

Prevalence and antimicrobial susceptibility of *Listeria* spp. isolated from bulk raw milk in north-eastern Poland

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Summary

The aim of the study was to determine the presence of *Listeria* spp. strains in the milk samples obtained from dairy farms in north-eastern Poland and to determine the profile of resistance to antibiotics recommended in the treatment of listeriosis.

500 samples of bulk milk were analyzed. Milk samples were obtained from dairy farms located in Warmia and Mazury region in Poland. Chronic mastitis, requiring frequent and long-term use of antibiotics has been documented in these herds. Isolation of *Listeria* spp. was performed according to the standard procedure PN-EN ISO 11290-1: 2017-07. Antibiotic resistance testing was performed by the disc diffusion method according to the Clinical & Laboratory Standards Institute (CLSI) recommendations.

In total, out of 500 samples of pooled milk, based on biochemical properties, 8 isolates were confirmed as belonging to the genus *Listeria* (1.6%). The further identification of *Listeria* strains on the basis of Microbact *Listeria* 12L showed that 3 strains (3/8, 37.5%) belonged to *L. monocytogenes* species and 5 strains (5/8, 62.5%) belonged to *L. innocua* species. The analysis of sensitivity to commonly used antimicrobial agents showed that all isolates, both belonging to *L. monocytogenes* and *L. innocua* species, were sensitive to ampicillin. Multidrug resistance, defined as resistance to at least three classes of antibiotics, was confirmed among four isolates (50%).

The studies undertaken revealed that raw milk can pose a risk for public health due to the prevalence of pathogenic *Listeria* spp. among which multidrug resistant strains are present. It is therefore necessary to rationalize the use of antibiotics and to monitor bacterial resistance in the food production environment.

Keywords: *Listeria* spp., contamination, antibiotic resistance, raw milk

Listeria spp. are widely distributed in the environment and are detected in samples of soil, plants, silage, manure, sewage or water (13). The environment of the digestive tract constitutes an ideal condition for the existence of this pathogen, therefore *Listeria* spp. is isolated from the intestine content of mammals, birds, fish, reptiles, amphibians, crustaceans and insects (34, 37). The presence of *Listeria* spp. both in animal and plant materials and in food is the consequence of its widespread occurrence in the environment, high resistance to physical and chemical factors, as well as the ability to survive and multiply in a wide range of temperatures. Dairy products made of unpasteurized

milk, ripened raw sausages, smoked fish and industrially produced Ready-To-Eat (RTE) are most commonly contaminated foods (23, 42, 50).

Bacteria of the genus *Listeria*, especially *L. monocytogenes*, are responsible for occasional but serious infections, both in humans and animals, causing a disease called listeriosis. In humans, two species are responsible for causing infection: *L. monocytogenes* and, less frequently, *L. ivanovii*. The elderly, pregnant women and newborns, allergy sufferers, diabetics, people with reduced immunity and after transplantation, i.e. people from the high-risk group of the so-called YOPI (Young, Old, Pregnant, Immunocompromised) are the

most susceptible to a severe course of disease (31, 53, 54). There are few reports on cases of listeriosis in humans caused by *L. ivanovii* (17, 46). Listeriosis can take the form of both sporadic disease and epidemic outbreaks with high mortality. In 2019, overall case mortality of listeriosis in the EU was high (17.6%) and increased compared to the levels noticed in 2018 (13.6%) and 2017 (15.6%). The number of reported deaths from listeriosis in relation to the rates of campylobacteriosis (0.03%) and salmonellosis (0.22%) is significantly higher, which makes listeriosis one of the most serious foodborne diseases. In 2019, 28 Member States reported 2,621 confirmed invasive cases of listeriosis in humans with an EU reporting rate of 0.46 per 100,000 inhabitants. According to a report of the European Food Safety Authority (EFSA), published in 2021, listeriosis ranks fifth among the most frequently reported zoonoses in Europe (10, 11).

Antibiotic therapy is one of the most effective forms of treatment of prolonged or severe bacterial infections occurring in humans and animals. However, the growing number of pathogens resistant to antimicrobial agents has a significant impact on their effectiveness. Currently, antibiotic therapy is the only treatment for listeriosis. The antibiotics of first choice are usually penicillin, ampicillin and amoxicillin. In patients sensitive to penicillin, treatment with trimethoprim/sulfamethoxazole (TMP/SMX) is alternatively used in listeriosis. Erythromycin (recommended for pregnant women), meropenem, linezolid, rifampin and vancomycin are other antibiotics recommended in the treatment of listeriosis (22, 30, 48).

The aim of the study was to determine the presence of *Listeria* strains in milk samples obtained from dairy farms in north-eastern Poland and to determine the profile of resistance to antibiotics recommended in treatment of listeriosis.

Material and methods

Isolation and identification. Milk samples were obtained from dairy farms located in Warmia and Mazury region in Poland. Chronic mastitis, requiring frequent and long-term use of antibiotics has been documented in these herds.

The 500 samples of bulk milk were stored at 4°C and analyzed within 6 hours after their collection. Isolation of *Listeria* spp. was performed according to the standard procedure (39). Briefly, 25 ml of each sample was introduced into 225 ml of broth medium half-Frazer (BioMaxima), and incubated aerobically at 30°C for 24 h ± 2 h. Next 0.1 ml of the culture was transferred to 10 ml of Frazer medium (BioMaxima) and subjected to further incubation at 37°C for 24 h ± 2 h. From the obtained culture two parallel isolations were performed onto agar Ottaviani *i* Agosti ALOA (BioMaxima) and Palcam agar (BioMaxima). After 24-48 hours of incubation at 37°C, the colonies were assessed for phenotypic features characteristic of *Listeria* spp. In order to determine the species affiliation of the isolated microorganisms, five colonies with morphological features typical of *Listeria* spp. were selected from all the inoculated plates

and were then plated on TSEYA agar (BioMaxima) and incubated for 18 h ± 2 h. Next Gram-stained microscope slides were prepared to determine the morphology of bacterial cells, and a biochemical series of MicrobactListeria12L (Oxoid-Argenta) were performed to determine the ability to hydrolyse esculin, to hemolyse and the utilization of 11 carbohydrates. The test was performed in accordance with the manufacturer's instructions. Based on the results of the analysis of biochemical series, the species of isolated strains were determined.

Antimicrobial susceptibility testing. Antibiotic resistance testing was performed by the disc diffusion method according to the Clinical & Laboratory Standards Institute (CLSI) recommendations. Each selected colony was suspended in physiological saline to obtain a density of 0.5 McFarland. The suspension obtained was spread on the surface of Mueller-Hinton agar medium (BioMaxima) with the addition of 5% defibrinated horse blood (BioMaxima) and 20 mg/L β-NAD (MH-F) (Oxoid), and then discs soaked with an antibiotic in given concentrations: erythromycin (15 µg), ampicillin (2 µg), meropenem (10 µg), trimethoprim-sulfamethoxazole (1.25-23.75 µg) were placed there. The plates were incubated at 37°C for 18 ± 2 h in aerobic atmosphere. Inhibition zones were designated according to the CLSI standards.

Results and discussion

Health condition of dairy cows, hygienic conditions of the farm and milking are the most important factors determining the microbiological quality of milk. In most cases, milk within the mammary gland does not contain pathogenic bacteria, although microorganisms such as *Brucella* spp. (15), *Clostridium* spp. (24), *Salmonella* spp. (8, 57), *Staphylococcus aureus* (8, 25, 34), *Bacillus cereus* (40), *Escherichia coli* (8, 36), *Listeria monocytogenes* (8, 16, 38) and *Campylobacter* spp. (5, 20, 58) were isolated from milk samples in different geographical regions. In recent years, increasing attention has been paid to listeriosis outbreaks related to the consumption of food contaminated by *L. monocytogenes*. This fact constitutes a significant risk of foodborne infections in humans due to consumption of dairy products. Milk and its products, due to the high content of nutrients, are popular among consumers, simultaneously in these products the development and multiplication of *Listeria* strains occur. According to data published by EFSA (10), unpasteurized milk and dairy products contaminated with *Listeria* spp. are the most common causes of listeriosis in humans. In the present study, out of 500 samples of pooled milk, 12 were positive for strains exhibiting the characteristic features of phenotypes for the genus *Listeria*-blue-green colonies surrounded or not by a cloudy zone around the colony. Identification of *Listeria* strains on the basis of MicrobactListeria12L reagent showed that 3 strains (3/8, 37.5%) belonged to *L. monocytogenes* species and 5 strains (5/8, 62.5%) belonged to *L. innocua* species. The overall prevalence rate of *Listeria* spp. was estimated at the level of 1.6%. There are nu-

Tab. 1. Antimicrobial susceptibility of *Listeria* spp. strains isolated from bulk raw milk

Strain	Number	Erythromycin (E)			Meropenem (MEM)			Trimethoprim-sulfametaxazole (SXT)			Ampicillin (AMP)		
		Reference Values – EUCAST	Test zone value (mm)	R/S	Reference Values – EUCAST	Test zone value (mm)	R/S	Reference Values – EUCAST	Test zone value (mm)	R/S	Reference Values – EUCAST	Test zone value (mm)	R/S
<i>L. monocytogenes</i>	1	S ≥ 25 R < 25	24	R	S ≥ 26 R < 26	30	S	S ≥ 29 R < 29	33	S	S ≥ 16 R < 16	22	S
	3 ^a		23	R		25	R		23	R		17	S
	5 ^a		18	R		24	R		19	R		19	S
<i>L. innocua</i>	2		24	R		28	S		27	R		22	S
	4		25	S		27	S		17	R		22	S
	6 ^a		23	R		22	R		24	R		19	S
	7 ^a		21	R		23	R		21	R		19	S
8	24		R	32		S	26		R	21		S	

Explanations: R – resistant; S – susceptible; a – multidrug resistance strain (MDR)

merous data on the contamination of milk and cheese by *Listeria* spp. (41), however the contamination levels vary significantly between different geographical regions ranging from 1.46% in Cyprus (6) to 23% in Mexico (51). Interestingly, in Syria, the prevalence of *Listeria* spp. was recorded in 16.2% of raw cow's milk samples and in 12.4% of sheep's milk samples and in none of the goat's milk samples (2). Taking into consideration the species identification, the findings of our study showed the prevalence of *L. monocytogenes* in 0.6% of bulk tank milk samples. Similar results, was recorded previously in Sweden at the level of 1.0% (52) and in Latvia at the level of 1.4% (26). In turn, a higher prevalence rates of *L. monocytogenes* were found in Iran (5%) (32), Morocco (5.9%) (12) and the U.S. (6.5%) (49). Simultaneously in our studies the majority of positive samples (5/8, 62.5%) we identified as *L. innocua* with the overall prevalence rate at the level of 1.0%. These findings are in accordance with the results obtained by Jamali et al. (21) who reported the prevalence rate of *L. innocua* at the level of 57.8%. The remaining isolates identified by these authors were as follows: *L. monocytogenes* – 21.7%, *L. welshimeri* – 12% and *L. seeligeri* – 8.4%. In contrast Al-mariri et al. (2) noticed *L. monocytogenes* as the most prevalent isolate (41.6%), while the remaining isolates were *L. innocua* (17.8%), *L. ivanovii* (14.2%), *L. welshimeri* (9.5%) and *L. grayi* (4.7%). Moreover *Listeria* spp. is also often isolated from different kinds of cheese. Rahimi et al. (41) found that 28.3% of cheese that had been made from raw milk were contaminated with *Listeria* spp., while Rudolf and Scherer (43) noted a high prevalence rate (8.0%) of *L. monocytogenes* in soft and semi-soft cheeses produced from pasteurized milk.

In recent years, special attention is paid to the constantly growing antibiotic resistance of microorganisms present in food, which is a significant problem in the protection of public health. Resistance to antibiotics is also noted among *Listeria* strains isolated

from food, including milk and milk products (3, 45), which is especially hazardous to humans because antibiotic therapy is necessary in more serious cases of listeriosis. The majority of *Listeria* strains are sensitive to amikacin, ciprofloxacin, chloramphenicol, clindamycin, doxycycline, rifampin, vancomycin, and co-trimoxazole (9, 22, 48). Ampicillin alone or in combination with gentamicin are the most common antibiotics used for the treatment of *Listeria* infection. However, in patients with allergies or conditions excluding penicillin therapy, second-line drugs including trimethoprim/sulfamethoxazole, erythromycin, vancomycin and fluoroquinolones are recommended (9, 48). In the treatment of pregnant women, erythromycin is a particularly recommended antibiotic. Despite recommendations given there is a real problem in the increasing frequency of resistance of *Listeria* spp. to antimicrobial agents commonly used in treatment of listeriosis, including a significant percentage of multidrug-resistant strains (9, 22, 48).

In this study all isolated strains of *L. monocytogenes* and *L. innocua* were sensitive to ampicillin (Tab. 1). These findings are in accordance with the previous studies conducted by Korsak and Szuplewska (28), Korsak et al. (27) and Babacan (4) showing that 100% of *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* strains obtained from food and food processing environments and *L. monocytogenes*, *L. innocua* and *L. ivanovii* isolated from cow's milk were sensitive to ampicillin. However some authors noticed the resistance of *Listeria* spp. to β -lactams. In the studies conducted by Skowron et al. (45) penicillin resistance was the most common in *Listeria* isolates originating from milk (44.4%). An extremely high resistance rate of *L. monocytogenes* strains isolated from dairy farms to ampicillin, at the level of 92%, was noted by Srinivasan et al. (47).

Treatment of listeriosis is commonly undertaken with a combination of ampicillin and gentamicin, but meropenem in monotherapy has been found active

(19, 29). Meropenem is a broad-spectrum β -lactam from the carbapenem group. The results of studies published by Carryn et al. (7) and Marco et al. (33) showed a significantly low minimal inhibitory concentration against *L. monocytogenes*. Similarly, *in vitro* studies stressed the high susceptibility of *Listeria* spp. to this antibiotic. The studies performed by Babacan (4) showed that all *Listeria* strains originating from cow milk were sensitive to meropenem. In our study, sensitivity to meropenem was found among 4 out of 8 *Listeria* strains (50%), as well as in one *L. monocytogenes* strain and in three *L. innocua* strains. Similar results were presented by Skowron et al. (44), who showed high resistance of isolated *L. monocytogenes* strains to meropenem at the level of 33.3%. Also Aksoy et al. (1) noticed 6.7% of *L. monocytogenes* strains resistant to meropenem. Moreover, in the present study high resistance was noticed in relation to erythromycin (100% of *L. monocytogenes* and 80% of *L. innocua*) and trimethoprim/sulfamethoxazole (66.6% of *L. monocytogenes* and 100% of *L. innocua*). Aksoy et al. (1) and Skowron et al. (44) noted 26.7% and 66.6% of trimethoprim/sulfamethoxazole resistant *Listeria* strains, respectively (1, 44). These findings suggest that systematic susceptibility testing should be performed before prescribing trimethoprim as a therapeutic alternative in case of first-line treatment failure or intolerance to β -lactams (35). Moreover in our studies an extremely high resistance was observed against erythromycin (7/8, 87.5%). Also Morvan et al. (35) observed the high rate of resistance to erythromycin among *L. monocytogenes* isolates obtained from clinical samples (35). According to Morvan et al. (35) resistance in this case could be due to a chromosomal mutation. In turn Skowron et al. (45) noticed only a single *L. monocytogenes* strain isolated from soft cheese resistant to this antimicrobial agent. Whereas Babacan (4) noticed that all *L. monocytogenes*, *L. innocua* and *L. ivanovii* isolates originating from cow's milk were sensitive to erythromycin and sulfamethoxazole/trimethoprim.

Relatively high resistance to meropenem, erythromycin and trimethoprim/sulfamethoxazole observed in our study may be a result of long-term antibiotic therapy of herds of dairy cows. In our study, 50% of all tested isolates (2 strains of *L. monocytogenes* and 2 strains of *L. innocua*) were described as multidrug-resistant with a E MEM SXT resistance pattern (Tab. 1). However, the level of drug resistance was higher in *L. monocytogenes* strains (66.7%) than in *L. innocua* (40%). A similar level of antibiotic resistance was presented by Aras and Ardi (3), where 66.7% of *L. monocytogenes* and 62.5% of *L. innocua* strains were resistant to at least three of antimicrobial agent tested. The increasing resistance to several groups of antibiotics has been significantly worrisome for the recent years.

The studies undertaken revealed that raw milk can pose a risk for public health due to the prevalence of pathogenic *Listeria* strains among which multidrug resistant strains are present. Moreover isolated strains showed high sensitivity only to one group of tested antibiotics, while the remaining antimicrobial agents showed limited use due to significant resistance rate.

The results obtained show a significant weakness in the treatment of both animals and humans. It is therefore necessary to rationalize the use of antibiotics and to monitor bacterial resistance in the food production environment. Due to the small number of strains against which AST (antimicrobial susceptibility testing) was applied, it is justified to continue research in the field of antibiotic resistance, especially among strains obtained from dairy cattle herds.

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