

GENOTYPIC MARKERS OF *YERSINIA ENTEROCOLITICA* O:9 ISOLATED FROM COWS POSITIVE IN SEROLOGICAL EXAMINATION FOR BOVINE BRUCELLOSIS

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Abstract

The aim of the study was to perform a molecular investigations for the presence of pathogenicity genotypic markers of *Y. enterocolitica* O:9 isolated from cattle, in which initially positive serological reactions for brucellosis were observed. Almost all isolates were *ail*-, *ystA*- and *myfA*-positive (n=19). On the other hand, one isolate, which harboured plasmid encoding gene *yadA* was *ail*-, *ystA*- and *myfA*-negative. The plasmid encoding *yadA* marker was present in half of the isolates tested. None of the examined isolates was *ystB*-positive. The results of the investigations revealed that the *Y. enterocolitica* O:9 isolates, related to false positive serological results for brucellosis, may be also potentially pathogenic for humans, due to the presence of chromosomal and plasmid-encoded molecular markers.

Key words: cows, brucellosis, *Yersinia enterocolitica* O:9, virulence markers, false positive serological results.

For many years the laboratory diagnosis of brucellosis has been generally based on serological tests. All animals classified as serologically positive are obligatorily slaughtered and subjected to bacteriological examination. However, the similarity of the O-antigenic side chain of *Brucella* lipopolisaccharide (LPS) with other microbes has restricted the specificity of serological diagnosis resulting in false positive serological results (FPSR). Most of the commonly reported FPSR are caused by infections with *Yersinia enterocolitica* O:9, as the bacterium possesses an identical O-antigen LPS chain to that present in *Brucella* sp. (1, 2, 13). For years this organism has been rarely observed in cattle, but it was very often found in the environment of pigs, causing diagnostic difficulties. In the last decade the presence of *Y. enterocolitica* O:9 has dramatically increased the rate of FPSR (16, 17, 23). The majority of *Y. enterocolitica* isolates from food and environmental sources are non-pathogenic types. On the other hand, yersiniosis caused by *Y. enterocolitica* most often causes diarrhoea, at times bloody, and occurs mostly in young children. In elderly persons and in patients with underlying conditions (iron overload, cirrhosis, diabetes, cancer, etc.) systemic forms of the disease are often observed. The bacterium is able to grow below 4°C and contaminates refrigerated food, a probable source of infection. Untreated water can also transmit the organism (10, 17). The *Y. enterocolitica*

species can be divided into six biotypes based on biochemical properties: 1A, which is generally regarded as nonpathogenic, and pathogenic 1B, 2, 3, 4, and 5 biotypes (21). In Europe, the majority of human pathogenic *Y. enterocolitica* belong to biotype 4 (serotype O:3) or, less commonly, biotype 2 (serotypes O:9, O:5,27) (17). Pigs are considered to be the primary reservoir of pathogenic types of *Y. enterocolitica*, mainly biotype 4 (serotype O:3). Biotype 2 (serotype O:9) has been isolated from other animal species, such as cattle, sheep, and goats (2, 3, 16, 17). Pathogenicity can be also determined using PCR methods. Among chromosomal virulence markers, a product of the *ail* gene promotes bacterial adhesion and invasion of epithelial cells, and a product of *myfA* gene promotes the colonisation of the intestine and allows the secretion of the heat-stable enterotoxin encoded by the *ystA* gene. The *ystB* gene encodes thermo resistant toxin YstB and can be found in the strains of biotype 1A, and, only occasionally, in the other biotypes. Pathogenic *Y. enterocolitica* strains also carry *yadA* gene, present in 70 kb plasmid named pYV, responsible for auto-agglutination, serum resistance, and adhesion (9, 10).

The paper presents the results of molecular investigations on the presence of pathogenicity genotypic markers of *Y. enterocolitica* O:9 isolated from cattle where initially positive serological reactions for brucellosis were ascertained.

Material and Methods

Bacterial strains. *Y. enterocolitica* O:9 (n=20) previously isolated from bovine lymph nodes according to self-modified ISO 10273:2003 were cultured on CIN agar at 30°C for 24-48 h prior to DNA extraction (22). As positive controls, *Y. enterocolitica* O:3/biotype 4 (IW90, DSMZ 9676) and *Y. enterocolitica* O:9/biotype 2 (IW91, DSMZ 11067) were used.

DNA extraction. One individual colony of each strain was suspended in 50 µl of sterile, DNase and RNase free deionised water (ICN Biomedicals). The suspensions were heated at 99°C for 5 min, chilled on ice, and then centrifuged at 13,000 x g for 1 min to pellet the cellular debris. The supernatant (5 µl) was subsequently used as a source of DNA template. The purity and concentration of DNA preparations were measured spectrophotometrically at 260 and 280 nm (GeneQuant 1300, GE Healthcare).

PCR assays. Each DNA amplification was performed in the 50 µl reaction mixture consisting of DNA template, 1X PCR buffer (Fermentas), 200 µM of dNTPs, 4 mM, 1 U of Taq DNA polymerase, nucleotide primers for chromosomal and plasmid virulence markers, and water. Sequences, characteristics, and concentration of the primers used in this study are shown in Table 1.

Multiplex PCR (for *ail* and *ystA* genes) and single PCRs were run in a thermocycler (T3, Biometra) under the following conditions: initial DNA denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 57°C for 1 min, and 70°C for 1 min (*ail*, *ystA* and *ystB* genes); initial DNA denaturation at 94°C for 5 min, followed by 55 cycles of 94°C for 1.5 min, 58°C for 1.5 min, and 72°C for 1 min (*yadA* and *myfA*); initial DNA denaturation at 94°C for 5 min, followed by 36 cycles of 94°C for 1 min, 62°C for 45 s, and 72°C for 45 s (*16S rRNA*); initial DNA denaturation at 95°C for 5

min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min (*per*). The final extension step was done at 70°C/72°C for 5 min. After staining with ethidium bromide for 0.5 min and washing in distilled water, the gels were photographed under UV light. The sizes of the PCR amplicons were compared to the 100 bp DNA marker (Fermentas).

Results

During the investigation, the presence of the *16S rRNA* gene typical for *Yersinia* species, and *per* gene characteristic for O:9 serotype, generating the amplicons of 330 bp and 312 bp, respectively, were observed in 20 isolates originating from cattle in which initially positive serological reactions for brucellosis were observed (Table 2). *Y. enterocolitica* O:3/biotype 4 reference strain was *16S rRNA* positive only, whereas the analysis of agarose electrophoresis of reference *Y. enterocolitica* O:9/biotype 2 showed two products of 330 and 312 bp. Almost all isolates (n=19) were *ail*-, *ystA*- and *myfA*-positive. On the other hand, one isolate, which harboured plasmid-encoded gene *yadA* was *ail*-, *ystA*- and *myfA*-negative. The plasmid encoding *yadA* marker was present in half of the isolates tested. None of the examined isolates was *ystB*-positive (Table 2).

Discussion

Serological examinations, based on detection of significant levels of specific antibodies, play a dominant role in diagnosis of brucellosis. On the other hand, a definitive diagnosis of *Brucella* infections can be made only by isolation and identification of the microorganism and its molecular markers.

Table 1
Sequences and characteristics of the primers used in the study

Primer name	Sequence (5'→3')	Target gene	Amplicon (bp)	Concentration (µM)	Reference
ailF	TGG TTA TGC GCA AAG CCA TGT	<i>ail</i>	356	0.1	(4)
ailR	TGG AAG TGG GTT GAA TTG CA				
ystAF	GTC TTC ATT TGG AGG ATT CGG C				
ystAR	AAT CAC TAC TGA CTT CGG CTG G				
ystBF	GTA CAT TAG GCC AAG AGA CG	<i>ystB</i>	146	0.1	(19)
ystBR	GCA ACA TAC CTC ACA ACA CC				
yadAF	TAA GAT CAG TGT CTC TGC GGC A	<i>yadA</i>	747	0.2	(8)
yadAR	TAG TTA TTT GCG ATC CCT AGC AC			0.2	
myfA	CAG ATA CAC CTG CCT TCC ATC T	<i>myfA</i>	272	0.1	(7)
myfB	CTC GAC ATA TTC CTC AAC ACG C			0.1	
Y1	AAT ACC GCA TAA CGT CTT CG	<i>16S rRNA</i>	330	0.1	(20)
Y2	CTT CTT CTG CGA GTA ACG TC			0.1	
per-12	GCA GAC GGGGGC AAA AGT A	<i>per</i>	312	0.05	(11)
per-14	CAC CTT GAT TTT TAA ATG GCA T			0.05	

Table 2
Results of molecular examination of chromosomal and plasmid markers of *Y. enterocolitica* O:9 isolates.

No. of isolate	Results of PCR for:						
	<i>ail</i>	<i>ystA</i>	<i>ystB</i>	<i>yadA</i>	<i>myf</i>	<i>16S rRNA</i>	<i>per</i>
1	+	+	-	-	+	+	+
2	+	+	-	-	+	+	+
3	+	+	-	-	+	+	+
4	+	+	-	-	+	+	+
5	+	+	-	+	+	+	+
6	+	+	-	+	+	+	+
7	+	+	-	-	+	+	+
8	+	+	-	-	+	+	+
9	+	+	-	+	+	+	+
10	+	+	-	-	+	+	+
11	+	+	-	-	+	+	+
12	+	+	-	+	+	+	+
13	+	+	-	+	+	+	+
14	+	+	-	-	+	+	+
15	+	+	-	+	+	+	+
16	-	-	-	+	-	+	+
17	+	+	-	+	+	+	+
18	+	+	-	+	+	+	+
19	+	+	-	-	+	+	+
20	+	+	-	+	+	+	+

One of the most important problems, which should always be born in mind, is the possibility of cross reactions between *Brucella* sp. and *Y. enterocolitica* O:9, which are difficult to distinguish from specific anti-*Brucella* reactions. The study by Szulowski *et al.* (18) shows that each year, in Poland, a small number of cows are classified as positive for brucellosis and slaughtered, but *B. abortus* is not isolated. On the other hand, according to the official instruction (6), samples from such animals are examined for *Y. enterocolitica*, this microorganism is often isolated from seropositive cows. In France, Gerbier *et al.* (2) who investigated the epidemiology of FPSR in bovine brucellosis conclude that these reactions appear to be due, at least partially, to *Y. enterocolitica* O:9. Hilbink *et al.* (3) indicated the link between non-specific serological reactions against *B. abortus* and the presence of *Y. enterocolitica* O:9 in ruminants in New Zealand.

Yersiniosis is the third most commonly reported zoonosis in the EU, despite the continuous decreasing five-year trend since 2006 with 6,776 confirmed human cases in the EU. The most common serotypes in human infections were O:3 and O:9. In

animals, *Y. enterocolitica* was most frequently detected in pigs, but it was also isolated from cattle, sheep, dogs, horses, and some wildlife species (17). According to the scientific opinion published by the BIOHAZ Panel in 2007, it is well-documented that pigs can harbour human pathogenic *Y. enterocolitica* with a very high prevalence, especially biotype 4 (serotype O:3) (16). Reservoirs other than pigs may also play a role in the epidemiology of human yersiniosis. The evidence suggests that ruminants (*e.g.* cattle) may play a role as reservoirs for biotype 2 (serotypes O:9 and O:5,27) (2, 3, 15). Until now, there were no studies on the virulence markers of *Y. enterocolitica* O:9 isolated from cattle, in which initially positive serological reactions for brucellosis were reported. In the study, it was demonstrated that almost all isolates were *ail*-, *ystA*- and *myfA*-positive (n=19). On the other hand, one isolate, which harboured plasmid-encoded gene *yadA* was *ail*-, *ystA*-, and *myfA*-negative. The plasmid encoding *yadA* marker was present in half of the isolates tested. None of the examined isolates was *ystB*-positive. Platt-Samoraj *et al.* (14) found the presence of *ail*, *ystA*, and *ystB* virulence markers in *Y. enterocolitica* strains isolated

from aborting sows and aborted fetuses. Among 45 *Y. enterocolitica* isolates, 40 belonging to biotype 1A, contained only the *ystB* gene, the remaining five, belonging to bioserotype 4/O:3, carried *ail* and *ystA* genes. Thoerner *et al.* (19) examined 33 strains of *Y. enterocolitica* O:9 from animals, 11 from humans, and two from food. Almost all of them were *ystA*- and *ail*-positive. None of them was *ystB*-positive. Among plasmid-borne genes, *yadA* marker was present in 29 *Y. enterocolitica* isolates, and those findings are similar to the results obtained in the present study. Different observations were described by Kot *et al.* (9). The authors examined 6 *Y. enterocolitica* O:9 strains isolated from humans (one) and pigs (five). Two of them were *ystA*+, *ail*+, *myfA*+, and *yadA*+, and one was *ystA*+, *ail*+, and *myfA*+. Three remaining strains belonging to O:9 serotype gave *ystA*+, *ystB*+, *ail*+, and *myfA*+ genotypic pattern, and were isolated from pigs. In the study, none of the isolates was *ystB*-positive. This result is similar to the findings of other authors, who found that *ail*+ and *ystA*+ positive *Y. enterocolitica* strains are almost always *ystB*-negative (8, 19).

The results of the presented investigations reveal that the significance of *Y. enterocolitica* O:9 in cattle is not limited to complication of diagnosis of brucellosis. The strains of *Y. enterocolitica* O:9 isolated from cattle slaughtered following positive results for brucellosis, due to the presence of chromosomal and plasmid encoded molecular markers may be also potentially pathogenic for humans. Such examinations should be continued to provide a better knowledge concerning the pathogenicity patterns of the microorganism, and to bring a progress in molecular epidemiology.

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