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Character of *Listeria* spp. isolated from livestock products and their related environmental areas

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Abstract

This study was carried out to investigate the characters of *Listeria monocytogenes* isolated from food, animal feces, dry cattle food, and the environment in Seoul and Kyonggi province during the period from 1998 to 2003. Serotyping of 70 *L. monocytogenes* isolates was performed according to the manufacturer's instruction. Minimum inhibitory concentrations (MICs) were determined by the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute. All the isolates were tested against 20 antimicrobial agents. The serotypes of the 70 *L. monocytogenes* isolates were 1/2c (62.8%), 1/2a (20%) and 1/2b (17.2%). Of the 70 *L. monocytogenes* isolates, 67.1%, 57.1%, 11.4%, 5.7%, 2.8%, 1.4% and 1.4% were resistant to tetracycline (Te), minocycline (Mi), norfloxacin (Nor), ciprofloxacin (Cip), neomycin (N), chloramphenicol (C) and cephalothin (Cf), respectively. However, all isolates were 100% sensitive to antibiotics such as amikacin, ampicillin, erythromycin, gentamycin, imipenem, kanamycin, ofloxcin, streptomycin, penicillin, trimethoprim, trimethoprim/sulfamethoxazole, tobramycin, and vancomycin. Multiple resistance patterns of the isolates were observed in TeMiNor Cip (1.4%), TeMiNor (7.1%), TeMiCip (2.9%), TeMiN (1.4%) and TeMi (44.3%). The results of this study indicate that many *L. monocytogenes* isolates are resistant to antimicrobial agents including Te and Mi. The possibility that the isolates could increasingly acquire multiple antimicrobial resistant properties cannot be precluded.

Key words : *Listeria monocytogenes*, Antibiotic susceptibilities, Serotype

INTRODUCTION

Listeria species is ubiquitous bacteria widely distributed in the environment (Charpentier and Courvalin, 1999; Gianfranceschi et al, 2003; Lukinmaa et al, 2003). Among the seven species of *Listeria*, only *Listeria monocytogenes* (*L. monocytogenes*) is commonly pathogenic for humans. It can cause serious infections such as meningitis or septicemia in newborns, immunocompromised patients and the elderly or lead to abortion (Charpentier et al, 1995; Charpentier and Courvalin, 1999; Miettinen et al, 1999; Lukinmaa et al, 2003). Although human listeriosis occurs only sporadically (Charpentier et al, 1999) several outbreaks have been observed during the last two decades (Charpentier et al, 1995; Charpentier and Courvalin, 1999). It is now established that food borne transmission constitutes the main route of acquisition of listeriosis (Charpentier and Courvalin, 1999; Miettinen et al, 1999; Lukinmaa et al, 2003). Despite efficient antibiotic therapy, listeriosis represents a public health problem since it is fatal in up to 30% of cases (Charpentier and Courvalin, 1999; Gianfranceschi et al, 2003; Lukinmaa et al, 2003).

The most common route of infection by *L. monocytogenes* is via the gastrointestinal tract (Poyart-Salmeron et al, 1990; Farber and Peterkin, 1991; Charpentier et al, 1995), as evidenced by several outbreaks of listeriosis caused by the ingestion of contaminated food materials

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(Miettinen et al, 1999; Lukinmaa et al, 2003). In addition, *L. monocytogenes* is a widespread, saprophytic bacterium that can be found not only in soil, on plants, and animal waste (Gianfranceschi et al, 2003) but also as a persistent organism in food and dairy processing environments (Chasseignaux et al, 2002). All of these environments are potential sources for contamination of fresh and prepared foods with *L. monocytogenes*, which, in turn, poses a significant public health risk in terms of the potential for listeriosis outbreaks. In order for these risks to be minimized, subtyping of *L. monocytogenes* isolates has been undertaken in several laboratories in recent years to begin to identifying type specific factors contributing to virulence, persistence, and/or transmissibility of the bacterium relative to its outbreak potential (Lukinmaa et al, 2003).

Because of the importance of *L. monocytogenes* epidemiology to human health, a number of discriminatory subtyping methods have been described for this organism (Borucki and Call, 2003; Borucki et al, 2003). Although pulsed field gel electrophoresis is the most commonly employed subtyping technique, new subtyping technologies are constantly introduced and tested in hopes of increasing the resolution, speed, and reproducibility of *L. monocytogenes* subtyping. The most recent examples of this are multilocus sequence subtyping (Salcedo et al, 2003) and microarray genomic analysis (Borucki et al, 2003). It is important that the strains included in these studies are initially characterized by using a universally accepted method such as serotyping. Serotyping makes it possible to compare results from different studies while providing a biological context for phylogenetic or phenetic relationships that are described by a new method.

Antimicrobial agents are administered at therapeutic and subtherapeutic levels to food producing animals, not only to prevent disease, but also to accelerate weight gain and to improve feed efficiency (Bower and Daeschel, 1999). Since the 1950s, this practice has exerted a selection pressure for antimicrobial resistance genes, which has led to the emergence of antibiotic resistant bacteria associated with food producing animals and foods (Klein et al, 1998; Georgnaras and von Holy, 2001). Of major concern is that human pathogens are also exhibiting resistance to antibiotics commonly used for treatment of human bacterial infections (Georgnaras and von Holy, 2001; Safdar and Armstrong, 2003). Recent studies have suggested a link between the use of antimicrobial agents in food producing animals and the emergence of human pathogens with decreased susceptibilities or complete resistance to antibiotics (Bower and Daeschel, 1999; Georgnaras and von Holy, 2001). This is probably due to cross resistance between the antimicrobial agents used exclusively in food producing animals and those used to treat humans (Charpentier and Courvalin, 1999; Georgnaras and von Holy, 2001). Although vancomycin is not used in veterinary medicine, it is used to treat Gram positive bacterial infections in humans, including methicillin resistant *Staphylococcus aureus* (Mulazimoglu et al, 1996; Georgnaras and von Holy, 2001). The emergence of vancomycin resistant Gram positive bacteria, therefore, is of major medical concern.

In general, isolates of *L. monocytogenes*, as well as strains of other *Listeria* spp. are susceptible to a wide range of antimicrobial agents except cephalosporins and fosfomycin (Hof et al, 1997; Charpentier and Courvalin, 1999). The choice of treatment for listeriosis remains the administration of ampicillin or penicillin G combined with an aminoglycoside, classically gentamicin (Charpentier and Courvalin, 1999; Safdar and Armstrong, 2003). The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co trimoxazole, is considered to be a second choice therapy. The most active agent in the combination seems to be trimethoprim, which is synergized by sulfamethoxazole (Schuchat et al, 1991; Charpentier and Courvalin, 1999). Most isolates from clinical as well as food borne and environmental sources are susceptible to the antibiotics active against gram positive bacteria. The first *L. monocytogenes* strains resistant to antibiotics were reported in 1988 (Charpentier and Courvalin, 1999). The strains were resistant to $>10\mu g$ of tetracycline per ml. The first multiresistant strain of *L. monocytogenes* was isolated in France in 1988 (Charpentier et al, 1995; Charpentier and Courvalin, 1999). Since then, other strains

of *Listeria* spp. isolated from food or the envir-onment or in sporadic cases of human listeriosis resistant to one or several antibiotics have been described (Charpentier et al, 1995; Charpentier and Courvalin, 1999).

In this study, we investigated the serotypes and susceptibilities of isolates of *Listeria* spp. which were isolated from livestock products and their related environmental areas in Korea. Susceptibilities to antimicrobial agents used in Korean food producing animals industry were determined. In addition, susceptibilities of the Gram positive isolates to vancomycin were investigated.

MATERIALS AND METHODS

Microorganism identification

Listeria strains were isolated from various livestock products such as poultry meats, pork, beef and hamburger, and the environment such as feces, dry animal food and knives at slaughter house in Seoul and Kyonggi province during the period from 1998 to 2003. To detect *Listeria* strains in food products, we followed the ISO11290 96 (Anonymous, 1996; Gianfranceschi et al, 2003) procedure.

In order to detect *Listeria* strains in environmental samples, we used the method described by Anoymous (1996) and Gianfranceschi et al (2003).

All strains were biochemically identified by using the commercial API *Listeria* system (bioMérieux, Fance). All isolates were stored at −70℃ in Tryptone Soya Broth (Oxoid, Basingstocke, UK) supplementd with 15% (vol/vol) glycerol.

Serotyping

All *L. monocytogenes* isolates were serotyped by using antisera against O and H antigens according to the manufacturer's instructions (Denka Seiken Co., Tokyo, Japan).

Antimicrobial susceptibility

The antimicrobial susceptibilities of *L. monocytogenesis* isolates to were tested with the disk agar method standardized by the Clinical and Laboratory Standards Institute (CLSI, 2008). CLSI has not yet provided specific guidelines for the testing of *Listeria* (Allerberger, 2003). Usually susceptibility testing is performed according to CLSI guidelines for bacteria that grow aerobically using Mueller Hinton agar with 5% horse blood. For trimethoprim/Sulfamethoxazole, the blood is lysed (Allerberger, 2003).

The antimicrobial susceptibility for 103 strains from 1998 to 2003 was performed by an agar plate antibiotic disk diffusion method (Kirby Bauer technique) (Safdar and Armstrong, 2003; CLSI, 2008).

All the isolates were tested against 20 antimicrobial agents as follows; ampicillin (Am), amikacin (An), cephalothin (Cf), chloramphenicol (C), ciprofloxacin (Cip), erythromycin (E), gentamicin (Gm), imipenem (Ipm), kanamycin (K), minocycline (Mi), neomycin (N), norfloxacin (Nor), ofloxacin (Ofx), penicillin (P), streptomycin (S), tetracycline (Te), tobramycin (Nn), trimethoprim (Tmp), trimethoprim/sulfamethoxazole (Sxt), van-

Table 1. The number of *Listeria* spp. isolates by type of food and environmental samples

Sourse	*L. monocytogenes* (%)				*L. innocua* (%)	*L. welshimeri* (%)
	No. of isolates	Serotype 1/2a	Serotype 1/2b	Serotype 1/2c		
Poultry meat	43 (61.4)	13 (18.6)	7 (10)	23 (32.8)	8 (32.0)	7 (87.5)
Pork meat	9 (12.9)		3 (4.3)	6 (8.6)	2 (8.0)	1 (12.5)
Hamburger	9 (12.9)		2 (2.9)	7 (10)		
Cattle feces	6 (8.6)			6 (8.6)	10 (40)	
Frozen pollack	1 (1.4)	1 (1.4)			2 (8.0)	
Knife of pig slaughter-house	1 (1.4)			1 (1.4)		
Dry cattle food	1 (1.4)			1 (1.4)		
Beef meat					2 (8.0)	
Swine feces					1 (4.0)	
Total	70 (100)	14 (20.0)	12 (17.2)	44 (62.8)	25 (100)	8 (100)

comycin (Va). All antimicrobial disks were purchased from BBL Co. (BBL Co, Sparks, USA).

Minimum inhibitory concentrations (MICs) were determined by the microdilution method according to the guidelines of the CLSI (2008). MICs were performed for the positive strains determined by agar plate antibiotic disk diffusion method. Cation adjusted Mueller Hinton Broth (Becton, Dickinson and Cmpany, Sparks, USA) was supplemented with 2~5% lysed horse blood, as specified by the CLSI guidelines (CLSI, 2008). MICs were read after incubation at 37°C for 18h, except for the vancomycin MICs, which were read after 24h (Ploy et al, 1998; Geornaras and von Holy, 2001). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control reference strains for the precision and accuracy of the susceptibility test procedure (CLSI, 2008). All antimicrobial agents used for MICs were obtained from Sigma (Sigma, St Louis, USA).

RESULTS

The number of *Listeria* spp. isolates

One hundred three *Listeria* strains were isolated from poultry meats, pork, beef and hamburger, and the environment such as feces, dry animal food and knives at slaughter house in Seoul and Kyonggi province during the period from 1998 to 2003 (Table 1). Among 70 *L. monocytogenes*, 61.4% were isolated from poultry meat, 12.9% from hamburger and pork meat respectively, 8.6% from cattle feces, and 1.4% from frozen Pollack, knife at a pig slaughter house and dry cattle food, respectively. Among 25 *L. innocua*, 40.0% were isolated from cattle feces, 32.0% from poultry meat, 8.0% from pork meat, frozen Pollack and beef, respectively, and 4.0% from swine feces. Among 8 *L. welshimeri*, 87.5% were isolated from poultry, and 12.5% from pork meat.

Serotypes of *L. monocytogenes*

The serotypes of *L. monocytogenes* isolates are shown in Table 1. In the food samples, the serotypes were 1/2c (51.4%) in poultry meat (32.8%), pork meat (10.0%) and hamburger (8.6%), 1/2a (20.0%) in poultry meat (18.6%) and frozen Pollack (1.4%), and 1/2b (17.2%) in poultry meat (10.0%), hamburger (4.3%) and pork meat (2.9%). In the environmental samples, the serotype was 1/2c (11.4%) in cattle feces (8.6%), knives used at the pig slaughter house (1.4%) and dry cattle food (1.4%).

Antibiotic resistance *Listeria* species

To our knowledge, among CLSI listed MIC breakpoints for resistance and susceptibility are not available for the antimicrobial agents tested in the present study, excluding Am and P. Therefore, the diameter (in millimeters) of each zone around the antibiotic disk was measured and interpreted according to method of Bauer et al (1966) recommended by Abrahim et al (1998).

Among the 70 *L. monocytogenes* isolates, 47 strains (67.1%) were resistant to Te (MIC, 64~128μg/ml), 40 strains (57.1%) to Mi (8~32μg/ml), 8 strains (11.4%) to Nor (16μg/ml), 4 strains (5.7%) to Cip (4μg/ml), 2 strains (2.8%) to N (4μg/ml), 1 strain (1.4%) to C (16μg/

Table 2. Antimicrobial resistance of *Listeria* isolates

Antimicrobial	*L. monocytogenes*		*L. innocua*		*L. welshmeri*	
Agent	No. (%) of resistant isolates	MIC (μg/ml)	No. (%) of resistant isolates	MIC (μg/ml)	No. (%) of resistant isolates	MIC (μg/ml)
Tetracycline	47 (67.1)	64-128	22 (88.0)	64-128	4 (50)	64-128
Minocycline	40 (57.1)	8-32	7 (28.0)	8-16		
Norfloxacin	8 (11.4)	16	6 (24.0)	16		
Ciprofloxacin	4 (5.7)	4	3 (12.0)	4		
Neomycin	2 (2.8)	4	1 (4.0)	4		
Chloramphenicol	1 (1.4)	16	1 (4.0)	32		
Cephalothin	1 (1.4)	32				
Streptomycin			11 (44.0)	32-64		
Ofloxacin			1 (4.0)	8		

Table 3. Multi-drug resistant patterns of isolates of *Listeria* spp.

Isolates (No. of strains tested)	Resistant pattern	No. (%) of resistant isolates
L. monocytogenes (70)	Te · Mi · Nor · Cip	1 (1.4)
	Te · Mi · Nor	5 (7.1)
	Te · Mi · Cip	2 (2.9)
	Te · Mi · N	1 (1.4)
	Te · Mi	31 (44.3)
L. innocua (25)	Te · S · Mi · Nor · Cip	1 (4.0)
	Te · S · Nor · Cip · N	1 (4.0)
	Te · S · Mi	4 (16.0)
	Te · Mi · Nor	1 (4.0)
	Te · Nor · C	1 (4.0)
	Te · Nor · Ofx	1 (4.0)
	Te · S	4 (16.0)
	Te · Mi	1 (4.0)
	Te · Nor	1 (4.0)
	Te · Cip	1 (4.0)

ml) and 1 strain (1.4%) to Cf (32μg/ml). All isolates of *L. monocytogenes* were 100% sensitive to Am, An, E, Gm, Ipm, K, Nn, Ofx, P, S, Sxt, Tmp and Va. Among the 25 *L. innocua* isolates, 22 strains (88.0%) were resistant to Te (64～128μg/ml), 11 strains (44.0%) to S (32～64μg/ml), 7 strains (28.0%) to Mi (8～16μg/ml), 6 strains (24.0%) to Nor (16μg/ml), 3 strains (12.0%) to Cip (4μg/ml), 1 strain (4.0%) to N (4μg/ml), 1 strain (4.0%) to C (32μg/ml) and 1 strain (4.0%) to Ofx (8μg/ml). All isolates of *L. innocua* were 100% sensitive to Am, An, Cf, E, Gm, Ipm, K, Nn, P, Sxt, Tmp, and Va. Among the 8 *L. welshimeri*, 4 strains (50.0%) were resistant to Te (MIC, 64～128μg/ml), but all *L. welshimeri* were 100% sensitive to the other antibiotics tested (Table 2).

Multiple resistance patterns

The multiple resistance patterns (MRP's) of *L. monocytogenes* isolates were observed in Te · Mi · Nor · Cip (1.4%), Te · Mi · Nor (7.1%), Te · Mi · Cip (2.9%), Te · Mi · N (1.4%), and Te · Mi (44.3%). For *L innocua* isolates MRP's were observed for Te · S · Mi · Nor · Cip (4.0%), Te · S · Nor · Cip · N (4.0%), Te · S · Mi (16.0%), Te · Mi · Nor (4.0%), Te · Nor · C (4.0%), Te · Nor · Ofx (4.0%), Te · S (16.0%), Te · Mi (4.0%), Te · Nor (4.0%), Te · Cip (4.0%) (Table 3).

DISCUSSION

Due to the diversity and recent introduction of antibiotics, infections caused by drug resistant bacteria did not represent a medical problem until the early 1980s. However, the evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by overprescription of drugs in clinical settings and their heavy use as growth promoters among farm animals (Bower and Daeschel, 1999; Charpentier and Courvalin, 1999; Georgnaras and von Holy, 2001). Since bacteria have the remarkable ability to develop resistance to every antibiotic, it is reasonable to anticipate that even bacterial species such as *Listeria*, which are still considered to be susceptible to almost all antibiotics, will eventually evolve toward multi resistance (Charpentier and Courvalin, 1999).

Listeria strains are serotyped according to variation in the somatic (O) and flagellar (H) antigens (Allerberger, 2003; Borucki and Call, 2003). Thirteen serotypes are known for *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Serotyping antigens are shared among *L. monocytogenes*, *L. innocua*, *L. seeligeri* and *L. welshimeri*. Serotyping, although not allowing speciation, serves a useful purpose for confirming the genus diagnosis *Listeria* and for allowing a first level subtyping for epidemiological purposes (Allerberger, 2003). The introduction of a commercial kit for serotyping *Listeria* (Denka Seiken, Tokyo, Japan) greatly improved the availability of this method (Miettinen et al, 1999; Allerberger, 2003; Lukinmaa et al, 2003). Interestingly, although serotype 1/2a is the most frequently isolated from food, serotype 4b causes the majority of human epidemics (Borucki and Call, 2003). Therefore, it is likely that serotype designation is associated with virulence potential. Our serological tests reveled that only a few serotypes (1/2a, 1/2b and 1/2c) were present in Korea, the others were not detected. The prevalent serotype was 1/2c in food and the environment. In fact, the predominance of a few serotypes and their percentages distribution among the different types of samples tested is consistent with the findings of other

authors (Gianfranceschi et al, 2003): 95% of the strains of food borne and clinical *L. monocytogenes* linked to outbreaks or sporadic cases of listeriosis in various parts of the world are of the 1/2a, 1/2b and 4b serotypes (Gianfranceschi et al, 2003; Lukinmaa et al, 2003).

Our results have shown that approxmately 70% and 60% of 70 *L. monocytogenes* were resistant to Te and Mi, respectively, and approximately 90% and 30% of 25 *L. innocua* to Te and Mi, respectively. Furthermore, Mi resistance was always associated with Te resistance. The relatively high incidence of Te resistance in *Listeria* isolates may be due to extensive use of these antibiotics worldwide, in particular in animal feeds (Charpentier et al, 1995). Charpentier and Courvalin (1999) reported that Te resistance is the most frequent resistance trait in *L. monocytogenes* isolates from human. It has been proposed that enterococci constitute a reservoir of resistance gene for *L. monocytogenes* and that the gastrointestinal tract of human and animals is the most probable site for acquisition by *Listeria* species of conjugative plasmids and transposons from *Enterococcus* and *Streptococcus* species (Charpentier et al, 1995; Charpentier and Courvalin, 1999). In a recent study of 685 strains collected in France from human sources in 1994 and 1995, one *L. monocytogenes* strain was resistant to Cip (Tsakaris et al, 1997). *L. monocytogenes* resistant to high levels of Tmp, was detected in France (Charpentier et al, 1995). Resistance to S was observed in several clinical, and food and environmental strains of *L. monocytogenes* from France (Charpentier et al, 1995), Italy (Facinelli et al, 1991) and Switzerland (Facinelli et al, 1991; arpentier et al, 1995), and in food and environment strains of *L. innocua* from Italy (Charpentier et al, 1995). Resistant to E (MIC $>32\mu g/ml$) was detected in a study on antibiotic susceptibilities clinical strains isolated in the United Kingdom between 1987 and 1990 (MacGowan et al, 1990). However, our results showed that more than approximately 6% of *L. monocytogenes* isolates were resistant to Nor and Cip. No *L. monocytogenes* isolates were resistant to S and Tmp. Also, more than approximately 15% of *L. innocua* isolates were resistnat to S, Nor and Cip. No *L. innocua* isolates were resistant to Tmp. Only Te resistance was detected in 50% of *L. welshimeri*. The last antimicrobial agent, Va, is a glycopeptide which is active against Gram positive bacteria and is widely used in human medicine to treat infections by staphylococci and enterococci (Klein et al, 1998; Georgnaras and von Holy, 2001). Va is also the antibiotic of choice for the treatment of methicillineresistant *S.* aureus infections (Georgnaras and von Holy, 2001). Recent studies, however, have reported the emergence of methicilline-resistant *S aureus* strains with reduced susceptilbilities to Va from patients in Michigan, New Jersey, New York, France and Japan (Ploy et al, 1998; Tenoner et al, 1998; Rotun et al, 1999). Furthermore, Biavasco et al (1996) reported that via *Enterococcus faecium* LS10, glycopeptide resistance was transferred to *L. monocytogenes*, *L. ivanovii*, and *L. welshimeri* recipients. However, all *L. monocytogenes*, *L. innocua* and *L. welshimeri* isolates, in the present study, were fortunately susceptible to Va. No cases of resistance to P, Gm and Va have been reported for strains of *Listeria* spp. originating from humans, food, or the environment (Charpentier et al, 1995; Charpentier and Courvalin, 1999; Georgnaras and von Holy, 2001; Safdar et al, 2003).

Multiple antibiotic resistance in *L. monocytogenes* and *L. innocua* which appeared to be transferable to *Enterococcus, Streptococcus* and *Staphylococcus* were reported for clinical strains, and food and environment strains in France (Poyart-Salmeron et al, 1990; Quentin et al, 1990), Greece (Tsakaris et al, 1997), Italy (Facinelli et al, 1991) and Switzerland (Hadorn et al, 1993). Our results showed that among 70 *L. monocytogenes*, 75.7% was resistant to at least one antibiotics and 57.1% was resistant to more than 2 antibiotics. Furthermore, the multiple resistance patterns of *L. innocua* isolates were more complex than those of *L. monocytogenes*. The results of this study indicate that most *L. monocytogenes* and *L. innocua* isolates from Korea are resistant to antimicrobial agents including Te and Mi. Furthermore, it is reasonable to speculate they will increasingly acquire multiple antimicrobial resistant properties. The assumption that species of *Listeria* are susceptible to almost all antimicrobial agents needs to be reassessed, as does the risk of food borne infections due to resistant

L. monocytogenes strains.

Consequently, large scale studies to evaluate the incidence of all *Listeria* spp. isolated from foods of animals origin and their related environmental sites and antimicrobial resistance trends are warranted.

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