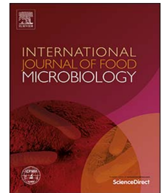
International Journal of Food Microbiology 269 (2018) 46–51

Contents lists available at ScienceDirect

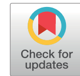
International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro



Short communication

Characterisation of ail-positive *Yersinia enterocolitica* of different biotypes using HRMA



Agata Bancerz-Kisiel[a,*], Anna Szczerba-Turek[a], Aleksandra Platt-Samoraj[a], Maria Michalczyk[b], Wojciech Szweda[a]

[a] Department of Epizootiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 2, 10-719 Olsztyn, Poland
[b] Department of Parasitology and Invasiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego 2, 10-719 Olsztyn, Poland

ARTICLE INFO

Keywords:
Yersinia enterocolitica
Biotype
ail gene
High resolution melting analysis
Single nucleotide polymorphism
Genotypes

ABSTRACT

Yersiniosis is one of the four most frequent foodborne zoonotic diseases in Europe, and ***Yersinia enterocolitica*** is the primary agent in human infections. The *ail* gene is an important chromosomal virulence marker of *Y. enterocolitica* which encodes Ail, a 17-kDa outer membrane protein that promotes attachment and invasion. In the present study, *ail*-positive *Y. enterocolitica* strains of different biotypes were examined using high resolution melting analysis (HRMA) and DNA sequencing. Genotype data relating to *Y. enterocolitica* strains isolated from different sources and belonging to different biotypes were compared. Applied method allowed efficient distinguishing of three genotypes and phylogenetic groups: *1A* – included non-pathogenic *Y. enterocolitica* strains; *1B* – consisted of highly pathogenic *Y. enterocolitica* strains and *2/4* – involved weakly pathogenic *Y. enterocolitica* strains. Amplicon genotyping based on HRMA supports rapid identification of *ail* SNPs correlated with biotype of examined *Y. enterocolitica* strains.

1. Introduction

Yersinia (Y.) enterocolitica is the main cause of yersiniosis in Europe and one of the four main causes of gastrointestinal diseases (EFSA, 2016). The pathogen is a gram-negative bacillus, widely distributed in nature, in aquatic and animal reservoirs, with pigs serving as a major reservoir of human-pathogenic strains (Bottone, 2015). The most important source of *Y. enterocolitica* infection is believed to be contaminated pork and pork products (Bucher et al., 2008). Foodborne outbreaks were also associated with consumption of untreated water, contaminated milk (unpasteurized or inadequately pasteurized), tofu or bean sprouts, and food contaminated by infected food handlers (Sabina et al., 2011). *Y. enterocolitica* is one of the most important foodborne pathogens in Europe (EFSA, 2016).

The species *Y. enterocolitica* has been differentiated into biotypes aiming to identify pathogenic strains by phenotypic traits (Kraushaar et al., 2011). The biotypes of *Y. enterocolitica* are divided into three groups according to the bacterial pathogenic properties: non-pathogenic biotype 1A, weakly pathogenic biotypes 2–5, and highly pathogenic biotype 1B (Bottone, 1997). According to Bottone (2015), the majority of *Y. enterocolitica* biotype 1A strains lack the virulence determinants of invasive strains and can induce infections only in immunocompromised individuals. By contrast, some authors suggest that these strains are emerging group of human pathogens (Huovinen et al., 2010; McNally et al., 2006).

The *ail* gene (attachment and invasion locus) is the chromosomal virulence marker of *Y. enterocolitica*, which encodes Ail, an outer membrane protein that promotes attachment and invasion (Sihvonen et al., 2011). The *ail* gene was always found in pathogenic strains of *Y. enterocolitica*, and used in a number of PCR (polymerase chain reaction) assays as target to quickly discrimination between pathogenic and non-pathogenic *Y. enterocolitica* strains (Kraushaar et al., 2011; Söderqvist et al., 2012; Ye et al., 2014). However, some newer reports have indicated that *ail* gene can be also detected in biotype 1A strains (Cheyne et al., 2010; Falcão et al., 2006; Joutsen et al., 2016, 2017; Sihvonen et al., 2011). According to these papers, methods for detection of potentially pathogenic *Y. enterocolitica* strains, relying only on *ail* searching, are not sufficient.

Single nucleotide polymorphism (SNP) is a DNA sequence changes caused by single nucleotide mutations. SNP is used as a tool for genotyping and is based on several techniques: restriction fragment length polymorphism (RFLP), direct sequencing or PCR-based TaqMan chemistry. Some of those techniques are expensive, laborious and time-consuming because they support the analysis of only one SNP per reaction. High-resolution melting analysis (HRMA) is a method for the detection of mutations, in which PCR and mutation scanning are carried

* Corresponding author at: University of Warmia and Mazury in Olsztyn, Department of Epizootiology, Oczapowskiego 2, 10-719 Olsztyn, Poland.
E-mail address: a.bancerz-kisiel@uwm.edu.pl (A. Bancerz-Kisiel).

https://doi.org/10.1016/j.ijfoodmicro.2018.01.016
Received 30 October 2017; Received in revised form 16 January 2018; Accepted 18 January 2018
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out simultaneously in a single procedure (Reed et al., 2007).

As the *ail* gene is present in *Y. enterocolitica* strains belonging to different biotypes, we decided to analyze *ail* harbouring *Y. enterocolitica* strains of biotypes 1A, 1B, 2 and 4, which were isolated in Poland from humans, pigs, wild boars and red deer. We applied HRMA and DNA sequencing to examine whether *ail* gene sequence has relationship to the biotype of investigated strain.

2. Material and methods

2.1. Experimental material

An ethical approval was not required as this study was retrospective and based only on bacterial strains. By courtesy of Regional Sanitary-Epidemiological Station in Olsztyn, National Institute of Public Health – National Institute of Hygiene in Poland and Department of Microbiology, Ludwik Rydygier Collegium Medicum in Bydgoszcz, human *Y. enterocolitica* strains were obtained. They were previously isolated from samples routinely submitted to these diagnostic laboratories. A collection of *Y. enterocolitica* strains isolated from pigs and wild animals were obtained from previous studies (Bancerz-Kisiel et al., 2012, 2015; Perkowska et al., 2011; Platt-Samoraj et al., 2017) which had been authorised by the Local Ethics Committee of the University of Warmia and Mazury in Olsztyn.

The experimental material consisted of 227 *Y. enterocolitica* strains, belonging to four different biotypes. All examined *Y. enterocolitica* strains had been first molecularly tested (triplex PCR – *ail, ystA, ystB*). Some of these results were previously published (Bancerz-Kisiel et al., 2015; Perkowska et al., 2011; Platt-Samoraj et al., 2017). The remaining strains were tested using triplex PCR at the first stage of this study, according to the methodology described by Bancerz-Kisiel et al. (2015), to identify *ail*-positive strains. *ystA* and *ystB* genes, encoding production of *Yersinia* stable enterotoxin A (YstA) and B (YstB), respectively, were also searched as a feature characteristic for *Y. enterocolitica* species. Only *ail*- and *ystA*-positive or *ail*- and *ystB*-positive strains were used. *Y. enterocolitica* strains of clinical origin were obtained from 99 human cases of yersiniosis, while 128 *Y. enterocolitica* strains isolated from animals came from healthy fattening pigs, as well as from hunted wild boars and red deer. We used 10 *Y. enterocolitica* biotype 1A strains (9 were not identified in terms of serotype; one strain belonged to serotype O:27); 13 *Y. enterocolitica* biotype 1B strains (serotype O:8); 21 *Y. enterocolitica* biotype 2 strains (serotype O:9), and 183 *Y. enterocolitica* biotype 4 strains (serotype O:3). Detailed characteristics of the *Y. enterocolitica* strains used in this study is presented in Table 1.

2.2. HRMA of ail

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 −20 °C for further analyses.

The HRMA was performed in the Rotor-Gene 6000™ real-time analyzer (Corbett Life Science, Sydney, Australia) with the use of the Eva Green saturating dye (Type-it HRM PCR Kit, Qiagen, Hilden, Germany). Defining an *ail* gene fragment suitable for HRMA 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. Primers that were applied in our previous studies to detect *ail*, proved to be effective: *ail*-1 (Forward) 5’TGGTTATGCGCAAAGCCATGT’3, and *ail*-2 (Reverse) 5’TGGAAGTGGGTTGAATTGCA’3. The 25 µl HRM-PCR mixture consisted of 12.5 µl of 2× HRM PCR Master Mix, 10.15 µl of RNase-free water, 1.75 µl of the primer mix (final concentration 0.7 µM each) and 0.6 µl of DNA (50 ng/reaction). A negative control containing all reagents, but without DNA was used in each run. The following HRM-PCR profile was performed: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 52 °C for 30 s. The melt analysis was conducted by acquiring fluorescence data at the temperature ramp of 65 °C to 90 °C at 0.1 °C intervals. Melting curves were normalized and analyzed using RotorGene 6000 Series Software 1.7. The genotypes were automatically assigned based on the shape of the HRM curves with the use of the Rotor-Gene software and by visual examination.

Table 1
Detailed characteristics of the *Y. enterocolitica* strains used in study.

Biotype	No. of strains	Serotype	No. of strains	Source	No. of strains	Virulence markers		
						ail	*ystA*	*ystB*
1A	10	O:27	1	Wild boars	1	+	ND	+
		NI	9	Wild boars	9	+	ND	+
1B	13	O:8	13	Humans	13	+	+	ND
2	21	O:9	21	Humans	5	+	+	ND
				Pigs	15	+	+	ND
				Red deer	1	+	+	ND
4	183	O:3	183	Humans	81	+	+	ND
				Pigs	101	+	+	ND
				Wild boars	1	+	+	ND

NI – not identified serotype.
ND – not detected.

2.3. Sequencing and phylogenetic analysis

To verify genotyping results three samples each of the different melting curves and Tm values, and all variations were chosen for direct sequencing (Genomed Sp. z o.o., Warsaw, Poland). Sequence data from the examined *Y. enterocolitica* strains were compared with the nucleotide sequence of the previously identified *ail* gene using BLASTN version 2.2.18. (Altschul et al., 1997). Multiple sequence alignment was carried out in ClustalW (Larkin et al., 2007) incorporated in the freeware Computational Evolutionary Biology package MEGA version 5.2.1. (Tamura et al., 2011). Nucleotide sequences were identified using BioEdit v.7.2.0. software. Phylogenetic analysis was carried out using the UPGMA (Sneath and Sokal, 1973) and the Maximum Composite Likelihood methods (Tamura et al., 2004).

The nucleotide sequences of *Y. enterocolitica* strains isolated from wild boars and analyzed in this study are available in the GenBank database under accession numbers KM253257.1 – KM253267.1. The remaining *ail* sequences of *Y. enterocolitica* strains, belonging to other biotypes and analyzed in this study, would be published soon. All of the other representative *ail* sequences used in this study for phylogenetic comparison were obtained from GenBank [FN812733, FN812735, FN812736].

3. Results

HRM curves were compared based on shape and T_m values, they were normalized and difference graphs were plotted. The results of this analysis were used to identify three genotypes (*1A, 1B* and *2/4*) and 3 variations (L70 PL, 6PSB PL, 98ITC PL) of the examined nucleotide sequence (Fig. 1). Direct sequencing revealed that *ail* nucleotide sequences obtained from amplicons had the length of 339 bp (base pairs) according to the National Centre for Biotechnology Information (NCBI). They were linked in position 2,007,823–2,008,161 in the *Y. enterocolitica* subsp. enterocolitica 8081 genome [GenBank AM286415], where the gene responsible for the production of an attachment invasion locus protein is encoded.

A sequence alignment of *Y. enterocolitica* strains belonging to biotype 1B (genotype *1B*) revealed 99.8% similarity between examined *ail* gene nucleotide sequences and *Y. enterocolitica* AM286415 sequence. Only one mutation was detected in tested nucleotide sequences – transition *G2007848T* (Fig. 2A). By contrast, an analysis of *Y. enterocolitica* strains belonging to biotype 1A (genotype *1A*), revealed the

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