (enterobacterial repetitive intergenic consensus) – PCR based approaches have been found to be relatively simple and cost-effective for the genus *Listeria* and have shown to generate DNA fingerprints comparable to PFGE that allow discrimination within a single bacterial species (Jersek et al., 1999;

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Chen et al., 2010). REP and ERIC sequences act as primer binding sites to amplify the genome of a variety of bacteria by PCR (Woods et al., 1993).

L. monocytogenes is ubiquitous and widely distributed in the environment. It has been isolated from a variety of sources, including soil, vegetation, food of animal origin, such as meat and dairy products, silage, fecal material, sewage and water (Orndorff et al., 2006; Gandhi and Chikindas, 2007). The incidence of listeriosis may varies between 0.1 and 11.3 per million in different countries (Anon., 2004). However, no such type of evidence is available in India and the epidemiological data available till date are not adequate for assessing the extent of infection in human beings (Barbuddhe et al., 2012). Only a few reports on the incidence of *L. monocytogenes* have been documented for food (Aurora et al., 2008; Kalorey et al., 2008) and clinical samples (Dhanashree et al., 2003; Kaur et al., 2007) from India. However, to the best of our knowledge no published information on the prevalence of *L. monocytogenes* in fresh water bodies like lakes, rivers etc., of India are available. The river Ganges, the most sacred river of India, is one of the ten large rivers of the World. In Varanasi it travels some 7 km distance with a half-moon curvature between its two tributaries, Assi in the south and Varuna in the north. Around 60,000 people take holy dip in the river daily and almost all of them sip the water. Also, out of the total potable water demand of 220 million liters per day (MLD), river Ganges serves as the source for nearly 110 MLD of raw water drawn from its Bhadaini ghat water intake (Mishra, 2005). Therefore, studies on river Ganges with reference to a pathogen like *L. monocytogenes* is imperative as a major part of Indian population depends on river Ganges for bathing, drinking and sustenance.

Considering the above facts, in this study, we characterized *L. monocytogenes* isolated from the river Ganges water, human clinical and milk samples for the presence of virulence genes and their response to antibiotic susceptibility. We also used ERIC- and REP-PCR techniques to generate DNA fingerprints of *L. monocytogenes* isolated from various sources and to see clonal relationships among them.

2. Materials and methods

2.1. Study site and sample collection

A total of 712 samples were collected from Varanasi, Uttar Pradesh, India, during June 2009–July 2010 and tested, of which 100 were water samples from the river Ganges, 300 samples were from humans and 312 samples were from milk and milk products. The Ganges water samples were collected ten meters away from the bank of the river and one meter deep at around 9.00 am from four sites along ghats (Ravidas ghat, Assi ghat, Bhadaini ghat and Dr. Rajendra Prasad ghat) of Varanasi (25°20′N and 83°E), India (Fig. 1). The ghat is a flight of stones steps originating from city lanes and reaching up to the river water front. Samples were also collected 100 m upstream from the reference point. Taking the outfall of Assi drain as the reference point, Ravidas ghat, Assi ghat, Bhadaini ghat and Dr. Rajendra Prasad ghat (Dr. R. P. ghat) are around 1 m, 650 m, 1.3 km and 2.6 km downstreams. All human clinical samples were collected from private and government hospitals, pregnant women with bad obstetric history like repeated abortion, still births and pre-term labor, of which 75 each were from vaginal swabs, cervical swabs, placental tissues and blood, respectively. Out of the 168 milk samples, 120 were from cow milk and 48 from pasteurized milk and out of the 144 milk products, 48 each were from cheese, butter and ice-cream, respectively. All the samples were collected aseptically, quickly transported to the laboratory under chilled condition and were processed within 24 h of collection.

2.2. River water quality measurements

The parameters related to water quality of river Ganges such as pH, temperature, turbidity, dissolved oxygen (DO), biological oxygen demand (BOD) and fecal coliform count (FCC) are regularly determined by Swatcha Ganga Research Laboratory (SGRL), Varanasi, India, as per the standard protocol of American Public Health Association (APHA, 1992). The data obtained by SGRL during June 2009–July 2010 have been used for ascertaining the pollution level in the river water at Varanasi which is primarily due to discharge of domestic sewage.

2.3. Isolation and identification of L. monocytogenes

L. monocytogenes were isolated from the samples following the standard double enrichment method as described by ISO 11290:1 with slight modifications (Anon., 1997). Briefly, 25 ml of water, 15 ml of milk, 5 g or ml of milk product, placental bit and blood, and cervical or vaginal swab were separately inoculated into 225, 135, 45 and 10 ml of half-Fraser broth (Difco, USA), respectively and incubated for 24 h at 30 °C. Second enrichment was done by adding 0.1 ml from the overnight grown culture into 10 ml of full concentration of selective agents (Fraser broth, Difco, USA) and incubated for 48 h at 37 °C with subsequent spreading on PALCAM agar (Difco, USA) and incubated again for 48 h at 37 °C. Gray-greenish colonies with black sunken center and black halo were picked up and confirmed by Gram staining, biochemical tests such as catalase test, methyl red–Voges–Proskauer (MR–VP) reaction, nitrate reduction and motility at 20–25 °C, acid production from rhamnose, xylose, mannitol, α-methyl-D-mannopyranoside and CAMP test with *Staphylococcus aureus* and *Rhodococcus equi* (Seeliger and Jones, 1986). *L. monocytogenes* strain MTCC1143, *S. aureus* strain MTCC1144 and *R. 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 as working stock in the laboratory.

2.4. Antibiotic susceptibility test

All *L. monocytogenes* isolates were tested for antimicrobial susceptibility for 10 commonly used antibiotics in veterinary and human therapy (Lyon et al., 2008; Nwachukwu et al., 2010), using the disc diffusion method of Bauer et al. (1966). Antibiotics discs (Oxoid, UK) 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 zone of clearance was recorded and interpreted following the guideline of the Clinical and Laboratory Standards Institute (CLSI, 2006) for Gram-positive bacteria.

2.5. DNA isolation

Chromosomal DNA was extracted from *L. monocytogenes* isolates grown overnight at 37 °C with shaking at 200 oscillations per min in brain heart infusion broth (BHIB, Difco, USA) using the method of Moon et al. (2007) with modification. Bacterial cells from 10 ml of culture were pelleted by centrifugation at 8000 rpm at 4 °C for 15 min, washed three times in normal saline solution (0.9%), suspended in 500 μl of lysozyme solution [10 mM Tris–HCl (pH 8), 2.5 mg lysozyme (Sigma) per ml] and 5 μl of lysostaphin (100 μg/μl; Sigma) and incubated for 2 h at 37 °C. Thereafter 250 μl of Tris–EDTA buffer [50 mM Tris–HCl (pH 8) and 100 mM EDTA (Sigma)], 100 μl of SDS [1% (w/v)], 50 μl of proteinase K solution (1 mg/ml; Sigma) were added and