International Journal of Microbial Resource Technology, Vol.1, No.1 (Jan 2012), 1-4

Original research paper

Isolation, Identification and Antibiotic Susceptibility Profiling of Antimicrobial Resistant *Listeria monocytogenes* from Dairy Milk

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Accepted: 15 August 2011, Available online 10 January, 2012

Abstract

The objective of the present study was to evaluate the antibiotic susceptibility of 25 Listeria monocytogenes isolates isolated from raw cattle milk towards 15 antibiotics presently used in veterinary and human therapy. Antibiotic susceptibility tests were performed by using the Kirby Bauer disk diffusion method. The isolates were tested for their susceptibility to 15 different common antimicrobial drugs. A total of 115 milk samples from various sources were cultured for incidence of L. monocytogenes. 25 isolates of L. monocytogenes were isolated from a total of the 115 samples. Several isolates from milk samples were found resistant to Nalidixic acid, Amoxycillin+sulbactum, Cloxacillin, Erythromycin, Kanamycin and Vancomycin. On the other hand, several isolates were found susceptible to the Ofloxacin, Ampicillin, Tetracycline, Oxacillin, Streptomycin, Sulphafurazole and Ciprofloxacin. The present study provides preliminary information on incidence of L. monocytogenes as milk contaminants which may act as vehicles for the transmission of drug resistance.

Keywords: *Antimicrobial drug resistance, L. monocytogenes, dairy cattle, food infections and food contamination.*

1. Introduction

In food producing animals, antibiotics are used for the prevention and treatment of bacteria associated infectious diseases as well as for growth promotion purposes (Phillips *et al.,* 2004). Food safety is a current rising public health worry worldwide. Various pathogens are developing resistance to most presently used antibiotics and there are increasingly frequent reports of pathogens which are resistant to almost all existing antibiotics (Levy, 2004). An undesired outcome of antimicrobial use in animals is the possible expansion of antimicrobial-resistant food borne bacterial pathogens and consequently transmission to humans as food contaminants (EFSA, 2008a). In addition, spontaneous mutation in food borne bacteria or the spread of resistant bacteria in the absence of selective pressure may also contribute to the antimicrobial resistance burden in food (EFSA, 2008b). Since food borne listeriosis was first documented in 1981 (Schlech *et al.,* 1983), numerous food borne outbreaks of *L. monocytogenes* have been documented worldwide (Denny and McLauchlin, 2008). Meat, poultry, dairy, and vegetable products have all been implicated as vehicles of listeriosis (Gianfranceschi *et al.,* 2006).

The important uniqueness of *Listeria* spp. contributing to food-borne transmission are the capability to grow up at a low temperature (Walker *et al.,* 1990), survive osmotic stress, and survive mild preservation treatment. Its exclusive capability of growth in food stored in a refrigerator increases the possibility of *Listeria* infection from contaminated cold foods. The unnecessary use of antimicrobials has led to the appearance of antimicrobial-resistant bacteria. In addition, antimicrobials used as growth promoters in animal feed have resulted in the dissemination of antimicrobial-resistant bacteria into the environment. The importance of raw milk and dairy products as a vehicle for the transmission of various diseases especially in countries where hygienic standards are not strictly enforced has been well documented (Meyer-Broseta *et al.,* 2003). Milk and dairy products are

two specific food categories with respect to the risk assessment for listeriosis. Currently there is limited information regarding the prevalence and antimicrobial susceptibility patterns of *Listeria* spp. in foods in India.

L. monocytogenes can be transmitted by the consumption of raw (unpasteurized) milk and other milk products, homemade raw milk products and by asymptomatic personnel handling of such products, particularly related to the infectious diseases. Therefore, *Listeria* spp. can survive in the final product when present in milk used to make cheese. The objective of the present work was to investigate the incidence and prevalence of *L. monocytogenes* and their antimicrobial susceptibility profiling.

2. Material and Methods

2.1 Food Samples

115 samples of raw cattle milk were obtained from local dairy farms in Meerut and Babugarh Cantt, Hapur and examined for the presence of *Listeria monocytogenes*. All samples were immediately transferred to the microbiology laboratory, C.C.S University, Meerut in portable insulated cold-boxes. The samples were analyzed on the day they were collected.

2.2 Isolation

The isolation was performed according to the protocol of ISO 11290-1:1996 Horizontal method for detection and enumeration of *Listeria monocytogenes* in food and animal feeding stuffs (Anonymous, 2004). 25 mL of each sample was taken in an aseptic manner and homogenized in 225 mL of half fraser enrichment broth. Following 24 h of incubation at 30 °C, 0.1 mL of primary enrichment was transferred to 10 mL of fraser broth, incubated at 35 °C for 48 h. A loopful of the second enrichment was streaked on Palcam agar (Himedia, India) and incubated at 35 ºC for 48 h. The plates were examined for typical *Listeria* colonies; sunken black colonies on Palcam Agar. Further isolates were confirmed on chromogenic *Listeria* monocytogenes differential Agar (Himedia, India). *Listeria* species hydrolyse the chromogenic substrate which produces greenish-blue coloured colonies. Differentiation of *Listeria monocytogenes* from other *Listeria* species is based on phosphatidylinositol-specific phospholipase C (PIPLC) activity. Phospholipase C enzyme hydrolyses the purified substrate added to the medium resulting in an opaque halo around *Listeria monocytogenes* colonies. Confirmed colonies were preserved for further studies.

2.3 Biochemical Identification

3.1 Biochemical Identification with commercial kit (Himedia, India):

The biochemical tests were performed with Himedia Identification kit which includes Voges-Proskauer reactions, Catalase, Nitrate reduction, Esculin, Voges Proskauer's, Methyl red, and 7 different carbohydrates utilization test- Xylose, Lactose, Glucose, α-Methyl-D-Mannoside, Rhamnose, Sucrose and Mannitol.

2.4 Antimicrobial Susceptibility

The antimicrobial susceptibility was performed by the standard Kirby Bauer disk diffusion method on Mueller–Hinton agar (Himedia, India). The isolates were tested for their susceptibility to 15 different antimicrobials drugs (Himedia, India): Ofloxacin (5 mcg), Ampicillin (25 mcg), Tetracycline (30 mcg), Kanamycin (30 mcg), Erythromycin (10 mcg), Azithromycin (15 mcg), Nalidixic acid (30 mcg), Amoxycillin+ Sulbactam (30/15 mcg), Streptomycin (25 mcg), Sulphafurazole (25 mcg), Ciprofloxacin (30 mcg), Rifampicin (30 mcg), Vancomycin (5mcg), Oxacillin (5mcg), Cloxacillin (10mcg). By the standard method of inoculation, the top of a single and well isolated colony was touched with a sterile loop and the growth was inoculated into 2 ml Mueller-Hinton broth. The broth culture was then allowed to incubate at 37 °C for 4 hours to obtain young culture. The turbidity of actively growing broth cultures was then adjusted to a 0.5 McFarland standard and then a sterile cotton swab was dipped into adjusted suspension within 15 minutes and excess broth purged by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The swab was then spread evenly over the entire surface of the Mueller Hinton plate to obtain uniform growth. The plates were then allowed to dry for 3 to 5 minutes. The antibiotic disks were gently applied to ensure their contact with the inoculated Mueller-Hinton agar surface, and incubated at 37 °C. The plates were observed after 18-24 h and the zones of inhibition were measured by antibiotic susceptibility scale (Himedia, India) to the nearest millimeter. The zone diameter for individual antimicrobial agents was then translated into susceptible and resistant categories according to the interpretation table (supplied by the Himedia, India).

3. Results

A total of 115 milk samples from various sources were cultured for incidence of *L. monocytogenes*, *L. monocytogenes was* isolated from a total of 25 (21.73%) of the 115 samples (Table1) as they showed growth on Palcam agar and chromogenic Listeria differential agar (Fig. 1).

When these 25 isolates were subjected to further morphological (Gram staining) and biochemical characterization, heamolysis, Coagulase, Catalase and by rapid identification kits (Himedia, India), 25 isolates showed positive result

Fig-1: Colonies of *L. monocytogenes* on PALCAM agar and *Listeria* chromogenic media.

Table-1: Incidence of *L. monocytogenes* from different milk samples.

Source	No of Isolates	Number of sample positive for *L. monocytogenes*
Cow milk	20	9
Buffalo milk	60	11
Goat milk	15	3
Sheep milk	20	2
Total	115	25

3.1 *Antimicrobial susceptibility*

Almost 80-90% of the isolates were showed multiple drug resistance to majority of the antimicrobial agents tested. Several isolates from milk samples were found resistant to Nalidixic acid, Amoxycillin+sulbactum, Cloxacillin, Erythromycin, Kanamycin and Vancomycin. On the other hand several isolates were found susceptible to the Ofloxacin, Ampicillin, Tetracycline Oxacillin, Streptomycin, Sulphafurazole and Ciprofloxacin. Various isolates also exhibited intermediate resistance to Erythromycin, Vancomycin, Streptomycin and Cloxacillin.

4. Discussion

In the present study, 25 dairy product samples were positive for *Listeria* spp. This result is in agreement with the results reported by Sharef *et al.* (2006) and Arslan and Özdemir (2008). The high occurrence of *Listeria* spp. in raw milk could be due to environmental contamination with infected animal wastes or unhygienic food production and storage practices. Among all the various milk, raw milk samples (Cow and Buffalo) had the highest prevalence rate of *Listeria* spp. Although the prevalence of *Listeria* spp. may vary in different dairy products, it has been shown that *Listeria* isolates can be found more frequently in raw milk samples (Arslan and Özdemir, 2008). *L. monocytogenes* were detected in 3.6% raw milk samples (Gaya *et al.*, 1998). In a study in the USA, 35 of 450 raw milk samples (7.8%) were positive for *Listeria* spp. (Abou-Eleinin *et al.,* 2000). In the same way, in a study conducted in Turkey, *Listeria* species were isolated from 33.1% of homemade white cheese samples. The sources of *Listeria* spp. in raw milk have been shown to be fecal and environmental contamination during milking, storage and transport, infected animals in dairy farms or poor silage quality (Bemrah *et al.,* 2001). Therefore, the contamination source of *Listeria* spp. in raw milk in this study is likely insufficient hygiene during milking, milk storage or transportation. The results of this study show that raw milk is an important source for *Listeria* infection. Further studies are needed to confirm and explore this relationship. The results of antimicrobial susceptibility testing in the present study indicate that there is a high resistance of *Listeria* spp. Antibiotic resistance in *Listeria* species is due to the acquisition of mobile genetic elements such as self-transferable and mobilizable plasmids and conjugative transposons (Charpentier *et al.,* 1995). Since listeriosis is transmitted primarily via foods, the presence of antimicrobial-resistant *Listeria* in raw food products has an important public health implication especially in developing countries, where there is widespread and uncontrolled use of antibiotics. Due to the high number of antimicrobial- resistant isolates, we recommend that in vitro antimicrobial susceptibility testing of *Listeria* be performed and appropriate treatment be instituted especially for those cases of food-borne listeriosis with severe or prolonged symptoms or in immunocompromised patients. In conclusion, the presence of *Listeria* spp. in a variety of raw milk and dairy products indicate the potential risk of infection with *Listeria* in people consuming raw milk, unpasteurized milk, or traditional dairy products. These high-risk groups should avoid previously prepared unpasteurized dairy products.

Acknowledgements
Authors are grateful to Department of Microbiology, C.C.S. University, Meerut for providing necessary facilities to carry out the present work.

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