

Prevalence of *Listeria monocytogenes* in milk, meat and foodstuff of animal origin and the phenotype of antibiotic resistance of isolated strains

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ABSTRACT: In 2000–2002 samples of raw materials (milk and beef, 518 samples), ready-to-cook foods and final products from milk (30 and 200 samples) and from meat (105 and 110 samples) and swabs from surfaces in two meat-processing plants (41 samples) were examined for the presence of *Listeria monocytogenes* (*L. monocytogenes*). 70 isolates were tested using the standard microdilution method for the susceptibility to 12 antimicrobial drugs, minimum inhibitory concentration (MIC) characteristics (MIC_m, MIC₅₀, MIC₉₀) were determined. *L. monocytogenes* was isolated from raw milk samples (15 samples). It was not isolated from any of the semi-finished and final milk products except for one sample of pasteurized milk. Furthermore *L. monocytogenes* was isolated from samples of raw beef, meat emulsion, fermented dry meat products and from swabs from production equipment. *In vitro* testing of susceptibility showed the considerable effectiveness of examined antimicrobial substances with streptomycin and norfloxacin being least effective (MIC_m = 4 µg/ml). Apart from the intermediate resistance (MIC = 1–2 µg/ml) and resistance (MIC = 4 µg/ml) to clindamycin (37 and 5 strains, respectively), all strains were susceptible to the tested substances. While the presence of *L. monocytogenes* in foodstuffs, in particular, are serious, current tests of susceptibility of *L. monocytogenes* isolates indicate low probability untreatable infections as a result of resistant strains from foods or transfer of resistance to other microorganisms in the Czech Republic.

Keywords: pathogenic bacteria; acquired resistance; food safety

Diseases caused by *Listeria monocytogenes* (*L. monocytogenes*) in humans belong to notifiable infections in the Czech Republic. In the last decade, the number of infections has nearly doubled from 9 cases in 1993 to 20 and more in 2000–2002 (Epidat, 2002, 2003). Even though cases of listeriosis, in comparison to other infections, are not so frequent, it is a disease under close surveillance because of its serious character and high mortality which is 30% and more in predisposed persons (Cox, 1989). According to WHO (2002), epidemics of listeriosis are exceptional and cases are rarely sporadic.

The most common route of infection of humans is consumption of foods contaminated by *L. monocy-*

togenes (Vasavada, 1988; Jay, 1996). It is logical that one of the hygienic requirements for raw materials and foods of animal origin is the absence of pathogenic microorganisms, including *L. monocytogenes* (Regulation 91/1999 Sb., CZ, 1999). This microorganism, nevertheless, has been found in foods of animal origin (Jedlickova et al., 1991; Karpiskova, 1998). Milk products (cheese) are these foods considered as risk foodstuffs (Marth and Ryser, 1990; Greenwood et al., 1991). Despite this, cases of food-borne listeriosis, have more often occurred following consumption of meat and meat products (Jay, 1996; Pitt et al., 1999). *L. monocytogenes* is apparently rather a rare milk pathogen. Its presence was con-

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firmed in only 2 of the tested 111 bulk milk samples (1.8%) in the Czech Republic (Schlegelova et al., 2002). A similar result was published by Jensen et al. (1996) who demonstrated *L. monocytogenes* in only 1.2% of the 36 199 herds tested in 1972–1994. From the results of typing by a combination of several methods the authors conclude that some of the bovine strains may belong to types known to cause human infections. There have been a number of different reports on the behaviour of *L. monocytogenes* during the heat processing of milk (Doyle et al., 1987; Farber et al., 1988; Pitt et al., 1999). Anyhow, strains of *L. monocytogenes* were demonstrated in pasteurised milk and in milk products (Marth and Ryser, 1990; Greenwood et al., 1991; Binderova and Rysanek, 1999).

Another problem is the fact that strains of *L. monocytogenes*, previously susceptible, become resistant to one or more to antimicrobial drugs under the pressure of use of antimicrobials (Teuber, 1999). Furthermore, transmission of resistance among bacterial species has been demonstrated. Thus, transmission from *L. monocytogenes* to *Staphylococcus aureus* was described by Lemaitre et al. (1998) and from *Escherichia coli* to *S. aureus* and *L. monocytogenes* by Trieu-Cuot et al. (1993). Enterococci and streptococci, in particular, represent a reservoir of genes of resistance for *L. monocytogenes*. The gastrointestinal tract of humans being most probably the site where the transmission of genes of resistance from *Enterococcus* spp. and *Streptococcus* spp. to *Listeria* spp. takes place (Poyart-Salmeron et al., 1990). Resistance of such strains to antimicrobial drugs would pose a very serious problem. Transmission of resistant strains as well as individual genes of resistance among animal and human populations is considered to be very probable (Aarestrup et al., 2000).

The objective of this study was to obtain information on the prevalence of the pathogenic bacterium *L. monocytogenes* in bovine milk, meat and samples of foodstuffs and the prevalence of resistance to antimicrobial drugs among isolates.

MATERIAL AND METHODS

Samples

Selected samples: (a) raw bulk milk (18 farms, 278 samples), (b) raw milk from the tanker trailers (77 samples), (c) raw milk from the silo prior

to pasteurization (20 samples), (d) raw milk from the balance tank of the pasteurizer (14 samples), (e) pasteurized milk (20 samples), (f) corresponding samples of semi-finished (10 samples) and (g) final products from three milk processing plants (200 samples) were collected under sterile conditions during 2000–2002.

During the same period selected samples from beef meat were collected: (a) swabs from beef carcasses immediately following evisceration (12 samples representing 60 swabs), (b) samples of cooled processed meat (7°C; 56 samples, production of smoked and cooked meat products), (c) frozen processed meat (–18°C; 61 samples, production of fermented dry salami and sausages), (d) meat emulsion (105 samples) and (e) corresponding samples of final products (110 samples) in four meat-processing plants. Two types of final products investigated smoked and cooked meat products with heat processing at 70°C for 10 minutes (Fine sausages, salami and “Tišnovská” sausage) and fermented dry meat products without heat processing in the technology (“Čertovka” sausage, “Dunajská” sausage, “Mexičan”, “Poličan” and “Lovecký” salami).

In two meat-processing plants (with different technology) swabs from production equipment and hands of meat carvers (41 samples) were repeatedly collected. The samples for analysis were obtained by mixing 5 swabs from an area of 10 cm² and agitated into 20 ml of a selective culture broth (half Fraser broth). The same method was used for the swabs from the cervical and perianal region of individual carcasses.

Isolates

Samples were examined in accordance with EN ISO 112 90-1. The samples were repeatedly propagated in the Fraser broth, cultured on selective media Palcam and Oxford (Oxoid, Hampshire, U.K.). Suspect colonies of *Listeria* spp. were confirmed using a chromogenic culture medium Rapid L'mono (Sanofi Diagnostics Pasteur, France) and identified using the API *Listeria* system (bio Mérieux, La Balme les Grottes, France).

The isolated strains were kept in ITEST Cryobanks B (ITEST Hradec Kralove, Czech Republic) at –70°C. Prior to resistance tests, strains were resuscitated on blood agar plates. Then, four to five colonies were resuspended in 2 ml of the Mueller-Hinton broth (Oxoid, Hampshire, U.K.) and further diluted

Table 1. Composition and extent of dilution series of antimicrobial agents in microtitration plates (MP) for *L. monocytogenes* including interpretation criteria for resistance assesment in terms minimum inhibitory concentration

Antimicrobial agent	Range of dilution on MP (µg/ml)	Susceptible (µg/ml)	Intermediate/Resistant (µg/ml)
Ampicillin; sulbactam ^D	0.5; 0.25–64; 32	≤8; 4	16; 8/≥32; 16
Cephalothin ^{A,D}	0.25–32	≤8	16/≥32
Clindamycin ^{A,D}	0.25–32	≤0.5	1–2/≥4
Erythromycin ^D	0.25–32	≤0.5	1–2/≥8
Gentamicin ^{A,D}	0.25–32	≤4	8/≥16
Neomycin ^C	0.5–64	≤8	≥16
Oxacillin ^F	0.25–32		
Penicillin ^{B,A}	0.06–4	≤2	–/≥4
Streptomycin ^E	1–128	≤8	>16
Tetracycline ^{A,D}	0.25–32	≤4	8/≥16
Vancomycin ^A	0.25–32	≤4	8–16/≥32
Norfloxacin ^F	0.5–64		

^Ainterpretation criteria according to NCCLS (1999)^Bvalid for *L. monocytogenes*^Cinterpretation criteria according to Communiqué (1994)^Dinterpretation criteria according to Jorgensen et al. (1999)^Einterpretation criteria according to Report (2003)^Fnot determined for *L. monocytogenes*

as recommended by NCCLS (1999) with the same medium under spectrophotometric control to obtain suspensions with a density of approximately 5×10^6 CFU/ml. Sensitivity tests were done in all the strains.

Sensitivity to antimicrobial agents

Antimicrobial agents. Antimicrobial drugs for sensitivity tests were selected considering (a) antimicrobial drugs used most frequently for the treatment of bovine mastitis in the Czech Republic (cephalothin – CLT; clindamycin – CLI; neomycin – NEO; oxacillin – OXA; penicillin – PEN; streptomycin – STR; tetracycline – TTC), (b) regulations limiting the use of selected antibiotics for the treatment of animals (norfloxacin – NOR; chloramphenicol – CMP), (c) importance to the treatment of human infectious diseases (erythromycin – ERY; vancomycin – VAN). Ampicillin; sulbactam (AMS) and gentamicin (GEN) were also included into the tests.

Twofold dilution series of antimicrobial drug standards (Sigma, St. Louis, MO) (Table 1) were prepared as recommended by Hindler et al. (1992). The drugs were diluted with the Mueller-Hinton broth supplemented with Ca^{2+} and Mg^{2+} (CAMHB). For OXA, the medium was further supplemented with sodium chloride (20 g/l) as recommended by Tamashiro (1992a).

Microdilution technique for the determination of MIC. Microtitration plates (MP) for tests of sensitivity to antimicrobial drugs by the standard microdilution technique were prepared as described by Tamashiro (1992a,b).

Ten micro-litre volumes of standardised suspensions of the individual strains were pipetted into each well of MP containing 100 µl of the appropriate antimicrobial drug dilution series. The MP were incubated for 18 h at 35°C and turbidity, indicating the growth of bacteria, was read at 630 nm using the apparatus Morwell Plate Reader (Morwell Diagnostics GmbH, Switzerland). Each strain was tested simultaneously in two MP.

Control reference strain of *Staphylococcus aureus* ATCC 29213 and strain *Listeria monocytogenes* CCM 5580 (The Czech Collection of Microorganisms, Brno, Czech Republic) were tested with each batch of MP and once a week during the test period.

Evaluation

Prevalence of *L. monocytogenes* in selected samples was assessed. MIC and the characteristics of MIC_m, MIC₅₀, MIC₉₀ and the range of MIC were evaluated for individual bacterial strains and antimicrobial drugs. MIC_{m(modal)} represents the most commonly encountered values of MIC. MIC₅₀ and MIC₉₀ include 50% and 90% of strains with the given or lower MIC value. The isolates were classified as "sensitive", "intermediate resistant" or "resistant" using the interpretation criteria for MIC given in NCCLS (1999), or for AMS and ERY in Antibacterial Susceptibility Tests (Jorgensen et al., 1999), or for NEO and STR in Communiqué (1994) and Report (2003) (Table 1).

RESULTS AND DISCUSSION

Prevalence of *L. monocytogenes* in milk samples and dairy products

In study the pathogenic microorganism *L. monocytogenes* was isolated from 15 samples of raw milk and one sample of pasteurized milk. It was not found in any one of the collected samples of semi-finished

and final products of milk (Table 2). The source of *L. monocytogenes* in raw milk is mostly the gastrointestinal tract of animals and the environment, skin of the teats, in particular (Harvey and Gilmour, 1992; Sanaa et al., 1996). Shedding of *Listeria* into milk due to chronic mastitis (O'Donnell, 1995) is less frequent. When examining bulk milk samples we found a low prevalence (2.1%) of *L. monocytogenes*. A similar frequency of findings of *L. monocytogenes* (0–5%) in bulk tank milk samples has been reported from countries such as Austria 1.5% (Deutz et al., 1999), Spain 3.6% (Gaya et al., 1998), India 1.7% (Adesinyun et al., 1996), USA 4.1% (Rohrbach et al., 1992) and Canada 1.9% (Fedio and Jackson, 1990). In the current study we isolated *L. monocytogenes* even from a balance tank of the pasteurizer and corresponding sample of pasteurized milk collected immediately after pasteurization (72.6°C, 15 seconds) under conditions high-temperature short-time milk pasteurization (HTST). There are different opinions on the survival after pasteurization treatment. At present, however, it is presumed that *L. monocytogenes* is killed by heating to 72°C at least 15 seconds (Pitt et al., 1999). It is, nevertheless, true that so-called pasteurized milk was responsible for several epidemics of listeriosis (Jay, 1996; Pitt et al., 1999). Contamination after pasteurization or faults of technology during pasteurization (inadequate temperatures or duration of pasteurization, technical faults) are held responsible for the presence of *L. monocytogenes* in pasteurized milk. The temperature of pasteurization at the time of collecting the positive sample following pasteurization, however, was 72.6°C and secondary contamination

Table 2. Prevalence of *L. monocytogenes* in selected samples of milk and milk products

Samples	Numbers of samples examined	Numbers of samples contaminated	Numbers of <i>L. monocytogenes</i> isolates from selected samples
Raw bulk milk	278	6 (2.1%)	11
Raw milk from tanker trailer	77	4 (5.1%)	8
Raw milk from silo prior to pasteurization	20	3 (15.0%)	6
Raw milk from the balance tank of the pasteurizer	14	2 (14.0%)	3
Pasteurized milk	20	1 (5.0%)	3
Semi-finished products	10	0	0
Final products	200	0	0
Total	619	16 (2.6%)	31

from the environment was improbable, so there are several other explanations such as inadequate duration of pasteurization and/or technological faults. The fact that the microorganism was not found in any further corresponding sample of semi-finished and final products, in spite of its presence in the raw material, supports the importance of observing conditions of HTST pasteurization for the production of dairy foodstuffs. Production of the so-called cottage cheese under conditions not eliminating the possibility of secondary contamination by *L. monocytogenes* seems to be problematic with this respect (Hicks and Llund, 1991).

Prevalence of *L. monocytogenes* in swabs from meat and samples of meat products

As *L. monocytogenes* occurs in the intestinal tract of animals (Buncic, 1991; Renterghem et al., 1991), contamination of raw meat and other materials during slaughtering and cutting is possible (Adesiyun and Krishnan, 1995; Fenlon et al., 1996). This fact is responsible for findings of *L. monocytogenes* in samples from carcass swabs, raw meat and produc-

tion equipment (Table 3). *L. monocytogenes* has been found in different kinds of raw meat including beef. In the samples of beef examined there has been a relatively high frequency of positive findings amounting to, for example, 9.3% (Kwiatek et al., 1992), 8.6% (Pan et al., 1996) and 30% of samples (Amoril and Bhunia, 1999), and 20.8% (Sramova et al., 2000) and 12.5% of samples in the Czech Republic, respectively (Karpiskova, 1998). It is, therefore, not surprising that within the present study *L. monocytogenes* has been isolated from samples of fore and hind beef processed meat (Table 3). Findings of *L. monocytogenes* in swabs from tools and working surfaces (Table 3) witness the fact that contamination of meat and meat products is due to secondary soiling from the environment or equipment of meat-processing plants (Wendlandt and Bergann, 1994; Samelis and Metaxopoulos, 1999). Contamination generally increased during cutting, probably as a result of cross contamination (Correge, 1997).

Within the present study, *L. monocytogenes* was isolated from the meat emulsion to produce "Tišnovská" sausage (T. sausage) and "Mexičan" salami (M. salami). In one of these products (i.e., the T. sausage) *L. monocytogenes* was found even in

Table 3. Prevalence of *L. monocytogenes* in samples from carcasses, raw meat, meat emulsion, final products and in swabs from the production environment of the meat-processing plants with different technology

Samples	Meat-processing plants					
	production of smoked and cooked meat products			production of fermented dry meat products		
	numbers of samples examined	numbers of samples positive	numbers of isolates	numbers of samples examined	numbers of samples positive	numbers of isolates
Swabs from carcasses						
Swabs from the cervical area	3	0	0	3	0	0
Swabs from the perianal area	3	0	0	3	1	2
Raw meat						
Fore beef processed meat	53	1	1	18	3	4
Hind beef processed meat	3	1	2	43	3	4
Meat emulsion	65	1	1	40	3	3
Final products	65	0	0	45	8	14
Swabs from the production equipment	18	1	3	18	3	5
Swabs from hands of carving persons	2	0	0	3	0	0
Total	212	4	7	173	21	32

the raw material. Samples of the corresponding final products of the T. sausages and M. salami, however, were negative. As the technological process of production of the T. sausage includes heat processing (70°C for 10 min), it is probable that *L. monocytogenes* bacteria contaminating the semi-finished product were killed by the heat. The M. salami, however, belongs to fermented dry products without heat processing. Of 110 final meat products, *L. monocytogenes* has only been found in samples of fermented dry products without heat processing (8 samples of "Lovecký" salami and "Čertovka" sausage). Positive findings in this kind of products are in agreement with those by other authors (Kriem et al., 1998). The presence of *L. monocytogenes* and survival of microorganism in raw salami has frequently been discussed (Buncic et al., 1991; Dabrowski et al., 1999). A number of factors play a role in the composition of microflora of the product including the effect of starter cultures, in particular. It has been found that although numbers of *L. monocytogenes* decrease during the production, once storage of the product begins the bacteria survive for a long period (Buncic, 1991). We have confirmed that *L. monocytogenes* may survive during the production of these meat products. *L. monocytogenes* has not been found in any smoked and cooked meat products in this study. Other authors, however, did find the organism in such products (Caseiro et al., 1991; Schmidt

and Leistner, 1991; Wellhaeuser, 1992; Casolari et al., 1994; Sramova et al., 2000). Heat processing is an important technological procedure with smoked and cooked meat products and is responsible for destruction of any *L. monocytogenes* cells present. Its occurrence in the final product is then attributed to secondary contamination (Uyttendaele et al., 1999).

Tests of susceptibility to antimicrobial drugs

Excepting STR, all antimicrobial drugs tested within this study effectively prevented the growth of *L. monocytogenes* (Table 4). Inhibitory concentrations of drugs varied from ≤ 0.06 µg/ml (PEN) to ≤ 0.5 µg/ml (AMS, CLT, CLI, ERY, GEN, NEO, NOR, OXA, TET and VAN). Values of MIC_m, MIC₅₀, as well as MIC₉₀ were in agreement with values of MIC for susceptible strains with the exception of CLI. MIC CLI values amounted to intermediate resistance (1–2 µg/ml) and resistance (≥ 4 µg/ml) in 37 and 5 strains, respectively, isolated from meat, milk and meat products. Values of MIC_m, MIC₅₀ as well as MIC₉₀ for this antimicrobial drug amounted to those of intermediate resistant strains. Resistance to CLI in strains isolated from meat has been described by Barbuti et al. (1992) also.

Table 4. Minimum inhibitory concentration (MIC) characteristics (MIC₅₀, MIC₉₀ and MIC_m and the range of MIC) of antimicrobial agents for *L. monocytogenes* strains

Antimicrobial agents	Range MIC	MIC ₅₀	MIC ₉₀	MIC _m
	(µg/ml)			
Ampicillin; sulbactam	≤ 0.5 ; 0.25–1; 0.5	≤ 0.5 ; 0.25	0.5; 0.25	0.5; 0.25
Cephalotin	≤ 0.25 –2	2	2	2
Clindamycin	≤ 0.25 –4	1	2	2
Erythromycin	≤ 0.25 –0.5	≤ 0.25	0.25	0.25
Gentamicin	≤ 0.25 –1	≤ 0.25	0.5	0.25
Neomycin	≤ 0.5 –4	1	2	0.5
Norfloxacin	≤ 0.5 –16	4	8	4
Oxacillin	≤ 0.25 –4	1	2	2
Penicillin	≤ 0.06 –2	0.125	0.25	0.06
Streptomycin	≤ 1 –16	4	8	4
Tetracycline	≤ 0.25 –2	0.5	1	0.5
Vancomycin	≤ 0.25 –1	1	1	1

No interpretation criteria of phenotypic evaluation of *L. monocytogenes* strains regarding resistance to OXA have been determined. Values of MIC of this antimicrobial drug varied in a wider extent than in two other β -lactam antibiotics (0.25–4 $\mu\text{g/ml}$) and the MIC₉₀ value equalled to 2 $\mu\text{g/ml}$. Barbuti et al. (1992) classified OXA as an antimicrobial drug to which strains of *L. monocytogenes* show variable response. Concerning NOR, there are no other interpretation criteria than for isolates from urine and the urinary tract. It is, therefore, not possible to classify the strains as susceptible or resistant. In the range of MIC for NOR we even found values of 16 $\mu\text{g/ml}$ that are typical for resistant strains of isolates from cases of urinary tract infection.

Also other authors found this microorganism to be considerably susceptible *in vitro* to frequently used antimicrobials (Abraham et al., 1998; Marco et al., 2000). Many antimicrobials highly effective *in vitro*, however, show lower efficacy against this microorganism *in vivo*. It is due to their poor ability to penetrate phagocytes and cause destruction of ingested *L. monocytogenes* bacteria or their bacteriostatic rather than bactericidal effect (Rivera et al., 1993). The antibiotic of choice due to its best therapeutic effects in human medicine is a combination of synergistic drugs such as penicillin (ampicillin) and aminoglycosides (Charpentier and Courvalin, 1999). Patients sensitive to penicillin are given trimethoprim (alone or in a combination with sulfamethoxazole), tetracycline, erythromycin or chloramphenicol (Poyart-Salmeron et al., 1990). Strains resistant both to ampicillin, penicillin and gentamicin have already been isolated (Rota et al., 1996). None of the isolates of *L. monocytogenes* in our study was resistant to the β -lactam antimicrobial drugs or the tested aminoglycosides, which along with β -lactam antimicrobial drugs are frequently used to treat animal infections. *Listeria* spp. are naturally resistant or have intermediate resistance to many recent cephalosporins (Rota et al., 1996; Troxler et al., 2000). As we have also confirmed, this does not apply to first generation cephalosporins (Wong et al., 1990).

The efficacy of the aminoglycoside antibiotic agent STR against *L. monocytogenes* was lower (Table 4), but quite comparable to other Gram positive bacteria isolated from raw materials and foods (apart from the *Enterococcus* spp.). Strains of *Staphylococcus aureus*, coagulase-negative staphylococci and *Bacillus cereus* were characterised by MIC₅₀ STR values varying in the range of 8–16 $\mu\text{g/ml}$ (Schlegelova et al.,

2002). Abuin et al. (1994) also found lower efficacy of STR (MIC₅₀ = 16 $\mu\text{g/ml}$) in strains of *L. monocytogenes* as well as the MIC₉₀ alongside to values of resistant strains (MIC_R = 32 $\mu\text{g/ml}$) so that at least 10% of strains isolated from foods and the environment of food processing plants were resistant to STR. Abraham et al. (1998) found up to 100% of strains from fresh made sausages to be resistant to STR.

Findings of resistant as well as poly-resistant strains of *L. monocytogenes* to antimicrobial drugs are rather sporadic. What is, however, serious is the fact that such strains have been isolated from foods. Walsh et al. (2001) frequently found resistance to TET and PEN in strains isolated from foods. Strains resistant to ERY were also isolated from foods (Barbuti et al., 1992; Rota et al., 1996). Transmission of resistance to ERY (the gene *ermC*) in strains isolated from foods was found among *Listeria* spp. and *Enterococcus faecalis* (Roberts et al., 1996). Strains of *Enterococcus* spp., on the other hand, may serve also as a donor of resistance to VAN for *L. monocytogenes* (Biavasco et al., 1996). Resistance to the glycopeptide antibiotic VAN is monitored very carefully (Aarestrup et al., 1998; van den Bogaard and Stobberingh, 1999) particularly in view of possible transmission of genes encoding resistance to VAN to MRSA strains which are difficult to control by therapeutic interventions (Chambers, 1988). Resistance to VAN was not demonstrated in any of our isolates.

While findings of *L. monocytogenes* in milk, in meat and foodstuffs made from them, in particular, are serious, tests of antibiotic susceptibility of *L. monocytogenes* isolates indicate low probability of infections as a result of resistant strains from foods or transmission of resistance to other micro-organisms in the Czech Republic.

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