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Antimicrobial susceptibilities of *Listeria monocytogenes* strains isolated from food and food processing environment in Poland

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ABSTRACT

A total of 471 *Listeria monocytogenes* isolates from different types of food and food-related sources in Poland during 2004–2010 were examined. This number includes 200 isolates from fish, 144 from fresh and frozen vegetables, 43 ready-to-eat products (deli foods, cold cuts), 13 from dairy products, 16 from raw meats, 15 from confectionery products and 40 directly from processing plants. All isolates were subjected to serotyping and lineage assays using PCR, and antimicrobial susceptibility using E-test and a broth microdilution method. Of all isolates, 256 (54.4%), 120 (25.5%), 59 (12.5%), 36 (7.6%) were identified as serotypes 1/2a (or 3a), 1/2c (or 3c), 1/2b (or 3b or 7), and 4b (or 4d or 4e), respectively. A direct correlation between the most common serotypes and three *L. monocytogenes* lineages was also observed. All *L. monocytogenes* isolates belonged to lineages I (20.2%) and II (79.8%). All strains were sensitive to ampicillin, amoxicillin, gentamicin, erythromycin, trimethoprim, rifampicin, vancomycin, chloramphenicol and sulfamethoxazol. Two of the *L. monocytogenes* strains (0.42%) showed phenotypic resistance. One strain was resistant to tetracycline and minocycline due to the presence of *tet*(M). It did not carry gene *int*, which may indicate that the *tet*(M) gene in this strain was not integrated in the transposon Tn*916*-Tn*1545* family. The resistance of the second strain to ciprofloxacin and norfloxacin was attributed to active efflux associated with overexpression of gene *lde*. Our data indicate the low prevalence of antimicrobial resistance among *L. monocytogenes* isolates from food and food-related sources in Poland.

1. Introduction

Antimicrobial resistant bacteria are a biological hazard associated with increased human morbidity and mortality and are a serious public health concern. The use of antimicrobial agents in animals, plant production and the production of other sources of food and feed has adverse public health consequences in that it creates a reservoir of resistant bacteria and of bacteria-borne resistance genes that can be transmitted to humans through the food chain. (EFSA, 2008; White et al., 2002). The use of antimicrobials at subtherapeutic levels in food-producing animals has long been viewed as undesirable e.g. the (Swann report, 1969). Since January 2006 the use of antimicrobials as growth promoting agents has been banned within the EU in order to reduce the numbers of resistant bacteria in farm animals (Regulation (EC) No, 1831/2003). In fact, emerging antimicrobial resistance phenotypes have been recognized among multiple zoonotic pathogens including *Salmonella enterica* serovar Typhimurium DT104, *Escherichia coli, Campylobacter jejuni*, *Listeria monocytogenes*, and *Yersinia enterocolitica* (White et al., 2002).

L. monocytogenes is a Gram-positive pathogen that can cause listeriosis in humans and animals, e.g., cattle, sheep, poultry, birds, etc. In humans, severe illness mainly occurs in the unborn child, infants, the elderly and those with compromised immune systems (Hof, 2004). These organisms are among the most important causes of death from foodborne infections in industrialized countries (EFSA, 2012). *L. monocytogenes* is estimated to cause nearly 1600 illnesses each year in the United States, with more than 1,400 hospitalizations and 250 deaths (Scallan et al., 2011). In the European Union the number of confirmed cases of listeriosis in 2010 was 1601. The overall notification rate of listeriosis was 0.35 cases per 100,000 population, with the country-specific notification rates, e.g. 1.33 in Denmark and 0.15 in Poland. The reported case-fatality rate was high, 17.0% (EFSA, 2012).

This opportunistic microorganism is ubiquitous in the environment, especially in plant matter and soil. The principal reservoirs are soil, forage and water. Other reservoirs include infected domestic and wild animals. The main route of transmission to both humans and animals is believed to be through consumption of contaminated food or feed (Farber and Peterkin, 1991; Gandhi and Chikindas, 2007; White et al., 2002).

With the exception of natural *in vitro* resistance to first generation quinolones, fosfomycin, and third-generation cephalosporins, *L. monocytogenes* is widely susceptible to clinically-relevant classes of antibiotics active against Gram-positive bacteria (Hof, 2003;

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Troxler et al., 2000). The first *L. monocytogenes* strains resistant to antibiotics were reported in 1988. The strains were resistant to >10 μg of tetracycline per ml (Poyart-Salmeron et al., 1990). The first multiresistant strain was isolated from a patient with meningoencephalitis in France in 1988 (Poyart-Salmeron et al., 1990). Since then, other strains of *L. monocytogenes* isolated from food or the environment or in sporadic cases of human and animal listeriosis resistant to one or several antibiotics have been described (Charpentier and Courvalin, 1999; Conter et al., 2009; Granier et al., 2011; Li et al., 2007; Morvan et al., 2010; Prazak et al., 2002; Rodas-Suárez et al., 2006; Safdar and Armstrong, 2003; Srinivasan et al., 2005; Vela et al., 2001; Walsh et al., 2001; Yan et al., 2010; Zhang et al., 2007). The levels of resistance are varied and influenced by antimicrobial use in humans and animals, as well as geographical differences. In view of these variations it would be advisable to implement a system monitoring the antibiotic resistance patterns of *L. monocytogenes* in food and environmental sources from different areas.

L. monocytogenes has 13 serotypes that differ in virulence potential (Kathariou, 2002). At least 95% of strains isolated from food and 98% of clinical strains isolated from human listerosis cases belong to serotypes 1/2a, 1/2b, 1/2c, and 4b. Among these serotypes, 1/2a is the most prevalent in food, and 4b is the most frequently detected serotype from human listeriosis cases (Ayaz and Erol, 2010). These serotypes can be grouped into genetic lineage I (serotypes 1/2a, 3b, 3c, and 4b) from human clinical listeriosis, lineage II (serotypes 1/2a, 1/2c, and 3a) from food isolates and clinical cases, and less common lineage III (serotypes 4a and 4c, as well as some 4b) from animal and environmental specimens (Nadon et al., 2001).

In spite of the importance of data on the incidence of drug resistant *L. monocytogenes* strains there is very little published information regarding the antimicrobial susceptibility of *L. monocytogenes* isolated from food and food-processing environments in Poland and no large-scale studies of this kind have been carried out at all. For this reason monitoring the occurrence of cases of resistance, even though relatively rare, should nevertheless be pursued. Consequently, the main objectives of this study were to: (i) evaluate the susceptibility of a large collection of *L. monocytogenes* strains isolated from different food products and food processing environments in the years 2004–2010 to 13 antibiotics used in the treatment of listeriosis and in veterinary practice, (ii) to determine the possible dissemination of drug-resistant strains in the environments embraced by the research, and also (iii) to identify the molecular mechanisms of resistance. An additional aim was to determine the frequency of the occurrence of specific serotypes and the correlation between the most frequent serotypes and lineage.

2. Materials and methods

2.1. Sample collection

The samples were collected from large retail outlets, smaller units and food-producing factories over a seven year period — from 2004 to 2010. Samples were collected in 5 cities in the central and north-east areas of Poland.

All food samples were transported to the laboratories inside portable insulated cold boxes, while the swabs were in sterile tubes. The samples were immediately subjected to microbiological analysis, which was carried out in accredited laboratories.

A total of 471 of *L. monocytogenes* isolates recovered from different types of food and food-related sources were examined. This number includes 200 isolates from fresh and smoked fish, 144 from fresh and frozen vegetables, 43 from ready-to-eat products (deli foods, cold cuts), 13 from dairy products, 16 from raw meats, 15 from confectionery products and 40 directly from processing plants (food-contact surfaces and instruments, and nonfood-contact surfaces such as floor).

2.2. Isolation and identifiacation of Listeria monocytogenes

The strains were isolated by standard procedure (ISO PN-EN ISO, 11290-1:1999/A1, 2005). 25 g of each food sample was taken in an aseptic manner and homogenized in 225 ml of Half Fraser Broth Base with Half Fraser Supplement. In the case of samples taken directly from food-processing plant environments, an area of approximately 100 cm^2 was swabbed using sterile swabs. Each swab was put in 100 ml Half Fraser Broth with supplement. Following 24±2 h incubation at 30 °C, 10 ml Fraser Broth was inoculated with 0.1 ml of the enrichment culture. After incubation for 48±2 h at 37 °C a loopful of the culture was surface streaked on ALOA agar nad Palcam agar plates. At least one colony from each plate suspected to be *Listeria* spp. was picked and cultured on Trypticase Soy agar Yeast agar (24 h, 37 °C). All the typical isolates were subjected to the following standard biochemical tests: Gram stain, catalyzed reaction, motility, beta-hemolysis reaction, acid prodution from rhamnose and xylose and CAMP test. The isolates that were characterized as *L. monocytogenes* were sent to the Department of Applied Microbiology, University of Warsaw.

To confirm the suspect isolates as *L.monocytogenes* the multiplex PCR method described by Huang et al. (2007) was applied. The following references strains were used: *L. monocytogenes* ATCC 13932, *L. grayi* ATCC 25401, *L. welshimeri* ATCC 35987, *L. seeligeri* ATCC 35967. *L. innocua* PZH 5/04 and *L. ivanovii* PZH 7/04 were from the collections of the National Institute of Public Health - National Institute of Hygiene (Warsaw, Poland).

The cultures were maintained in Trypticase soy-yeast extract agar (TSYEA) (Oxoid, Basingstoke, Hampshire, United Kingdom) at 4 °C throughout the study period and stored at −80 °C in brain heart infusion broth (BHI, Oxoid) containing 20% glycerol.

2.3. Identification of L. monocytogenes serotypes and lineages by PCR

After being confirmed as *L. monocytogenes* species, the isolates were serotyped by multiplex PCR according to the method described by Doumith et al. (2004) using primers lmo0737 (906 bp), lmo1118 (691 bp), ORF2819 (471 bp), and ORF2110 (597 bp) (Genomed, Warsaw, Poland). The lineages of the isolates were analyzed under the same primer and reaction conditions as the multiplex PCR method developed by Ward et al. (2004).

2.4. Antimicrobial susceptibility determination

The antimicrobial resistance of *L. monocytogenes* isolates to 13 antimicrobial agents was determined using E-test (AB Biodisk, Solna, Sweden) (norfloxacin, minocycline, trimethoprim, rifampicin, gentamicin, sulfamethoxazole) and a broth microdilution method (ampicillin, amoxicillin, tetracycline, ciprofloxacin, chloramphenicol, erythromycin, vancomycin). Standard antimicrobial agents (ampicillin, amoxicillin, tetracycline, chloramphenicol, vancomycin, ciprofloxacin, erythromycin) tested in the microdilution method were supplied as powders by Sigma-Aldrich, St. Louis, USA.

The E-test was performed using E-strips following the manufacturer's instructions on Mueller-Hinton agar plate containing 5% sheep blood (Oxoid) and incubated at 37 °C for 18 to 20 h.

The microdilution method was performed according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). Briefly, 100 μl of various concentrations of each of the seven antimicrobial agents diluted in cation-adjusted Mueller–Hinton broth (Becton, Dickinson and Company, Sparks, USA) supplemented with 5% lysed horse blood were dispensed into a 96-well plastic microdilution tray. Since 100 μl of bacterial suspension was added to each well, the concentration of each dilution of antimicrobial agent was twice the desired final concentration in the well. The inoculum contained 5×10^5 of cfu/ml. MICs were read after 18–20 h incubation at 37 °C.