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Original article

Bioserotypes and virulence markers of *Y. enterocolitica* strains isolated from roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*)

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Abstract

Free-living animals are an important environmental reservoir of pathogens dangerous for other animal species and humans. One of those is *Yersinia (Y.) enterocolitica*, the causative agent of yersiniosis – foodborne, enzootic disease, significant for public health. The purpose of the study was to identify bioserotypes and virulence markers of *Y. enterocolitica* strains isolated from roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) obtained during the 2010/2011 hunting season in north-eastern Poland. From among 48 rectal swabs obtained from 24 roe deer, two strains of *Y. enterocolitica* from one animal were isolated. Although both belonged to biotype 1A they were identified as different serotypes. The strain obtained from cold culture (PSB) belonged to serotype O:5, while the strain isolated from warm culture (ITC) was regarded as nonidentified (NI), what may suggest mixed infection in that animal. The presence of *ystB* gene, coding for YstB enterotoxin, directly related to *Y. enterocolitica* pathogenicity was detected in both strains using triplex PCR. The effect of the examination of 32 swabs obtained from 16 red deer was the isolation of two *Y. enterocolitica* strains from two different animals. Both belonged to biotype 1A with NI serotype, but were originated from different types of culture. They gave positive results in case of products of a size corresponding to the *ystB* gene. No amplicons corresponding to *ail* and *ystA* genes were found. Roe deer and red deer may carry and shed *Y. enterocolitica*, what seems to be important in aspect of an environmental reservoir of this pathogen. The *Y. enterocolitica* strains isolated from wild ruminants had the amplicons of the *ystB* gene, what suggest they can be potential source of *Y. enterocolitica* infection for humans.

Key words: roe deer, red deer, *Yersinia enterocolitica*, reservoir, virulence markers

Introduction

Wild ruminants are an interesting research topic because they live in small populations, in hard-to-reach areas and it is very difficult to collect material samples from them. They could also be an environmental reservoir of pathogens dangerous for humans and other animal species. One of these pathogens is *Yersinia (Y.) enterocolitica*, a Gram-negative bacillus belonging to the *Enterobacteriaceae* family, and the causative agent of yersiniosis. In animals this disease is usually asymptomatic or may be associated with mild diarrhea. In humans however, it may provoke a variety of clinical signs and induce a life-threatening conditions, especially in young children (Bottone 1997). Yersiniosis is therefore a zoonosis, important in public health aspect, what has been confirmed by the recent EFSA report (European Food Safety Authority, 2012).

Wild animal meat is increasingly consumed because of its taste and health properties. Looking at the alimentary route as a major in yersiniosis infection, it should be stated that not properly cooked meat originating from wild ruminants may be potential source of infection for humans. Furthermore, evisceration of hunted animals without an appropriate hygienic measures may expose hunters to the infection with *Y. enterocolitica*.

Out of the six known biotypes, the *Y. enterocolitica* strains belonging to biotypes 1B and 2-5 are considered as pathogenic for animals and humans. They have the pYV (plasmid *Yersinia* virulence), the *ail* (attachment-invasion locus) and *ystA* (*Yersinia* stable toxin A) chromosomal genes, coding respectively Ail, an outer membrane protein that promotes attachment and invasion as well as YstA, an enterotoxin production. The 1A biotype strains lacking the classical virulence markers were regarded as non-pathogenic. However, recently it was demonstrated that most of them have the *ystB* gene, coding for YstB enterotoxin, which is the most probable reason for diarrhea in the course of the yersiniosis caused by this biotype (Singh et al. 2004, Tennant et al. 2005, Platt-Samoraj et al. 2006). It seems that the standard procedure of biotype and serotype determination is an insufficient criterion to assess the pathogenic potential of *Y. enterocolitica* strains (Singh et al. 2003, 2005, Tennant et al. 2005).

The purpose of the present study was to identify the bioserotypes and virulence markers of *Y. enterocolitica* strains isolated from roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) obtained during the 2010/2011 hunting season in north-eastern Poland.

Materials and Methods

Bacterial strains and culture conditions

The materials for the study consisted of 48 rectal swabs from 24 roe deer and 32 rectal swabs from 16 red deer of various age, obtained in north-eastern Poland. Two samples were taken from each animal to determine the ability of *Y. enterocolitica* to grow under low temperature conditions. One swab from each animal was then placed in a test tube with 9 ml of ITC – irgasan, ticarcillin, and potassium chlorate medium (warm culture, prepared according to PN-EN ISO 10273, incubation at 25°C for 48 h). Simultaneously, the another swab from each animal was placed in a test tube with 9 ml of PSB – peptone, sorbitol, and bile salts medium (cold culture, prepared according to PN-EN ISO 10273, incubation at 4°C for 3 weeks). Next, 0.5 ml of each culture was transferred into 4.5 ml of 0.5% KOH in 0.5% NaCl for 20 s, after which a loopful was streaked onto a CIN (cefsulodin, irgasan, and novobiocin) plate and incubated at 30°C for 48 h. Further biochemical identification of 1-5 typical colonies from each CIN plate was carried out according to the PN-EN ISO 10273 standard, to make a preliminary selection of potentially pathogenic *Y. enterocolitica* strains.

Serotype and biotype determination

The determination of the serologic group of the strains examined was performed using the slide agglutination test. Live bacterial cells from the 24-h blood agar culture (Graso Biotech, Starogard Gdański, Poland) were used as an antigen, and the sera for the most common somatic antigens O:3, O:5, O:8, and O:9 came from ITEST company (Hradec Kralove, Czech Republic). The cells of the tested strain were suspended in a drop of 0.85% NaCl placed on a glass slide and then connected with a drop of serum placed nearby and mixed with the bacteriological oese. After shaking for 1 min, agglutination with one of the four sera used was considered as a positive result. If there was no agglutination with any serum, the strain was regarded as nonidentified (NI). Biotype determination of the examined strains was made in accordance with the PN-EN ISO 10273 standard.

DNA isolation

Genomic DNA isolation was performed with the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions and was stored at -20°C for further analyses.

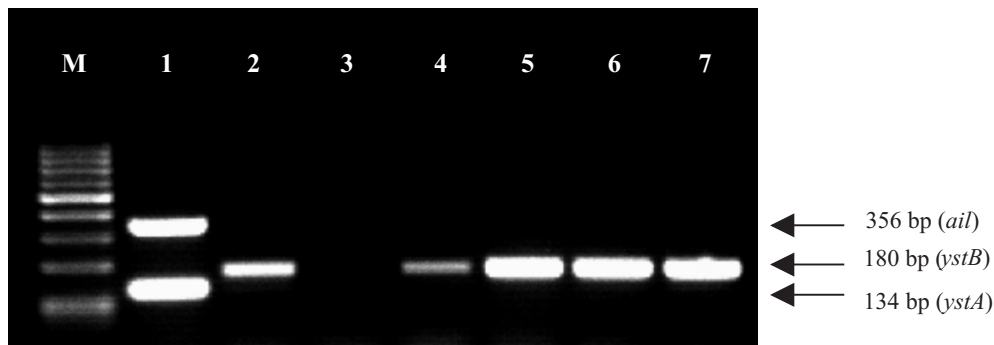


Fig. 1. Electrophoretic separation of *ail*, *ystA* and *ystB* gene amplification products from *Y. enterocolitica* strains in triplex PCR; M – size marker GeneRuler 100bp DNA Ladder Plus (Fermentas); Lane 1 – positive control, contained DNA isolated from a reference strain of *Y. enterocolitica* O:8 (ATCC 23715); Lane 2 – positive control, contained DNA isolated from a reference strain of *Y. enterocolitica* O:5; Lane 3 – zero control, did not contain bacterial DNA, contains *ail*, *ystA* and *ystB* gene primers; Lanes 4 – 5 – products of amplification of *ystB* gene fragments from *Y. enterocolitica* strains isolated from red deer; Lanes 6 – 7 – products of amplification of *ystB* gene fragments from *Y. enterocolitica* strains isolated from roe deer.

Primers and triplex PCR conditions

Triplex PCR included the amplification of three genes: *ail*, *ystA*, and *ystB*. The sequences of the primers (synthesized in the DNA Sequencing Laboratory of the Biochemistry and Biophysics Institute of the Polish Academy of Sciences, Oligo, Warsaw), and triplex PCR conditions were published previously (Bancerz-Kisiel et al. 2012). One modification was applied, temperature of primer connection was 45°C. Triplex PCR was carried out using a HotStarTaq Plus DNA polymerase (Qiagen GmbH, Hilden, Germany) and a HotStarTaq Plus Master Mix Kit (Qiagen). The 20- μ l reaction mixture contained about 120 ng of isolated DNA (from 1 to 3 μ l), 10 μ l of HotStarTaq Plus Master Mix 2x, 2 μ l of CoralLoad Concentrate 10x, and 0.1 μ l of each of the primers (final concentration 0.5 μ M); the total was supplemented by up to 20 μ l of RNase-free water. Three controls were applied to each reaction: two positive, with DNA isolated from the reference strains O:8 and O:5, and one negative, without DNA. Once the reaction was over, electrophoresis separation was conducted in 2% agarose gel containing 0.5 μ g/ml ethidium bromide in order to visualize the DNA fragments of the *Y. enterocolitica* strains obtained as a result of triplex PCR. The size of the products was evaluated by means of a comparison with the standard mass of GeneRuler 100-bp Ladder Plus (Fermentas UAB, Vilnius, Lithuania). The following products of the reaction were searched for: fragments of the *ail* gene of 356 bp size, the *ystA* gene of 134 bp size, and the *ystB* gene of 180 bp size, respectively. Electrophoresis results were recorded using the GelDoc gel evidence system (Bio-Rad Laboratories, Milan, Italy). To confirm the specificity of the amplicons obtained, they were purified using a CleanUp kit (A&ABiotechnology) and sequenced (Genomed, Warsaw, Poland).

Results

Bacteriological examinations of the 48 swabs obtained from 24 roe deer enabled the isolation of two *Y. enterocolitica* strains. It was 4.16% of tested samples and animals, because both strains were isolated from the same animal (female), but from two kinds of culture. Although both strains belonged to biotype 1A, they were identified as different serotypes. The strain obtained from PSB belonged to serotype O:5, while the strain isolated from ITC was regarded as NI, what may suggest a mixed infection in that animal. The presence of genes directly related to *Y. enterocolitica* pathogenicity was evaluated in both strains using triplex PCR with three pairs of primers for the *ail*, *ystA*, and *ystB* genes. No amplicons corresponding to *ail* and *ystA* genes were observed, whereas reaction products of a size corresponding to the *ystB* gene were found in both of the samples tested (Fig. 1).

Similar examinations of 32 swabs obtained from 16 red deer enabled the isolation of two *Y. enterocolitica* strains from two different animals (males), which constituted 6.25% of the tested samples and 12.5% of the tested animals. Both strains belonged to biotype 1A with a NI serotype, but originated from different types of culture. The application of triplex PCR using primers for the *ail*, *ystA*, and *ystB* genes gave positive results in the case of products of a size corresponding to the *ystB* gene, no amplicons corresponding to *ail* and *ystA* genes were observed (Fig. 1).

Table 1 shows the triplex PCR results, including the age and sex of animals from which strains were originated, type of culture, biotype and serotype of these strains.

Table 1. Specification of the *Y. enterocolitica* strains isolated from roe deer and red deer.

Source	Sex	Culture	Biotype	Serotype	Virulence markers		
					<i>ail</i>	<i>ystA</i>	<i>ystB</i>
Roe deer	female	warm	1A	NI	–	–	+
	cold	1A	O:5	–	–	+	
Red deer	male	warm	1A	NI	–	–	+
	male	cold	1A	NI	–	–	+

NI – nonidentified

Discussion

The problem of *Y. enterocolitica* reservoirs in wild animals was recognized relatively early as well in Europe as in the rest of the world. Nonetheless, the results recorded in our studies are one of few reports on the prevalence and detailed characteristics of *Y. enterocolitica* in wild ruminants.

The studies carried out by Henderson (1984) were one of the first in this research area; 176 *Y. enterocolitica* strains (19.1%) were isolated from 922 fecal samples collected from farmed and feral red deer in New Zealand. On that basis the author assumed that red deer are one of the main reservoirs of *Y. enterocolitica* in an environment. A similar percentage of positive results was reported by Shayegani et al. (1986) in New York State (United States of America). Among the samples collected from 145 white-tailed deer (*Odocoileus virginianus*) *Y. enterocolitica* strains of various serotypes were isolated in 27 cases (18.6%). However, the results of recent studies conducted in the Ohio State (United States of America) by French et al. (2010) indicate that the level of *Y. enterocolitica* isolation from white-tailed deer amounts to 30%. Unfortunately, the samples for bacteriological investigations were pooled by the authors what obscures the picture of current situation concerning *Y. enterocolitica* infections in this animal species.

Pagano et al. (1985) conducted one of the first studies on the prevalence of *Yersinia spp.* in wild animals in Europe. The study was carried out with 60 fecal samples collected from red deer and 13 from roe deer (Stelvio National Park in Italy). Two *Y. enterocolitica* strains with different biotypes and serotypes were isolated, from which the first originated from a deer (biotype 1A, serotype O:5) and the second from a feeding ground (biotype 1A, serotype O:22). The most recent studies carried out in Italy by Magnino et al. (2011) in 2005-2009 (Lombardy and Emilia-Romagna) yielded the isolation of *Y. enterocolitica* biotype 1A from 19 out of 237 roe deer examined, which constituted 8.02% of the animals

tested. The bacteriological investigations performed by the same authors on 11 samples collected from red deer did not yield any positive result. An attempt to evaluate the prevalence of *Yersinia spp.* in free-ranging red deer in Norway was undertaken by Aschfalk et al. (2008). *Yersinia spp.* were isolated in 10 out of 170 tested animals and *Y. enterocolitica* comprised 8 strains (4.7%) with three belonging to 1A/O:5 bioserotype, three – to 1A/O:8 bioserotype, and two – to 1A/O:6 bioserotype. In Poland, studies on the prevalence of *Yersinia spp.* in wild animals have so far been carried out only by Koronkiewicz et al. (2004) on 450 animals (wild boars, roe deers and red deers). *Yersinia spp.* were isolated only from wild boars (2.4%) and red deer (18.2%), but unfortunately without giving any information on species, biotype, serotype and molecular characteristics. Against the background of European and world reports the results obtained in our study bring some interesting observations in this research area. Noteworthy is primarily a higher percentage of positive results for red deer (12.5%) and slightly less when it comes to roe deer (4.16%). Research reaffirmed the need to perform two types of culture because *Y. enterocolitica* strains were isolated with both, warm and cold culture, and the exact bioserotype characteristic allowed the demonstration of mixed infection in roe deer.

Apart from our research, the only studies which included the application of molecular techniques to evaluate the prevalence and characteristics of *Yersinia spp.* genus in wild ruminants were conducted in Switzerland by Joutsen et al. (2012). These authors showed that *Y. enterocolitica* biotype 1A was found in 8 out of 77 (10.4%) red deer tested and in 7 out of 55 (12.72%) roe deer examined. The majority of these isolated strains and all strains isolated in our studies had *ystB* gene encoding the production of YstB enterotoxin. The recent reports have indicated the increasing importance of biotype 1A in the pathogenesis of yersiniosis and revealed the occurrence of clinical cases caused by *Y. enterocolitica* strains capable of producing the enterotoxin YstB (Singh et al. 2003, Mc Nally et al. 2004, Platt-Samoraj et al. 2006). This

is particularly important in the context of *ystB* positive *Y. enterocolitica* strains, isolated from wild ruminants, which meat can be eaten raw (Tatar rissole) or almost raw (blood or medium fried steak).

The presence of *Y. enterocolitica* in rectal swabs collected from clinically healthy wild red deer and roe deer indicates the carrier state of this pathogen and its fecal shedding into the environment. By comparing the results of our studies with the previous reports on the prevalence of *Y. enterocolitica* in wild boars (Bancerz-Kisiel et al. 2009) and wild ducks (Bancerz-Kisiel et al. 2012) it is assumed that wild animals are one of the reservoirs of potentially pathogenic *Y. enterocolitica* strains. The isolation of *Y. enterocolitica* from wild ruminants proves that they constitute a potential source of infection for humans.

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