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Genotypes of *Listeria monocytogenes* Strains Isolated from 2000 to 2002 in Poland

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Abstract

Pulsed field gel electrophoresis (PFGE), multiplex PCR and multilocus sequence typing (MLST) methods were used for genotyping study of seventy-three *L. monocytogenes* isolates collected in Poland between 2000 and 2002 from human, food, environment and a diseased goat. The multiplex PCR, which is an alternative method to classical serotyping, divided the isolates into four PCR groups, IIa (42.5%), IIb (27.4%), IIc (4.1%) and IVb (26%). The isolates displayed 33 distinct PFGE profiles. Twenty eight strains were further characterised by MLST based on sequence analyses of seven housekeeping genes. The combined sequence analyses revealed a total of 10 different allelic profiles from which 3 were not described earlier. It is intended that results obtained in this study will be the first data of a national database of *L. monocytogenes* genotypes in Poland.

Key words: *Listeria monocytogenes*, genotypes, PFGE, MLST, PCR

Introduction

Listeria monocytogenes is the causative agent of listeriosis, which occurs as sporadic and epidemic cases, both essentially related to the consumption of contaminated foods (Mead *et al.*, 1999). Neonates, pregnant women, the elderly, immunosuppressed transplant recipients and others with impaired cell-mediated immunity belong to a group with the greatest risk of development of listeriosis. Most frequent forms of listeriosis include meningoencephalitis, bacteremia and perinatal infections (Doganay, 2003). Although morbidity is relatively low (2–10 reported cases per million of population per year) listeriosis is characterised by a high mortality rate – 20–30%. Therefore listeriosis remains a great public health concern (de Valk *et al.*, 2003).

Various methods have been used to gain insight into clonal relationships between *L. monocytogenes* isolates for epidemiological investigation and surveillance purposes. These include serotyping (Seeliger and Hohne, 1979), bacteriophage typing (Loesser and Busse, 1990), multilocus enzyme electrophoresis – MLEE (Bibb *et al.*, 1990), ribotyping (Wiedmann *et al.*, 1996), random amplified polymorphic DNA differences – RAPD (Mazurier and Wernars, 1992) and DNA macrorestrition combined with pulsed field gel electrophoresis – PFGE (Brosch *et al.*, 1994). Recently multilocus sequence typing method – MLST (Salcedo *et al.*, 2003) and multiplex PCR (mPCR) – based serotyping (Doumith *et al.*, 2004a; Doumith *et al.*, 2005) have been developed.

Currently, PFGE constitutes the standard subtyping method for *Listeria* typing due to its reproducibility and high discriminatory power (de Valk *et al.*, 2003). Electronic databases of PFGE profiles are being created to detect new emerging clones and to trace infection clusters in the future (*e.g.* Swaminathan *et al.*, 2001; Lukinmaa *et al.*, 2003; Wagner and Allerberger, 2003).

Lately, Mędrala *et al.* (2003) have reported results of typing by RAPD and PFGE using SmaI endonuclease of strains originated from a Polish fish-processing plant. The aim of the present study was to gain

insight into diversity and clonal relationships between *L. monocytogenes* strains isolated in Poland from different sources using PFGE, multiplex PCR and MLST typing methods. It is intended that results obtained in this study will be the first data of a national database of *L. monocytogenes* genotypes.

Experimental

Materials and Methods

Isolates tested. A total of 73 isolates from clinical material samples ($n = 14$), food ($n = 50$), environment ($n = 8$) and a diseased goat ($n = 1$) were examined in the study. The strains isolated over the period from 2000 to 2002, without an evident epidemiological link among them, were received from 4 laboratories in Warsaw and from laboratories in Szczecin, Puławy, Gdańsk and Bydgoszcz. Strains were confirmed as *L. monocytogenes* by Api Listeria system (BioMérieux) and by determination of the haemolytic activity on a TSA agar plate supplemented with horse blood.

PFGE analysis. Isolation of whole bacterial chromosomes, macrorestriction of DNA with ApaI and AscI endonucleases and PFGE (CHEF-DR III system, Bio-Rad) were performed according to standardized protocol (Graves and Swaminathan, 2001). Gel images of PFGE profiles were analysed using BioNumerics software version 3.0 (Applied Maths). Pattern comparisons were made on band positions using Dice band-based similarity coefficient (maximum optimization 0.5 %; maximum position tolerance 1%). Two patterns were considered as different when they differed by only one band. Strain profiles were clustered by Unweighted Pair Group Method using Averages (UPGMA).

mPCR typing. mPCR typing method was that described by Doumith *et al.* (2004a). The developed method allows the separating of isolates of the species *L. monocytogenes* into four distinct PCR profiles in correlation with serovars. PCR profiles designated IIa, IIc, IIb and IVb correspond to *L. monocytogenes* isolates of serovars (1/2a-3a), (1/2c-3c), (1/2b-3b-7) and (4b-4d-4e) respectively. All remaining *Listeria* isolates are separated in the fifth PCR group designated L.

MLST analysis. The fragment sequences from the 7 housekeeping genes (*abcZ*, *dat*, *ldh*, *cat*, *dapE*, *bglA* and *lhkA)* were analysed as described by Salcedo *et al.* (2003). DNAStar software was used to compare obtained sequences. Sequences different even at a single nucleotide site were considered distinct alleles. Cluster analysis based on the joined sequences from the 7 genes were conducted using MEGA version 2.1 software (Kumar *et al*., 2001 – http://www.megasoftware.net/).

Results and Discussion

Of the 73 isolates tested by mPCR 31 represented PCR group IIa (42,5%), 20 – PCR group IIb (27,4%), 19 – PCR group IVb (26,0%) and 3 – PCR group IIc (4,1%). International data show that more than 90% of *L. monocytogenes* isolates from food and more than 98% of isolates from patients with listeriosis belong to only 4 of the 13 serotypes described – 1/2a, 1/2b, 4b and 1/2c (Doumith *et al.*, 2004a; Kathariou, 2002). Three isolates representing mPCR group IIa and 2 isolates of group IVb were classically serotyped and confirmed as serotypes 1/2a and 4b respectively (data not shown).

Thirty-three PFGE-profiles were obtained after DNA restriction with ApaI and 31 after restriction with AscI endonuclease (Table I). Four most prevalent pulsotypes (PTs) were represented by 11 (15.1%), 9 (12.3%), 5 (6.8%) and 4 (5.5%) isolates (total – 39.7%). However, the strains presenting these pulsotypes were received from one laboratory at the same time and thus may represent serial isolates of the same clone. According to data received from the laboratory, which provided strains presenting PT IX, the strains were originated from different sources including human. Theoretically it might be possible that epidemiological link between these strains really exists – environmental and food strains were isolated from samples of Odra River water and fish suggesting that listeriosis might have been developed after consumption of contaminated fish. Otherwise, accidental identities of profiles or samples contamination seem to be more probable.

The remaining pulsotypes were represented by 1 (23.3% of strains), 2 (24,7%) or 3 (12.3%) isolates suggesting quite great genetic diversity of the strains. PT XV was identical to PT of *L. monocytogenes* strain used as a control in Listeria Laboratory of Institut Pasteur, Paris (CDC reference strain H2446). Such identity of PTs of strains not linked by a source and time of isolation confirms observations on international prevalence of certain *L. monocytogenes* clones (Jacquet *et al.*, 1995). PTs V, XII, XXV and XXVIII are remaining examples of identity of strains isolated by different laboratories from different sources. The same PT was presented by isolates from milk (Puławy) and vegetables (Bydgoszcz) (2 times: PTs V and XXV). Two isolates from vegetables (Bydgoszcz – vegetables imported from Hungary) and 1 from fish (Szczecin) showed PT XII. Finally, both – an isolate from vegetables (Bydgoszcz – vegetables imported from Hungary) and a human one presented PT XXVIII.

Some human isolates, although different, may be related. The similarity of ApaI- and AscI-profiles of 2 IVb strains isolated in 2 different laboratories in Warsaw was 95%, similarity between 3 other IVb strains

Table I
Genotypes of *L. monocytogenes* isolates

Strains		mPCR profile	PFGE profile		Pulsotype
No	Origin (No)		ApaI	AscI	
2	F	IIa	1	20	I
2	F	IIa	2	25	II
1	F	IIa	3	31	III
1	H	IIa	4	27	IV
2	F	IIa	5	29	V
4	F	IIa	6	26	VI
2	F	IIa	7	26	VII
2	F	IIa	8	28	VIII
5	E (3), F (1), H (1)	IIa	9	22	IX
3	F	IIa	10	24	X
1	H	IIa	11	23	XI
3	F	IIa	12	30	XII
2	F	IIa	14	21	XIII
1	H	IIa	15	19	XIV
3	E	IIc	13	18	XV
9	F	IIb	16	5	XVI
1	H	IIb	17	5	XVII
1	F	IIb	18	6	XVIII
1	F	IIb	19	8	XIX
1	H	IIb	20	2	XX
1	H	IIb	21	1	XXI
1	H	IIb	22	7	XXII
2	E	IIb	31	9	XXIII
1	H	IIb	32	4	XXIV
2	F	IIb	33	3	XXV
1	F	IVb	23	13	XXVI
1	H	IVb	24	12	XXVII
2	F (1), H (1)	IVb	25	11	XXVIII
1	H	IVb	26	14	XXIX
11	F	IVb	27	10	XXX
1	A	IVb	28	17	XXXI
1	H	IVb	29	15	XXXII
1	H	IVb	30	16	XXXIII

F – food, H – human, E – environment, A – animal

Table II
Allelic profiles (STs) of *L. monocytogenes* isolates in comparison with PFGE and mPCR profiles

ST*	allele*							No of isolates	mPCR profile(s)	Pulsotype(s)
	abcZ	*bglA*	*cat*	*dap*	*dat*	*ldh*	*lhkA*			
6	3	1	1	1	3	2	3	6	IVb	XXVI, XXVIII, XXIX, XXX
11	3	9	9	3	3	2	5	3	IVb, IIb	XXXII, XXXIII, XX
14	4	4	4	3	2	2	5	6	IIb	XVI, XVII, XXI, XXII
15	2	1	11	3	3	6	7	1	IIb	XIX
19	5	7	3	5	1	3	6	1	IIa	XIII
21	5	8	5	7	6	1	1	5	IIa	IX, X, XI
22	6	5	6	4	1	1	1	1	IIc	XV
30	4	4	17	3	2	2	5	1	IIb	XVI
31	7	11	15	8	6	1	8	2	IIa	IV, V
32	11	1	12	19	3	6	7	2	IIb	XXIII, XXIV

* designations according to Salcedo *et al.* (2003) with exception of STs 30, 31, 32 and *cat* allele 17, which were not described in this article.

isolated in 3 Warsaw laboratories was 89% (ApaI) and 86% (AscI). Profiles of 2 IIa isolates (Szczecin and Warsaw) were similar in 89% (ApaI) and 80% (AscI), and profiles of 2 IIb isolates (2 Warsaw laboratories) – in 87% (ApaI) and 95% (AscI).

Number of identified alleles of sequenced genes was as follows: *abcZ* – 7, *bglA* – 7, *cat* – 10, *dapE* – 7, *dat* – 4, *ldh* – 4 and *lhkA* – 6 (Table II). All alleles but one of *cat* gene (17), were described previously by Salcedo *et al.* (2003). New allele corresponds to the previously described allele 1 (GenBank accession number AY158249) with a single substitution (C into an A in position 165). The isolates represented a total of 10 allelic profiles (ST), 3 of these profiles (30, 31, 32) were not described earlier (Salcedo *et al.*, 2003). The same isolates were divided by PFGE into 21 PTs. Similarity of PTs of isolates representing identical ST accounted for at least 80% with one exception – in the case of ST 11 the similarity between the PT of an isolate typed by mPCR as IIb and PTs of 2 other strains typed as IVb was less than 70%. MLST also failed

to distinguish isolates of different serotypes in the case of ST 22 characterising Spanish strain 1/2a (Salcedo *et al.*, 2003) and strain showing PCR-profile IIc (this study). On the other hand, one of the strains presenting identical PT (XVI) was distinguished by a specific ST (30) characterised by the presence of the new *cat* allele.

Dendrograms showing cluster analysis of PFGE-profiles as well as STs revealed that isolates tested could be divided into 2 major groups (data not shown). One of them included all isolates representing PCR profiles IIa and IIc and the second contained all isolates representing profiles IIb and IVb. Division of the isolates into two groups consisting of IIa, IIc and IIb, IVb isolates is in full agreement with the data obtained by many authors. A number of different typing studies suggested that 3 main lineages correlated with serovars exist within the *L. monocytogenes* species: lineage I comprised 1/2a, 1/2c and 3c serotypes, lineage II comprised 4b, 1/2b and 3b serotypes and lineage III comprised 4a and 4c serotypes (Wiedmann *et al.*, 1997; Doumith *et al.*, 2004b). Serotypes 4a and 4c were not represented among the strains tested.

Due to Polish law each case of listeriosis should be reported. According to data of Chief Sanitary Inspectorate and Department of Epidemiology of National Institute of Hygiene, Warsaw, Poland, the number of reported cases of listeriosis during the period of 1991–2000 accounted for 52. In 2001, 2002 and 2003 there were 9, 31 and 5 cases reported, respectively (Czarkowski *et al.*, 2003; Czarkowski *et al.*, 2004). These numbers might be largely underestimated. For comparison, in Finland, which has almost 8 times less habitants than Poland, during 11 years period (1990–2001) there were 314 cases of invasive listeriosis (20–50 cases per year) (Lukinmaa *et al.*, 2003). Similarly, Belgian Listeria Reference Centre receives between 30–50 human clinical strains of *L. monocytogenes* per year (Yde and Genicot, 2004).

L. monocytogenes is not a fastidious bacterium and should be isolated from clinical material samples quite easily. To gain reliable knowledge on prevalence of listeriosis in Poland, all cases of the disease must be reported. Furthermore, to trace outbreaks and follow long-term trends in the epidemiology of listeriosis, continuous typing of *L. monocytogenes* is necessary, preferably using internationally recognised typing methods according to standardised protocols. DNA macrorestriction and PFGE is one of such methods. mPCR and MLST offer a readily comparable data and might be very useful in long-term surveillance. mPCR is also useful as a first-line screening method to detect and trace an outbreak strain within numerous food isolates. It was mentioned earlier that in some countries national databases of listerial genotypes are being developed. Thus, Polish regional clinical and food laboratories are encouraged to collect isolated strains of *L. monocytogenes* for typing purposes. We hope that the data obtained in the present study will be the beginning of national database of *L. monocytogenes* genotypes.

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