A study of single nucleotide polymorphism in the *ystB* gene of *Yersinia enterocolitica* strains isolated from various wild animal species

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▌Abstract

Introduction and objective. *Y. enterocolitica* is the causative agent of yersiniosis. The objective of the article was a study of single nucleotide polymorphism in the *ystB* gene of *Y. enterocolitica* strains isolated from various wild animal species.
Materials and method. High-resolution melting (HRM) analysis was applied to identify single nucleotide polymorphism (SNP) of *ystB* gene fragments of 88 *Y. enterocolitica* biotype 1A strains isolated from wild boar, roe deer, red deer and wild ducks.
Results. HRM analysis revealed 14 different melting profiles – 4 of them were defined as regular genotypes (G1, G2, G3, G4), whereas 10 as variations. 24 of the examined *Y. enterocolitica* strains were classified as G1, 18 strains as a G2, 21 strains as a G3, and 15 strains as a G4. Nucleotide sequences classified as G1 revealed 100% similarity with the *Y. enterocolitica* D88145.1 sequence (NCBI). Analysis of G2 revealed one point mutation – transition T111A. One mutation was also found in G3, but SNP was placed in a different gene region – transition G193A. Two SNPs – transitions G92C and T111A – were identified in G4. Direct sequencing of 10 variations revealed 5 new variants of the *ystB* nucleotide sequence: V1 – transition G129A (3 strains); V2 – transitions T111A and G193A (2 strains); V3 – transitions C118T and G193A (1 strain); V4 – transitions C141A and G193A (2 strains); and V5 characterized by 19 SNPs: G83A, T93A, A109G, G114T, C116T, A123G, T134C, T142G, T144C, A150C, G162A, T165G, T170G, T174A, T177G, G178A, A179G, A184G and G193A (2 strains). The predominant genotype in isolates from wild ducks was G1; in red deer G2; in wild boar G3; in roe deer G1 and G4.
Conclusions. The proposed HRM method could be used to analyze *Y. enterocolitica* biotype 1A strains isolated from different sources, including humans.

▌Key words
HRM, SNP, wild animal species, *Y. enterocolitica*; *ystB*

INTRODUCTION AND OBJECTIVES

According to EFSA (European Food Safety Authority) reports, *Yersinia enterocolitica* is one of the most important foodborne pathogens. The epidemiology of *Y. enterocolitica* infections is complex and not fully elucidated, because the pathogen widely colonizes terrestrial and aquatic habitats, and various animals species [1, 2, 3, 4, 5, 6].

Not all *Y. enterocolitica* strains are pathogenic for humans and animals. Strains belonging to biotypes 1B and 2–5 are considered to be pathogenic, while biotype 1A strains without classical virulence markers are regarded as non-pathogenic [7, 8, 9]. Nonetheless, according to McNally et al. [10], biotype 1A strains are becoming the predominant pathogenic agent of yersiniosis (58% of the reported cases) in the Commonwealth countries, surpassing bioserotype 4/O:3 strains. This observation was confirmed by a recent case-control study of diarrheic patients in Finland, where the majority of isolated *Y. enterocolitica* strains belonged to biotype 1A [11].

It suggests that the previously determined pathogenicity criteria, based solely on bioserotype classification, may be insufficient.

The identification of virulence markers seems to be one of the reliable methods for pathogenicity determination. The *yst* gene, which encodes the production of enterotoxin Yst (*Yersinia* stable toxin), is one of the most important and genetically stable virulence markers of *Y. enterocolitica* strains. Yst is synthesized as a polypeptide chain composed of a 30-amino acid C-terminal domain and an 18-amino acid N-terminal signal sequence [12]. Yst has been divided into YstI enterotoxins (A, B and C) and the recently discovered YstII enterotoxin [9]. Classically, pathogenic strains possess the *ystA* gene that encodes YstA production [13].

Although *Y. enterocolitica* biotype 1A strains rarely produce YstA enterotoxin [14], more than 80% of them contain the *ystB* gene, which encodes the production of enterotoxin YstB – probably the main cause of diarrhea in clinical cases of yersiniosis caused by this biotype [13]. Rammamurthy et al. [15] demonstrated that 88.9% of *Y. enterocolitica* biotype 1A strains isolated from clinical cases of yersiniosis contributed to the accumulation of fluids in the intestines, which confirms their toxigenic potential. Therefore, *ystB* seems to be most appropriate virulence marker for determination of potential pathogenicity *Y. enterocolitica* biotype 1A strains.

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High-resolution melting (HRM) analysis is an emerging method for the detection of single nucleotide polymorphisms (SNPs) and for SNPs-based genotyping [16, 17]. In this method, the reduction in fluorescence is carefully monitored when a PCR product stained with a double-strand-specific fluorescent dye is heated through its melting temperature (T_m) [18]. HRM analysis is a single step procedure in a closed tube, and unlike in traditional melting analyses, its results are recorded in the shape of a melting curve [18].

The objective of the article was a study of single nucleotide polymorphism in the *ystB* gene of *Y. enterocolitica* strains isolated from various wild animal species.

MATERIALS AND METHOD

The study utilized 88 *Y. enterocolitica* biotype 1A isolates obtained from different wild animal species (Tab. 1). 41 *Y. enterocolitica* isolates were collected from harvested wild boar, roe deer, red deer and wild ducks immediately after the hunt. Forty-seven *Y. enterocolitica* isolates were obtained from three different regions of cold-stored carcasses of wild animals. Primary identification involved bacteriological methods, biotyping, serotyping and detection of virulence markers as described in previous papers [2, 3, 19]. Detailed characteristics of the isolated *Y. enterocolitica* strains are presented in Table 1.

Genomic DNA was isolated with the use of the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions, and it was stored at -20ºC for further analyses.

All HRM analyses were performed in the Rotor-Gene 6000™ real-time analyzer (Corbett Life Science, Sydney, Australia) using the PCR HRM curve analysis assay and Eva Green saturating dye (Type-it HRM PCR Kit, Qiagen, Hilden, Germany). Primer sequences used in the reaction: *ystB*-1 (Forward) 5'TGTCAGCATTTATTCTCAACT3' and *ystB*-2 (Reverse) 5'GCCGATAATGTATCATCAAG3' were previously described by Bancerz-Kisiel et al. [1]. 25 µl reaction mixtures were composed of 12.5 µl of 2x HRM PCR Master Mix, 10.15 µl of RNase-free water, 1.75 µl of the primer mix (final concentration of 0.7 µM each) and 0.6 µl of DNA (50 ng/reaction). The cycling conditions were: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 46 °C for 30 s, and 72 °C for 10 s. The amplified DNA was subjected to HRM at 0.1 °C increments at temperatures ranging from 65 °C – 90 °C.

Genotypes were identified based on the shape of the HRM curve observed in the Rotor-Gene software after normalization of HRM curves. RotorGene 6000 Series Software 1.7. was used for melting curve analysis. Samples of each of the four SNP genotypes and 10 variations were subjected to direct sequencing to verify genotyping results (Genomed Sp. z o.o., Warsaw, Poland). Sequence data from the examined strains were compared with the nucleotide sequence of *Y. enterocolitica* DNA for *Yersinia* Heat-stable Enterotoxin Type B complete cds, registered in GenBank under accession no. D88145.1, in the BLASTN vs. 2.2.18 program [20]. Multiple sequence alignment was performed in ClustalW [21] incorporated into the freeware Computational Evolutionary Biology package MEGA version 5.2.1. [22]. Nucleotide sequences were presented graphically in BioEdit v.7.2.0. software.

RESULTS

Defining a *ystB* gene fragment suitable for HRM was a compromise between minimizing the size of the fragment in order to simplify reaction and maximizing the size of the fragment so as to maximize the number of detected mutations. Initial experiments involved *ystB* gene fragments with a length of more than 179 bp (base pairs), which were used in the final stage of this study. The fragments were amplified by primers designed based on the *ystB* gene sequence (GenBank Accession No. D88145.1) in the Primer-BLAST programme available on the National Centre for Biotechnology Information (NCBI) website. These attempts, however, were unsuccessful, and the newly-designed primer pairs were unsuitable for *ystB* HRM analysis. Primers that were applied in previous studies by the authors of the presented study to detect *ystB,* proved to be effective.

HRM curves were compared based on shape and T_m values, they were normalized and difference graphs were plotted (Fig. 1). Examination of the normalized curves using normalization regions of 73–75 and 81–83 revealed the presence of 4 regular genotypes (G1, G2, G3, G4). 10 HRM curves with confidence percentage lower than 90 were defined as variations (one of them is presented on Fig. 1). 24 of the examined *Y. enterocolitica* strains were classified as G1, 18 strains as a G2, 21 strains as a G3, and 15 strains as a G4. The *ystB* nucleotide sequences from each of the 4 genotypes and nucleotide sequences of 10 variations were directly sequenced to verify HRM analysis. Direct sequencing revealed that the examined nucleotide sequences (after cutting and processing) had a length of 153 bp according to the NCBI. They were linked in position 41–194 in *Y. enterocolitica* DNA for *Yersinia* Heat-stable Enterotoxin Type B complete cds (GenBank Accession No. D88145.1).

A sequence alignment analysis of nucleotide sequences classified as G1 revealed 100% similarity with the *Y. enterocolitica* D88145.1 sequence (Fig. 2). Analysis of sequences of G2 revealed one point mutation in the examined region: transition T111A (Fig. 2). One mutation was also found in *ystB* nucleotide sequences of G3, but SNP was placed in a different gene region – transition G193A (Fig. 2). By contrast, 2 SNPs – transitions G92C and T111A – were identified in *ystB* nucleotide sequences of G4 (Fig. 2). Direct sequencing of 10 variations revealed that none of them were correlated with regular genotypes. 5 new variants of *ystB* nucleotide sequence were observed. 3 variations (V1) were characterized by one point mutation – transition G129A (Fig. 2). 2 SNPs were detected in the subsequent 5 variations, but they were located at different positions for a given group of strains: V2 – transitions: T111A and G193A; V3 – transitions: C118T and G193A; V4 – transitions: C141A and G193A (Fig. 2). In two variations (V5), 19 SNPs were found in the examined nucleotide sequences: G83A, T93A, A109G, G114T, C116T, A123G, T134C, T142G, T144C, A150C, G162A, T165G, T170G, T174A, T177G, G178A, A179G, A184G and G193A (Fig. 2). Variations were found only in *Y. enterocolitica* isolates from cold-stored carcasses of wild animals.

Y. enterocolitica strains from wild ducks were isolated only from samples collected immediately after harvest. 60% of these strains belonged to G1, and 40% – to G4 (Tab. 2). *Y. enterocolitica* strains isolated from roe deer immediately after harvest belonged to G2, whereas in the group of strains isolated from cold-stored carcasses, genotypic diversity was

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Table 1. Genotypes of *Y. enterocolitica* biotype 1A strains analyzed in the study

Strain	Host	Sampling site	Year of isolation	Serotype	ail	ystA	ystB	Genotype
Ka16PSB	wild duck	Cloaca	2010	O:8			+	4
Ka27PSB	wild duck	Cloaca	2010	O:8			+	4
Ka32PSB	wild duck	Cloaca	2010	O:5			+	1
Ka35PSB	wild duck	Cloaca	2010	O:8			+	1
Ka38PSB	wild duck	Cloaca	2010	NI*			+	1
K6ITC	roe deer	Rectum	2010	NI			+	2
K6PSB	roe deer	Rectum	2010	NI			+	2
J2ITC	red deer	Rectum	2010	O:5			+	1
J12PSB	red deer	Rectum	2010	NI			+	1
1PSB	wild boar	Rectum	2011	NI	+		+	4
5PSB	wild boar	Rectum	2011	NI			+	3
9PSB	wild boar	Rectum	2011	NI			+	3
16PSB	wild boar	Rectum	2011	O:8			+	3
17ITC	wild boar	Rectum	2011	O:27			+	3
64ITC	wild boar	Rectum	2012	NI	+		+	3
64PSB	wild boar	Rectum	2012	NI	+		+	3
68PSB	wild boar	rectum	2012	NI			+	3
69ITC	wild boar	rectum	2012	NI			+	1
69PSB	wild boar	rectum	2012	NI			+	3
76PSB	wild boar	rectum	2012	NI			+	4
77PSB	wild boar	rectum	2012	O:8			+	3
101PSB	wild boar	rectum	2012	NI	+		+	2
104ITC	wild boar	rectum	2012	NI	+		+	4
104PSB	wild boar	rectum	2012	NI	+		+	2
136PSB	wild boar	rectum	2012	NI			+	2
143ITC	wild boar	rectum	2012	O:27	+		+	2
143PSB	wild boar	rectum	2012	NI	+		+	2
148PSB	wild boar	rectum	2012	NI	+		+	2
149PSB	wild boar	rectum	2012	NI	+		+	2
150ITC	wild boar	rectum	2012	NI			+	4
150PSB	wild boar	rectum	2012	NI			+	4
152PSB	wild boar	rectum	2012	NI			+	4
156ITC	wild boar	rectum	2012	NI			+	3
159PSB	wild boar	rectum	2012	NI			+	3
177PSB	wild boar	rectum	2012	NI			+	4
179PSB	wild boar	rectum	2012	NI			+	2
206PSB	wild boar	rectum	2013	NI			+	3
224PSB	wild boar	rectum	2013	NI			+	3
248ITC	wild boar	rectum	2013	NI			+	1
256PSB	wild boar	rectum	2013	NI	+		+	1
258PSB	wild boar	rectum	2013	NI			+	1
1(T)PSB	roe deer	carcass; peritoneum	2013	O:8			+	V1
1(O)PSB	roe deer	carcass; perineum	2013	NI			+	V1
2(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	2
2(T)PSB	roe deer	carcass; peritoneum	2013	NI			+	1
2(O)PSB	roe deer	carcass; perineum	2013	NI			+	V1
6(T)PSB	roe deer	carcass; peritoneum	2013	NI			+	1
8(T)PSB	roe deer	carcass; peritoneum	2013	O:27			+	2
8(O)PSB	roe deer	carcass; perineum	2013	NI			+	3
9(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	V5
10(O)PSB	roe deer	carcass; perineum	2013	NI			+	2
11(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	4
11(O)PSB	roe deer	carcass; perineum	2013	NI			+	1
12(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	2
12(T)PSB	roe deer	carcass; peritoneum	2013	O:8			+	2
13(T)PSB	roe deer	carcass; peritoneum	2013	NI			+	V2
17(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	4
18(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	2
20(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	V2
24(M)PSB	roe deer	carcass; tonsil area	2013	NI			+	V5
32(M)PSB	red deer	carcass; tonsil area	2013	NI			+	2
33(T)PSB	red deer	carcass; peritoneum	2013	O:8			+	4
34(M)PSB	red deer	carcass; tonsil area	2013	NI			+	V3
34(T)PSB	red deer	carcass; peritoneum	2013	O:3			+	4
34(O)PSB	red deer	carcass; perineum	2013	NI			+	V4
35(M)PSB	red deer	carcass; tonsil area	2013	O:5			+	1
35(O)PSB	red deer	carcass; perineum	2013	O:3			+	1
36(T)PSB	red deer	carcass; peritoneum	2013	NI			+	4
36(O)PSB	red deer	carcass; perineum	2013	NI			+	3
43(O)PSB	wild boar	carcass; perineum	2013	NI			+	V4
46(M)PSB	wild boar	carcass; tonsil area	2013	O:8			+	3
46(T)PSB	wild boar	carcass; peritoneum	2013	O:8			+	1
46(O)PSB	wild boar	carcass; perineum	2013	NI			+	3
47(T)PSB	wild boar	carcass; peritoneum	2013	O:5			+	1
47(O)PSB	wild boar	carcass; perineum	2013	O:5			+	4
48(M)PSB	wild boar	carcass; tonsil area	2013	NI			+	2
48(O)PSB	wild boar	carcass; perineum	2013	NI			+	1
49(M)PSB	wild boar	carcass; tonsil area	2013	O:8			+	1
49(O)PSB	wild boar	carcass; perineum	2013	NI			+	1
50(T)PSB	wild boar	carcass; peritoneum	2013	O:5			+	1
50(O)PSB	wild boar	carcass; perineum	2013	O:8			+	1
51(T)ITC	wild boar	carcass; peritoneum	2013	O:5			+	3
51(M)PSB	wild boar	carcass; tonsil area	2013	O:5			+	1
51(T)PSB	wild boar	carcass; peritoneum	2013	O:5			+	3
51(O)PSB	wild boar	carcass; perineum	2013	NI			+	3
58(O)PSB	wild boar	carcass; perineum	2013	NI			+	1
59(M)PSB	wild boar	carcass; tonsil area	2013	NI			+	3
60(T)PSB	wild boar	carcass; peritoneum	2013	O:8			+	1

* NI – not identified

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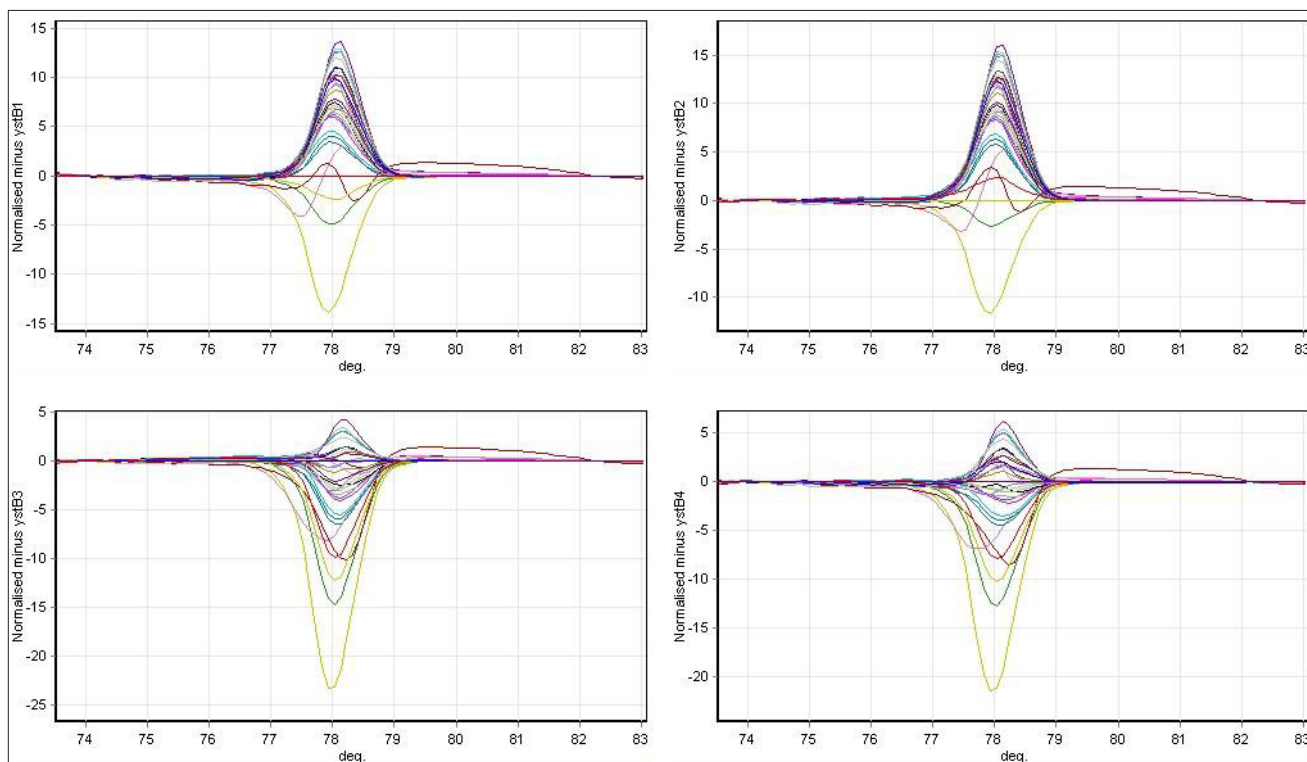
Figure 1. HRM difference graphs of *Y. enterocolitica ystB* gene.
Figure shows 4 genotypes (G1, G2, G3 and G4) and some variations of the examined nucleotide sequence *ystB* gene *Y. enterocolitica* strains isolated from wild animals detected using HRM

Figure 2. Single nucleotide polymorphism of *Y. enterocolitica ystB* gene.
Phylogenetic analysis of G1 nucleotide sequences *ystB* gene *Y. enterocolitica* strains isolated from wild animals revealed 100% similarity with the *Y. enterocolitica* D88145.1 sequence. Analysis of G2 nucleotide sequences *ystB* gene revealed one point mutation in the examined region: transition T111A. One mutation was also found in nucleotide sequences of G3– transition G193A. Two SNPs – transitions G92C and T111A – were identified in *ystB* nucleotide sequences of G4. Three variations were characterized by one point mutation – transition G129A. Two SNPs were detected in subsequent five variations, but they were located at different positions for a given group of strains – transitions: T111A and G193A; C118T and G193A; C141A and G193A. In two variations, 19 SNPs were found in the examined nucleotide sequences: G83A, T93A, A109G, G114T, C116T, A123G, T134C, T142G, T144C, A150C, G162A, T165G, T170G, T174A, T177G, G178A, A179G, A184G and G193A.

higher. More than 30% of those strains also represented G2, but a significant number of variations and some G1 and G4 were also noted (Tab. 2). *Y. enterocolitica* strains isolated from red deer immediately after harvest were classified to G4. Similar to roe deer, genotypic diversity was observed in red deer only in the group of strains isolated from cold-stored carcasses. More than 30% of those strains also represented G4, but a significant number of variations and some G1 were observed (Tab. 2). In the group of strains isolated from wild boar immediately after harvest, more than 40% belonged to G3, but a high number of G2 and G4 was noted (Tab. 2). By contrast, in the group of strains isolated from cold-stored wild boar carcasses, the predominant was G1 (52.6%), G3 was detected in 31.5% of examined strains (Tab. 2).

It should also be noted that different genotypes were identified in 11 individuals from which more than one *Y. enterocolitica* strain was isolated. In some cases, up to

3 genotypes were determined in samples collected from one animal; for example, 2(M)PSB, 2(T)PSB and 2(O)PSB. Detailed characteristics of those strains are shown in Table 1. Interestingly, the presence of *ail* gene in some of the examined *Y. enterocolitica* strains (*ail* is rarely detected in biotype 1A, *ystB* positive strains) did not affect on genotype. In this group of *ail* positive strains, all genotypes were detected.

DISCUSSION

In the presented study, 88 *Y. enterocolitica* strains were examined with the use of *ystB* SNPs detection by HRM analysis. According to best knowledge of the authos', this is one of the few studies where the HRM method was deployed to characterize *Y. enterocolitica* strains. Bancerz-Kisiel et al. [16] used HRM to detect possible mutations in the *ymoA* (*Yersinia*

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Table 2. Comparison of genotypes of *Y. enterocolitica* strains isolated from different wild animal species, immediately after the hunt and from cold-stored carcasses

| Source | | Strain No. | Genotype | | | | | | | | | |
| | | | 1 | | 2 | | 3 | | 4 | | Variation | |
			No.	%	No.	%	No.	%	No.	%	No.	%
Wild duck	after shooting	5	3	60.0	-	-	-	-	2	40.0	-	-
Roe deer	after shooting	2	-	-	2	100.0	-	-	-	-	-	-
	cold-stored	18	3	16.7	6	33.3	1	5.6	2	11.1	6	33.3
Red deer	after shooting	2	2	100.0	-	-	-	-	-	-	-	-
	cold-stored	10	2	20.0	1	10.0	1	10.0	3	30.0	3	30.0
Wild boar	after shooting	32	4	12.5	8	25.0	13	40.6	7	21.9	-	-
	cold-stored	19	10	52.6	1	5.3	6	31.5	1	5.3	1	5.3

modulator) gene region, and to evaluate their influence on the enterotoxic properties of *Y. enterocolitica* strains. By contrast, Souza and Falcao [23] used HRM analysis as the method for *Y. enterocolitica* genotyping. They were searching for SNPs detection in 50 *Y. enterocolitica* strains of biotypes 1A, 1B, 2, 3, 4, and 5. Different melting profiles were determined for 7 fragments of 50 genotyped *Y. enterocolitica* strains, and the SNPs identified in the *hsp60*-SNP1 fragment were identified only after heteroduplex formation. Souza and Falcao [23] developed a phylogenetic analysis based on HRM which confirmed the grouping of *Y. enterocolitica* biotypes into 3 clusters in view of their pathogenic potential.

The current study is the first to describe the application of the HRM method for genotypic the characteristics of *Y. enterocolitica* biotype 1A strains. The origin of *Y. enterocolitica* strains, isolated from various species of wild animals, should also be noted. SNPs detection in one of the most important for this biotype virulence markers – *ystB,* revealed 14 different melting profiles. Four of them were detected more frequently and were defined as regular genotypes, whereas 10 were defined as variations. Direct sequencing of variations revealed 5 new variants of the *ystB* nucleotide sequence, which differed from regular genotypes. Variations were detected only in *Y. enterocolitica* strains isolated from cold-stored carcasses. The above could indicate that *Y. enterocolitica* multiplies under optimal growth conditions, and new variants are probably created as a result of direct contact between microorganisms.

Our findings could also point to the predominance of some genotypes in *Y. enterocolitica* isolated from particular wild animals species. In isolates from wild ducks *Y. enterocolitica* was represented mainly by G1. *Y. enterocolitica* strains isolated from red deer belonged in majority to G2, while isolates from roe deer to G1 and G4. The predominant genotype in isolates from wild boars was G3. This is the first study described *Y. enterocolitica* strains isolated from wild animal in such detail, therefore, our results cannot be compared with other authors' findings. Further studies involving a higher number of samples is required to substantiate our observations.

The HRM-based method for genotyping *Y. enterocolitica ystB* positive strains revealed that a single animal can be a source of more than one genotype of *Y. enterocolitica.* Interestingly, the above observation was not always correlated with differences in the serotype of the examined isolates. Similar observations were made by Souza and Falcao [23], who demonstrated that strains with different serotypes produced identical HRM profiles. According to

the cited authors, despite considerable heterogeneity in *Y. enterocolitica* O antigens that determine serotype, strains are related genetically.

Authors of this study did not have an access to any *ystB* positive *Y. enterocolitica* biotype 1A strain, which would be isolated from clinical case of yersiniosis. These infections manifested by diarrhea are very rare, difficult to proper diagnose and then *Y. enterocolitica* biotype 1A isolation. However, comparing clinical isolates with other biotype 1A strains using HRM method developed in this study would be interesting in the context of widely discussed problem of diversified pathogenicity *Y. enterocolitica ystB* positive strains.

CONCLUSIONS

To conclude, the results of the HRM and sequences analysis indicate that *Y. enterocolitica* biotype 1A strains are highly diverse. SNP-based genotypes are correlated with the host species, but further work is needed to validate this observation. The proposed HRM method could be used in epidemiological investigation to expand existing knowledge of genetic variability of *Y. enterocolitica ystB* positive strains. In the view of the zoonotic properties of *Y. enterocolitica* this method could be also applied to analyze *Y. enterocolitica* strains isolated from human cases of yersiniosis. Undisputed advantage of this method would be their adoption to predicting pathogenic potential of examined *Y. enterocolitica* biotype 1A strains.

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