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ORIGINAL ARTICLE

Isolation, molecular detection and antibiogram of *Listeria monocytogenes* from human clinical cases and fish of Kashmir, India

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Abstract The present study investigated the occurrence, antibiogram and molecular epidemiology of *Listeria monocytogenes* from fish and human clinical cases in Kashmir, India. A total of 100 fresh samples of commonly eaten indigenous fish, *Cyprinus carpio* and *Schizothorax niger*, were tested for the presence of *L. monocytogenes*. Clinical samples comprising of blood (40) and CSF (20) were taken from patients with signs of encephalitis, meningitis/meningoencephalitis, septicemia and pyrexia of unknown origin. Following enrichment and plating on selective agar, conformation of the organisms was done on the basis of various biochemical tests followed by Christie, Atkins, Munch-Peterson (CAMP) test. *L. monocytogenes* was isolated from two fish and two clinical samples (blood, 1; CSF, 1) showing an overall prevalence of 2.5%. The prevalence was 3.33% in clinical samples and 2.0% in fish samples. The isolates were subjected to PCR assay for virulence associated with *hyl*A genes. The pathogenicity of the isolates was confirmed by demonstration of monocytosis and kerato-conjunctivitis in rabbits. *L. monocytogenes* isolated from human clinical cases were sensitive to gentamicin, ciprofloxacin, doxycycline, ceftriaxone and resistant to ampicillin, amoxycillin, cefpodoxime, streptomycin, norfloxacin and cefphotaxime. However, the isolates recovered from foods were sensitive to gentamicin, doxycycline, enrofloxacin, amoxycillin/clavulanic acid, ciprofloxacin, ampicillin/cloxacillin, ceftriaxone and resistant to streptomycin, cefpodoxime and cefphotaxime. Multi-drug resistant strains were found in the present study, representing a potential threat to human health.

Keywords *Listeria monocytogenes* · *hyl*-A · Clinical samples · Kerato-conjunctivitis · Monocytosis · PCR · Multi-drug resistant

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Introduction

Listeria monocytogenes is the etiological agent of listeriosis, a food-borne disease occurring primarily in immunocompromised individuals, causing septicemia and central nervous system infections and in pregnant women, who may suffer preterm delivery, miscarriage or stillbirth (Drevets and Bronze 2008: Lavi et al. 2008). Healthy adults may suffer a febrile gastroenteritis after ingesting large numbers of *L. monocytogenes* cells (Ooi and Lorber 2005). In the past decade, *L. monocytogenes* was involved in several outbreaks of listeriosis associated with consumption of dairy, fish, meat and vegetables with about 2,500 cases per year with 28% mortality (Mead et al. 1999). The mortality

rate is exceptionally high for a food-borne illness, being 34% for the intrauterine and postnatal cases and in average 36% for the nonperinatal cases (Siegman-igra et al. 2002; Swaminathan and Gerner-Smidt 2007; Drevets and Bronze 2008). Antibiotic resistance and inefficient emperical treatment of *Listeria* infection could be responsible for this increased mortality (Charpentier and Courvalin 1999).

The organism has already been isolated in tropical environment from fish in India (Moharem et al. 2007; Parihar et al. 2008). However, only a few surveys have been conducted to assess the presence of *L. monocytogenes* in fish in temperate regions. Human listeriosis in India has been reported by many workers (Bhujwala and Hingorani 1975; Gupta et al. 1997). However, the data available in our country is not adequate (Malik et al. 2002; Barbuddhe et al. 2004). The present study first of its kind in Kashmir, India, was designed to screen the presence of *L. monocytogenes* from human clinical cases and fish spp. of this temperate region.

Material and methods

Bacteria strains

The strains of *L. monocytogenes* (ATCC 19112) and *Staphylococcus aureus* (ATCC 11632) used in the study were procured from Himedia, India.

Sampling procedure

A total of 160 samples (fish, 100; clinical samples, 60) were taken in the present study for isolation and identification of *L. monocytogenes*. The fish samples were collected in UV-sterilized polyethylene zipped sachets from their respective places. They were transported to the laboratory immediately in ice packs and processed for the isolation of *L. monocytogenes* within 4–6 h of collection. Clinical samples comprising of blood (40) and CSF (20) were collected from patients showing signs of encephalitis, meningitis/meningoencephalitis, septicaemia and pyrexia of unknown origin (PUO). The samples were collected in sterile vials and brought to the laboratory immediately in ice packs except CSF samples which were transported at an ambient temperature.

Isolation and biochemical identification

Isolation of *L. monocytogenes* from fish samples was carried as per USDA protocol (McClain and Lee 1988) with slight modifications. The primary enrichment was carried out in University of Vermont Medium (UVM), containing nalidixic acid and acriflavine and the secondary enrichment was carried out in Fraser broth (FB), containing nalidixic acid, lithium chloride and acriflavine. Briefly, 25 g of each fish sample was thoroughly minced with homogenizer and inoculated into test tubes containing 225 ml of UVM broth and incubated at 30°C for 24 h. About 0.5 ml of the UVM enrichment was then transferred into 10 ml of Fraser broth and incubated at 37°C for 24 h. A loopfull of culture from the Fraser broth was streaked on *Listeria* selective agar and incubated at 37°C for 24 h. Greenish-yellow colonies typical of *Listeria* spp. were considered positive. At least five presumptive colonies from *Listeria* selective agar were streaked on PALCAM agar and incubated at 37°C for 24–36 h for confirmation of *L. monocytogenes*. The human clinical samples (blood and CSF) were streaked directly on 5% sheep blood agar and observed for development of characteristics beta-hemolysis. The presumptive colonies were further streaked on PALCAM agar and incubated at 37°C for 24–36 h for further confirmation of *L. monocytogenes*.

All the isolates were also subjected to standard biochemical tests such as catalase, oxidase, motility at 22°C and acid production from mannitol, rhamnose and xylose, nitrate reduction, urea hydrolysis, gelatin liquification, methyl red test and Voges–Proskauer test as per Cowan and Steel (1993). *L. monocytogenes* isolates identified biochemically were also tested for CAMP reaction with *S. aureus*.

Animal inoculation tests

The study was carried out on healthy New Zealand white rabbits procured from an organised rabbitry in India. The animals were treated humanely during the whole period of experimental study and the work was considered by the Institutional Animal Ethics Committee vide no. AU/FVSc/C-09/4073-68 on Ethical standards in Animal Experimentation. The animals were also tested for *Listeria* organism and were found to be negative on bacteriological and serological examination.

Demonstration of monocytosis in rabbits and Anton test

L. monocytogenes grown in Brain Heart Infusion Broth (BHIB) at 22°C for 24 h was inoculated intraperitoneally in healthy New Zealand white rabbits. Three groups of rabbits were taken and each group consisted of three rabbits (Group 1, Group 2 and Group 3). Group 1 rabbits were given *L. monocytogenes* infection isolated from humans and Group 2 rabbits were given *L. monocytogenes* infection isolated from fish, while as Group 3 rabbits were kept as control. Approximately 10^8 cells/ml/animal was used.

Blood was drawn from the animals prior and following the inoculations using disposable syringes fitted with 22-gauge needles from the marginal ear vein of each rabbit. Blood smears were made at regular intervals of 24 h up to 120 h post-inoculation and stained with Giemsa's stain. The slides were then observed under microscope for development of monocytosis.

The isolates were also tested for development of kerato-conjunctivitis by instillation of approximately 1 million actively motile *L.monocytogenes* in the eye of two adult healthy New Zealand white rabbits. The rabbits were observed for development of kerato-conjunctivitis up to 8 days following instillation. One rabbit was also kept as control.

Detection of virulence associated *hyl*A genes

All isolates confirmed by cultural, biochemical and in vivo pathogenicity test were screened for their virulent and non-virulent status by verifying the presence or absence of *Listeriolysin O* (*hly*A) gene. The amplification of *hly*A gene was carried out by following the protocol described by Notermans et al. (1991) with slight modifications. For standardization of PCR, a virulent strain of *L. monocytogenes* ATCC 19112 was used.

DNA extraction

Isolates of *L. monocytogenes* were grown overnight in brain heart infusion broth at 37°C. The obtained culture (approximately 1 ml) was centrifuged in a microcentrifuge (Sigma, USA) at 12,000 rpm for 10 min. The recovered pellet was resuspended in 100 μl sterilized DNAse and RNAse-free MilliQ water (Millipore, USA), heated in a boiling water bath for 10 min, and then snap chilled in crushed ice. We used 2.5 μl of the obtained lysate as template DNA in an amplification reaction volume of 25 μl.

PCR amplification

A pair of forward (5′-GCAGTTGCAAGCGCTTGG AGTGAA-3′) and reverse (5′-GCAACGTATCCTC CAGAGTGATCG-3′) primers were used for amplification of the 456 bp region of the *hly*A gene in 25 μl of reaction mixture containing 2.5 μl of lysate DNA, 2.5 μl of 10× PCR buffer, 0.5 μl of 10 mM, 2.0 μl of 25 mM $MgCl_2$, 3 μl of 10 ng/μl of the primers (forward+reverse), 0.1 μl of 5 U/μl *Taq* DNA polymerase and 14.4 μl MilliQ water. The amplification parameters included an initial denaturation at 94°C for 2 min followed by 35 cycles each of denaturation at 94°C for 15 s, annealing at 57°C for 30 s, extension at 72°C for 1.30 min and a final extension at 72°C for 5 min. The PCR products were analyzed by submersive agarose gel electrophoresis to resolve the amplified DNA fragments of the target gene.

Antibiotic sensitivity testing

All *Listeria* isolates were subjected to antibiotic sensitivity by disc diffusion method as described by Bauer et al. (1966).The test and control strains were seeded in Muller–Hinton agar and the following panel of antimicrobial discs and concentrations were used: enrofloxacin (10 μg), streptomycin (10 μg), amoxycillin (30 μg), cefpodoxime (10 μg), amoxycillin/clavulanic acid (30 μg), norfloxacin (10 μg), oxytetracycline (30 μg), cephotaxime (10 μg), gentamicin (10 μg), ampicillin (10 μg), doxycycline (30 μg), cephalexin (30 μg), amikacin (10 μg), ampicillin/cloxacillin (10 μg), ciprofloxacin (30 μg).

Results and discussion

L. monocytogenes have been frequently isolated from various clinical and food samples throughout the globe. In the present study, 60 clinical samples (blood, 40; CSF, 20) from patients with signs of meningitis, encephalitis, septicemia and pyrexia of unknown origin were tested for the isolation and identification of *L. monocytogenes.* The organism was isolated from one blood and one CSF sample showing an overall prevalence of 3.33%. The results are in concurrence with the findings of other workers who reported the prevalence of *L. monocytogenes* between 1.34% and 4% in India (Bhujwala et al. 1973; Bhujwala and Hingorani 1975; Stephen et al. 1978). In India, there are only few reported cases of human listeriosis. The epidemiological data available in our country is not adequate either because of failure to identify the isolate, low incidence rate or lack of awareness (Malik et al. 2002; Barbuddhe et al. 2004). The organism has also been isolated from 0.3% of clinical samples in Mangalore (Dhanashree et al. 2003a, b). A seroprevalence of 16% of listeriosis in women with spontaneous abortions has also been reported (Kaur et al. 2006). The organism was also isolated from an aborted fetus and the blood of mother by some workers (Bhujwala et al. 1973; Gupta et al. 1997; Gomber et al. 1998). Thomson et al. (1981) reported prevalence of listeriosis as 2.2% in meconium stained babies and 0.2% among total births. There has been a great variability in the reported cases of human listeriosis throughout the globe. The overall incidence of human listeriosis has been reported to vary from 2 to 10 cases per million in European countries (Goulet et al. 2008), and 3.4 to 4.4 per million in the United States (Sauders et al. 2005). The great variability in the incidence rate and in other epidemiological features between studies and other medical centres within studies suggest that many cases escape diagnosis (Siegman-igra et al. 2002).

Fish and fishery products have also been a source of *L. monocytogenes* infection to humans. In our study, 100 fresh fish samples were tested for the isolation and identification of *L. monocytogenes*. The organism was isolated from two fish samples showing an overall prevalence of 2.0%. These findings are in agreement with the findings of other research groups (Vaz-Velho et al. 1998; Autio et al. 1999; Handa et al. 2005; Moharem et al. 2007), who

reported the prevalence of *L. monocytogenes* in fish to be between 1.5% and 2.0%. Dhanashree et al. (2003a, b) isolated *L. monocytogenes* in 1.3% of fresh raw fish samples from Manglore India, whereas Dauphin et al. (2001) reported a prevalence of as high as 88% in fishes of the United Kingdom. The variability in the prevalence of *L. monocytogenes* in fishes in different geographical locations could partly be due to variability in sampling, transportation and isolation techniques (Soultos et al. 2007).

The pathogenic potential of *Listeria* isolates have been assessed by in vitro pathogenecity tests like beta-hemolysis on sheep or horse blood agar (Schonberg et al. 1989); CAMP test (Mckellar 1994). All isolates in this study produced a prominent zone of beta-hemolysis in blood agar plates in 24–36 h, as well as showed positive CAMP test with *S. aureus*. However, the isolates from human clinical cases produced a prominent zone of beta-hemolysis, while the isolates from foods produced only moderate to narrow zones of hemolysis on blood agar plates depicting higher virulence of the human isolates which could be due to passaging of the organism in the definitive hosts resulting in increased virulence. In his findings, Zaidi (1998) reported that a sixth passaged culture of *L. monocytogenes* 7973 resulted in reduced dose of LD_{50} compared to the unpassaged culture. The relationship between hemolysin production and pathogenic property of *Listeria* has been widely investigated. Ivanov et al. (1982) described a positive correlation between hemolytic activity and pathogenicity. The pathogenic nature of *L. monocytogenes* has been linked to the development of keratoconjunctivitis in laboratory animals like rabbits following instillation of live culture into the conjunctival sac. In the present study, an 18-h-old broth culture produced keratoconjunctivitis in healthy rabbits within 2–5 days by intraocular route. However, the human and the food isolates differed in their intensity with respect to the development of kerato-conjunctivits in experimental animals. The human isolates produced severe kerato-conjunctivitis starting from second day and becoming more severe on the fifth day postinoculation. The food isolates on the contrary produced only mild to moderate kerato-conjunctivits on day 5 postinoculation suggesting an increased virulence of the human isolates. Severe kerato-conjunctivitis leading to fatal purulent meningitis in rabbits has been reported by some workers (Seeliger and Finger 1976).

In the present study, monocytosis was demonstrated in all the rabbits following intraperitoneal inoculation of live cultures of *L. monocytogenes.* The human isolate produced an increase in the monocyte count from 2% (pre-inoculation level) to 10% (72 h post-inoculation). However, in case of food isolate the monocyte count increased up to 8% only. Similar observations have been made previously (Radostitis et al. 1994). Some researchers have linked the monocytosis producing factor to virulence on the plea that only the virulent strains of *L. monocytogenes* were able to produce monocytosis in rabbits, while as less virulent/avirulent strains of *Listeria* spp. like *L. ivanovii* failed to produce monocytosis (Hany et al. 1995).

The isolates of *L. monocytogenes* were also screened for their virulent and non-virulent status by verifying the presence or absence of *Listeriolysin O* (*hly*A) gene. The isolates produced a strong amplification of the target gene thereby confirming the isolates as *L. monocytogenes.* Several virulence associated genes have been sequenced and used as PCR targets for the detection of *L. monocytogenes,* however *Listeriolysin O* encoded by *hyl*A gene has been regarded as the most important virulence factor (Border et al. 1990).

The isolates were also tested for their sensitivity to various antibiotics. Among the human isolates the high in vitro sensitivity was shown by 100% of isolates for gentamicin, doxycycline, ciprofloxacin, ceftriaxone, 50% for each of enrofloxacin and amoxicillin/clavulanic acid. The isolates registered an intermediate response for cephalexin (100%) and ampicillin/cloxacillin (100%). The isolates were however found to be 100% resistant to ampicillin, oxytetracycline, amoxicillin, streptomycin, amikacin, cefpodoxime, erythromycin, norfloxacin and cefphotaxime. Isolates from foods were found to be 100% sensitive to gentamicin, doxycycline, enrofloxacin, amoxicillin/clavulanic acid, ciprofloxacin, ampicillin/cloxacillin, ceftriaxone, 80% sensitive to oxytetracycline, 60% sensitive for each of ampicillin and amikacin. The isolates showed an intermediate resistance for norfloxacin (80%) and cephalexin (60%). The isolates were however found to be resistant to cefpodoxime (100%), streptomycin (80%) and cefphotaxime (80%). The in vitro drug sensitivity pattern of the isolates under study showed considerably higher resistance of human isolates to antibiotics than the food isolates, which could be due to indiscriminate use of antibiotics in animals leading to its increased resistance as it gets transferred to humans either via food or by any other means. Although it was previously considered that multi-resistant strains of *Listeria* spp. are not commonly found in nature, evidence of emergence of multiresistant strains from various sources has been reported (Prazak et al. 2002; Srinivasan et al. 2005). Our results provide further provide the evidence of emergence of multi-resistant strains in nature, representing a potential threat to human health.

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