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Characterization of nonpathogenic *Listeria* species isolated from food and food processing environment

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ABSTRACT

A total of 127 *Listeria* isolates from food and food processing environments, including 75 *L. innocua*, 49 *L. welshimeri*, 2 *L. seeligeri* and 1 *L. grayi* were tested for susceptibility to eight antimicrobials, benzalkonium chloride (BC), cadmium and arsenic. The isolates were also screened for the presence of extrachromosomal genetic elements - plasmids, and their restriction pattern types were determined. All strains were susceptible to ampicillin, ciprofloxacin, erythromycin, gentamicin, rifampicin, trimethoprim and vancomycin. Two of the *L. innocua* isolates showed resistance to tetracycline and minocycline. The resistance was determined by the presence of chromosomal localization of *tet*(M) gene, which was not integrated in the transposon Tn*916*-Tn*1545* family. Of analyzed isolates, 18.11% and 55.91% isolates were resistant to BC and cadmium, respectively, but all were susceptible to arsenic. Resistance to BC was correlated with resistance to cadmium - all BC resistant isolates were also resistant to cadmium. On the other hand, 67.61% of cadmium-resistant isolates were susceptible to BC, suggesting that cadmium and BC resistance were not always concurrent in *Listeria* species. 48.03% of isolates contained plasmids. The size of most of the identified replicons was in the range of 50–90 kb. All plasmids were classified into 12 groups with identical restriction pattern (I–XII). Interestingly, plasmids belonging to the same group were determined in isolates of the same species. Only in one case, plasmids with I-type profile were identified in *L. innocua* and *L. welshimeri*. There was an association between resistance to BC and plasmid DNA presence: all resistant isolates carried a plasmid. A correlation between resistance to cadmium and plasmid carriage was also observed in *L. innocua* and *L. seeligeri* isolates, but among resistant *L. welshimeri*, 23.08% of isolates did not have plasmids. This may suggest that resistance is associated with determinants located within the chromosome. To elucidate the adaptation strategies and ecology of *Listeria* spp., it is important to have a better understanding of its resistance to antimicrobials and environmental toxicants such as heavy metals and disinfectants.

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1. Introduction

The genus *Listeria* (phylum *Firmicutes*), covering a group of Gram-positive, non-spore-forming, rod-shaped bacteria, contains 17 species. Phylogenetic analysis showed the existence of four well-supported clades within the genus comprising: (i) *L. monocytogenes, L. marthii, L. innocua, L. welshimeri, L. seeligeri and L. ivanovii,* which we refer to as *Listeria* sensu stricto, (ii) *L. fleischmannii*, *L. aquatica* and *L. floridensis*, (iii) *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis*, *L. grandensis*, *L. riparia*, *L. borriae*, *L. newyorkensis* and (iv) *L. grayi* (den Bakker et al., 2014; Weller et al., 2015).

Listeria spp. are widely distributed in many different environments, including soil, surface water, vegetation, sewage, animal feed, farm environments, food processing environments, urban and suburban environments. Two species, *L. monocytogenes* and *L. ivanovii,* are facultative intracellular pathogens, the etiological agents of listeriosis - a food-borne infection. The other fifteen species are harmless environmental saprophytes (Bertsch et al., 2013b; den Bakker et al., 2014; Graves et al., 2010; Halter et al., 2013; Leclercq et al., 2010; Weller et al., 2015). Nonetheless, some of them, including *L. ivanovii, L. seeligeri, L. innocua, L. welshimeri,* and *L. grayi,* have been occasionally implicated in human clinical cases reports, mainly in individuals with suppressed immune functions and/or underlying illnesses (Liu, 2013).

Apart from *L. grayi*, listeriae are naturally susceptible to various antimicrobial agents such as glycopeptides, tetracyclines, trimethoprim, penicillins, carbapenems, rifampicin, macrolides, lincosamides, chloramphenicol and naturally resistant to most cephalosporins, they also show reduced susceptibility to fluoroquinolones (Troxler et al., 2000).

Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC) are among the most commonly used disinfectants. They possess antimicrobial effect against a broad range of microorganisms e.g. bacteria, yeast, molds, algae, viruses and play a critical role in controlling the spread of environmentally transmitted pathogens in

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healthcare and food-processing environments, as well as in the home (Gerba, 2015). They exhibit greater activity against Gram-positive bacteria than against Gram-negative ones (Jennings et al., 2015). Bacteria are regularly exposed to sublethal concentrations of disinfectants, and this can lead to a selective pressure for acquisition of resistance determinants or for adoptation of initially susceptible bacteria (Hegstad et al., 2010).

Agricultural and industrial practices have a crucial role in polluting the environment with heavy metal ions. Microbial populations in this habitat adapt to different concentrations of heavy metals and become resistant (Hobman and Crossman, 2014). Resistance to antibiotics, disinfectants and heavy metals is important for bacterial survival in contaminated environments.

Plasmids, the natural vectors of horizontal gene transfer (HTG), play a key role in the dissemination. of genes of adaptive value (including virulence factors). Their transfer may lead to creation of highly virulent, multiresistant strains as well as new emerging pathogens (Schmidt and Hensel, 2004). *Listeria* spp. are important model for gene transfer studies since these bacteria are characterized by high conservation of chromosome genomes, which is a unique feature among the phylum *Firmicutes*. Relatively small amount of exogenous DNA in the chromosomes suggests that plasmids are major factors in determining the variability of the host strains (den Bakker et al., 2010).

A lot of studies have focused on resistance of *L. monocytogens* strains isolated from different sources to antimicrobial agents, disinfectants, and heavy metals. However, not to much is known about the resistance to these compounds among *Listeria* spp. strains, especially isolated from the food processing plant environment. Therefore, in this study we conducted a comprehensive characterization of collection of nonpathogenic *Listeria* spp., including *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* derived from food and food processing environments in Poland. We determined the susceptibility of these strains to different antimicrobials, arsenic, cadmium and benzalkonium chloride (quaternary ammonium compound commonly used as a disinfectant) and assessed a correlation between resistance to these compounds and identified extrachromosomal genetic elements - plasmids.

2. Materials and methods

2.1. Sample collection and bacterial isolation

All samples were collected from large retail outlets, smaller retail stores and food-producing factories between 2001 and 2010. The samples were transported to the laboratory in portable insulated cold boxes and the swabs were transported in sterile tubes. The samples were immediately subjected to microbiological analysis.

The strains were isolated according to the standard procedure ISO PN-EN ISO 11290-1:1999/A1:2005 as described in detail elsewhere (Korsak et al., 2012). The isolates were pre-identified by beta-hemolysis reaction, acid production from rhamnose and xylose and CAMP test, followed by the multiplex PCR method described by Huang et al. (2007). 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. *L. innocua* PZH 5/04 and *L. ivanovii* PZH 7/04 were obtained from the collections of the National Institute of Public Health - National Institute of Hygiene (Warsaw, Poland).

Cultures were maintained in brain-heart infusion agar (BHI; Oxoid, Basingstoke, Hampshire, United Kingdom) at 4 °C throughout the study period and stored at −80 °C in BHI broth containing 20% glycerol.

2.2. Determination of susceptibility

2.2.1. Determination of antimicrobial susceptibility

The resistance of *Listeria* spp. isolates to 8 antimicrobial agents: ampicillin (MIC range 0.125–2 μg/ml), ciprofloxacin (0.063–8 μg/ml), erythromycin (0.032–2 μg/ml), gentamicin (0.032–2 μg/ml), rifampicin (0.016–2 μg/ml), trimethoprim (0.032–2 μg/ml), vancomycin (0.125–8 μg/ml), and tetracycline (0.063–64 μg/ml) was determined using broth microdilution method (antimicrobials supplied as powders by Sigma-Aldrich, St. Louis, USA) according to the approved CLSI guidelines M45-A2 for *L. monocytogenes* (CLSI, 2010). Classification of strains as susceptible, intermediate and resistant was based on a protocol from previous susceptibility study with different *Listeria* species (Troxler et al., 2000). Minimal inhibitory concentration (MIC) values of the antimicrobials against the isolates classified as resistant were retested three times. In the case of strains resistant to tetracycline, the MICs for minocycline were also determined.

2.2.2. Determination of BC and heavy metal susceptibility

BC and heavy metals susceptibility of *Listeria* spp. was assessed as described previously by Mullapudi et al. (2008) with minor modifications. Briefly, inoculum was prepared by selecting *Listeria* colonies from BHI agar incubated for 24 h, and re-suspending them in sterile saline solution to obtain turbidity of 0.5 McFarland units (Densitometer II, Pilava-Lachema Diagnostika, Czech Republic), which corresponds to approximately 1.5×10^8 cfu/ml. To determine susceptibility to BC, a 3 μl volume of bacterial suspension was dropped in duplicate on cation adjusted Mueller Hinton 1.2% agar plates (Becton, Dickinson and Company) with 1.2% defibrinated sheep blood supplemented with variable concentrations of BC: 0, 2.5, 5, 10, 20 and 40 μg/ml. To determine susceptibility to cadmium and arsen, 3 μl of cell suspension was dropped in duplicate onto Iso-Sensitest agar (ISA) (Oxoid), supplemented with 35, 70, 140, 200 μg/ml anhydrous cadmium chloride (Sigma) or 25, 50, 100, 200, 300, 500 μg/ml sodium arsenite (Sigma).

Each plate contained the panel of test isolates, as well as the designated negative control strains. The plates were incubated at 37 °C for 48 h, and the quantity of growth on the test plates was compared with that on control ISA or Mueller Hinton plates.

Strains were considered resistant to BC, cadmium and arsenic if they yielded confluent growth on agar supplemented with 10 μg/ml of BC, 70 μg/ml cadmium chloride and 500 μg/ml sodium arsenite, respectively (the criteria for *L. monocytogens* were adopted, Mullapudi et al., 2008). MICs were determined in at least two independent replicates.

2.3. DNA isolation

2.3.1. Genomic DNA isolation

Total genomic DNA was extracted using Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit as recommended by the supplier (EurX, Gdańsk, Poland) or Chelex-100 (Bio—Rad, Hercules, USA) resin-based technique. Three to five colonies from BHI plate were suspended in 50 μl of 5% Chelex-100. The suspensions were mixed and briefly centrifuged. After incubation for 20 min at 95 °C the samples were cooled on ice for 5 min and centrifuged at 2.400 x *g* for 3 min (Eppendorf MiniSpin Plus Centrifuge). The resulting supernatants were used as DNA templates in the PCR mixture.

2.3.2. Plasmid isolation

All *Listeria* spp. isolates were grown overnight in BHI broth at 37 °C. A 4 ml volume of the culture was harvested by centrifugation (4 °C, 3 min, 2.400 x *g*, Eppendorf MiniSpin Plus Centrifuge). The pelleted cells were used immediately for plasmid DNA preparation or frozen at −70 °C for use on the following day. Plasmids were isolated according to the method described by Anderson and McKay (1983).

2.4. Genetic basis of resistance mechanisms

2.4.1. PCR of tetracycline resistance genes and integrase gene

Based on antimicrobial susceptibility results, tetracycline and minocycline resistant *Listeria* isolates were further characterized by detecting the presence of *tet*(M), *tet*(K), *tet*(L), *tet*(S), *tet*(T) and *int*-Tn

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