Detection of Pathogenic *Yersinia enterocolitica* Serotype O:3 by Biochemical, Serological, and PCR Methods

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Abstract

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In this study, the pathogenic *Y. enterocolitica* of serotype O:3 was monitored. The serotype is widely spread in Europe and has been linked to human yersiniosis. For the detection of pathogenic strains were used biochemical and serological methods as well as PCR methods based on the identification of virulence genes (*ail*, *rfbC*, *ystA*, *yadA*, *virF*). The occurrence of *Y. enterocolitica* O:3 strains was monitored in slaughter animals from a number of farms in the Czech Republic. A total of 3748 samples were collected coming from pigs (1388), cattle (633), poultry (902), and slaughter facilities (825). Fifty-two *Y. enterocolitica* O:3 isolates were identified by biochemical and serologic methods, and 53 *Y. enterocolitica* O:3 isolates were identified by PCR methods (46 isolates from pigs, 2 isolates from poultry, 3 isolates from cattle, and 2 isolates from a poultry slaughtering facility). All isolates of *Y. enterocolitica* O:3 carried genes *ail* and *rfbC*, 83% isolates carried gene *ystA*, 79% isolates carried gene *yadA* and 49% isolates carried gene *virF*. The use of PCR methods based on the identification of *ail* and *rfbC* genes provides for a sufficiently specific identification of pathogenic *Y. enterocolitica* O:3 strains with optimum time consumption compared to biochemical and serological methods. It is not recommendable to use other PCR methods (detection of the *ystA*, *yadA*, and *virF* genes) for the detection of pathogenic *Y. enterocolitica* strains because those methods are not very specific for the determination of pathogenicity.

Keywords: *Yersinia enterocolitica*; serotype O:3; PCR; virulence; genes; biochemical methods; cultivation; DNA

The list of bacterial agents causing food-borne diseases includes, besides the most frequently encountered *Salmonella* and *Campylobacter,* also *Yersinia enterocolitica. Y. enterocolitica* is a Gram-negative bacterium belonging to the genus *Yersinia*, family *Enterobacteriaceae*. Of the 12 species that comprise the genus *Yersinia*, three are important from the human pathogenicity point of view, namely *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica. Y. pestis* is the causative agent of the bubonic plague, and *Y. pseudotuberculosis* and *Y. enterocolitica* are intestinal pathogens (Boer 1992; Sprague & Neubauer 2005; Tennant *et al.* 2005).

Y. enterocolitica is divided into about 60 serotypes according to the variability of the O-antigen present in the outer membrane of bacteria (Skurnik *et al*. 1999). Of the 11 serotypes important from

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the human infections point of view, the most frequently occurring are serotypes O:3; O:9; O:5.27, and O:8. Pathogenic strains of O:4, O:8, O:13a/b, O:18, O:20, and O:21 serotypes are prevalent in the USA. In Europe and Japan, only O:3 and O:9 serotypes occur (Wannet *et al.* 2001).

Y. enterocolitica are widely distributed throughout the environment and have been isolated from raw milk, sewage-contaminated water, soil, sea-food, humans, and many warm-blooded animals such as poultry and, most importantly, pigs (Schmidt & Rodrick 2003). Therefore, pork meat is thought to be an important source of the infection (Lambertz & Danielsson-Tham 2005). Pathogenic strains of *Y. enterocolitica* are transmitted to man mainly from contaminated water or food (meat, milk or vegetables), more specifically raw or undercooked pork, and they may cause various infectious diseases (enteritis, enterocolitis, mesenteric lymphadenitis) (Schmidt & Rodrick 2003).

Until recently, pathogenic strains of *Y. enterocolitica* used to be identified on the basis of the detection of the virulence plasmid pYV (70 kb). Localised on the plasmid are virulent genes *yadA* and *virF* (Cornelis 1998; Thoerner *et al*. 2003; Platt-Samoraj *et al.* 2006). The *yadA* gene encodes the production of the YadA membrane protein, which functions as a protection of *Y. enterocolitica* cells against the activity of polymorphonuclear leukocytes (Ruckdeschel *et al*. 1996). The *virF* gene activates the transcription of *yop* genes that encode the secreted proteins (Yops) (Michiels *et al.* 1990). Proteins (Yops) protect bacterial cells against the activity of macrophages (Cornelis 1998). The plasmid pYV might be lost relatively easily when the bacteria are stored for a longer period of time, subjected to numerous passages or grown at temperatures above 37°C (Platt-Samoraj *et al*. 2006).

To determine pathogenicity, it is therefore better to use chromosomal genes (*ail, rfbC*, and *yst*) (Weynants *et al.* 1996; Wannet *et al*. 2001; Thoerner *et al*. 2003). The *ail* gene encodes Ail protein, it occurs only in the pathogenic strains of *Y. enterocolitica*. This protein participates in bacterial adhesion to mammalian cells, facilitates their invasion into eukaryotic cells, and intensifies their resistance to the bacteriocidal effects of complement (Platt-Samoraj *et al.* 2006). The *rfbC* gene is localised on the chromosome only in pathogenic strains of *Y. enterocolitica* serotype O:3 (Weynants *et al*. 1996). Also important for the pathogenicity reasons is the Yst thermostable enterotoxin, which is a protein with the molecular weight of 7494 Da. The enterotoxin is encoded by the *yst* gene, and facilitates the invasion of the microorganism into tissues by damaging the intestinal epithelium (Platt-Samoraj *et al*. 2006). Pathogenic strains of *Y. enterocolitica* produce the thermostable enterotoxin YstA (*ystA* gene*)*, which, sporadically, may also be produced by non-pathogenic strains of *Y. intermedia* and *Y. enterocolitica* of the biotype 1A (Thoerner *et al.* 2003; Singh & Virdi 2004; Platt-Samoraj *et al.* 2006).

The countries with the highest prevalence of yersiniosis include Germany, Scandinavian countries, and Belgium. In the Czech Republic (No. of cases/during 2000–2004: 231/2000, 301/2001, 403/2002, 372/2003, 498/2004), the number of the reported cases of yersiniosis is not as high as in some other European countries, such as Germany (No. of cases/during 2000–2004: 4.778/2000, 7.186/2001, 7.515/2002, 6.571/2003, 6.182/2004) (EFSA, Trends and sources of Zoonoses, Zoonotic Agents and Antimicrobial resistance in the European Union in 2004), In view of an increase in the incidence of yersiniosis in the Czech Republic (EPIDAT, CR), the risks presented by pathogenic strains of *Y. enterocolitica* and the need for an additional specific identification, the authors of this study focused on the monitoring of the pathogenic *Y. enterocolitica* of serotype O:3, which is widely spread across Europe and mentioned in connection with human yersiniosis (Gürtler *et al*. 2005).

MATERIAL AND METHODS

In 2005, a total of 3748 samples were collected in slaughterhouses (Czech Republic), of which 1388 samples were collected from pigs (tongues, tonsils, carcass halves, surface), 633 samples from cattle (tongues, carcass halves, skin), 902 from poultry (surface, intestines) and 825 samples from slaughter facilities.

Sterile cotton swabs were used to collect smear samples from the rectum, tongues, tonsils, and carcasses surfaces of the slaughtered pigs, cattle, and poultry, and from various surfaces of the slaughterhouse premises. As to pigs, samples were taken from the skin surface before bleeding, the surface of the tongue and tonsils after evisceration and, after the carcasses had been halved, from the carcass surfaces in the rectum region. With cattle, samples were taken from the skin surface before

bleeding, the surface of the tongue after evisceration and, after the carcasses had been halved, from the carcass surfaces in the rectum region. As to poultry, samples were collected from the carcass surfaces after washing (i.e. after scald and defeathering), from surfaces after evisceration, and from the intestinal (caecum) content. Samples from the slaughtering premises were taken at pig slaughter facilities for pigs, poultry, and cattle, and they were collected from the instruments (chopping knives, saws, knives), work surfaces, trays, etc. The smear samples collected were placed in the transport Amies Agar Gel Medium (Copan Italia S.p.A) and taken to the laboratory for immediate processing. The bacterial strains were recovered after selective enrichment in ITC Broth Base (HiMedia, Čaderský-Envitek, spol. s r. o., CR) for 48 h at 24°C and incubation on Selective Agar base (CIN) (HiMedia, Čaderský-Envitek, spol. s r. o., CR) for 24 h at 30°C (ČSN EN ISO 10273).

Pathogenic *Y. enterocolitica* O:3 strains were detected by biochemical and serological methods, and by PCR methods.

Culture methods, biochemical tests and serotyping. The isolation of *Y. enterocolitica* strains, their biochemical identification and pathogenicity determination were performed in accordance with ČSN EN ISO 10273 and Bottone (1997) (Table 1).

Suspected colonies were taken out of the pure culture obtained and used for preliminary tests (oxidase, urease production, glucose and lactose fermentation, hydrogen sulphide production, and gas formation from glucose). Colonies with positive urease and glucose, negative oxidase and lactose fermentation, and negative hydrogen sulphide production and gas formation from glucose were then selected. In these selected colonies, biochemical confirmation tests were performed (lysine decarboxylase and ornithine decarboxylase, sucrose, rhamnose, xylose and trehalose fermentation, and citrate). Colonies with typical characteristics (positive sucrose fermentation and ornithine decarboxylase, negative lysine decarboxylase and citrate and trehalose, rhamnose and xylose positive or negative) were tested for pathogenicity (esculin hydrolysis, pyrazinamidase activity, and salicin).

Biochemical identification of the strains was supplemented with biochemical reactions for *Y. enterocolitica* identification (indole, hydrogen sulphide, lysine, ornithine, urease, arginine, simmons citrate, malonate, phenylalanine, β-galactosidase, inositol, adonitol, cellobiose, sucrose, trehalose, mannitol, acetoin, esculin, sorbitol, rhamnose, melibiose, raffinose, dulcitol, glucose), which are part of the commercially available Enterotest24 (Pliva-Lachema, CR).

In the isolated pathogenic strains, the O:3 serotype was identified by slide agglutination using commercially available antiserum (O:3) (Itest plus, s. r. o., Hradec Králové, CR).

DNA extraction. Bacterial DNA was prepared by the phenol-chloroform method as described by Sambrook *et al.* (1989). Cultivated colonies of bacterial cells identified as *Y. enterocolitica* were suspended in Tris-HCl-EDTA buffer, pH 8.0, frozen,

Table 1. Interpretation of biochemical and pathogenicity tests of *Y. enterocolitica*

Test	Reaction	Test	Reaction
Species identification		**Species identification**	
Urease	+	Trehalose	+/–
Indole	–/+	Rhamnose	+/–
Glucose	+	Xylose	+/–
Gas formation from glucose	–	Simmons citrate	–
Lactose	–		
Hydrogen sulphide	–		
Oxidase	–	**Pathogenicity determination**	
Lysine decarboxylase	–	Eesculin hydrolysis	–
Ornithine decarboxylase	+	Pyrazinamidase activity	–
Sucrose	+	Salicin	–

thawed, and subsequently lysed by incubation with proteinase K at 55°C overnight. The released DNA was extracted twice with an equal volume of phenol:chloroform:isoamylalcohol (Serva, Germany) and once with chloroform. DNA concentrated by ethanol precipitation was dissolved in 40 µl TE buffer and stored at –20°C.

PCR methods. For the detection of (*ail, yadA*, *ystA, rfbC, virF*) genes, specific primers and PCR conditions as described by (Weynants *et al*. 1996; Lantz *et al*. 1998; Wannet *et al*. 2001; Thoerner *et al*. 2003) were used (Table 2). Specific DNA sequences of genes (*ail, yadA*, *ystA, rfbC, virF*) were amplified in a thermocycler (Mastercycler Personal, Germany). Reference bacterial strains *Yersinia enterocolitica* CCM 5671, *Yersinia rohdei* CCM 4075^T, and *Campylobacter jejuni* subsp. *jejuni* ATCCC 33560 (FCCM, CZ) were used in this study.

The reaction mixture (12.5 µl) contained 1 µl of DNA template, 4.2 µl H_2O, 6.3 µl PCR-Mix (PP MASTER MIX, Top-Bio, s. r. o., CR), 0.5 µl of each primer (0.01 nM/µl).

PCR products were analysed on 3% agarose gel (Serva, Germany) run at 120 V in a Tris-H_3BO_3-EDTA buffer, pH 8.3. The DNA fragments were stained with ethidium bromide and photographed using a UV-transilluminator (UltraLum, Claremont) to visualise the bands. The GeneRuler™ 50 bp DNA Ladder (MBI Fermentas, Lithuania) was used as a molecular weight marker.

RESULTS AND DISCUSSION

In 2005, 53 isolates of *Y. enterocolitica* serotype O:3 were isolated from a total of 3748 samples collected from slaughter animals and slaughter facilities. Fifty-two *Y. enterocolitica* O:3 isolates were identified by biochemical and serological methods, and 53 *Y. enterocolitica* O:3 isolates were identified by PCR methods (Table 2) based on the identification of *ail* and *rfbC* genes. The list of isolates *Y. enterocolitica* O:3 strains from individual sources is given in Table 3.

The highest incidence of isolates of *Y. enterocolitica* O:3 was found in pigs (3.3%), while in cattle and poultry their incidence was low (0.5% and 0.2%, respectively). Compared with the incidence among slaughter animals, the incidence rate of pathogenic strains in slaughter surroundings was negligible (Table 3). Pigs are generally reported as the main reservoir of pathogenic strains of *Y. enterocolitica*, which has been corroborated by a number of studies. For instance, studies from Germany report up to 64.4% incidence of pathogenic strains of *Y. enterocolitica* O:3 (Gürtler *et al*. 2005) in pork samples. In Italy, pathogenic strains of *Y. enterocolitica* have been reported in 14.7% of 150 samples collected from tonsils of pigs (Bonardi *et al*. 2003). In a Norwegian study investigating the prevalence of *Y. enterocolitica* pathogenic strain in 249 samples from five different slaughterhouses, 15.2% of the samples tested were positive for *Y. enterocolitica* pathogenic strains (Johannessen *et al*. 2000).

To detect the genes (*ail, yadA, rfbC, ystA, virF*) using the PCR methods, specific sequences of genes were amplified and individual amplified fragments were detected by agarose gel electrophoresis (Figure 1).

The occurrence of virulence genes (*ail, yadA, rfbC, ystA, virF*) in isolates of *Y. enterocolitica* O:3 and reference strains is shown in Table 4.

According to literary data, the *ail* gene occurs only in pathogenic strains of *Y. enterocolitica*, and the *rfbC* gene in pathogenic strains of *Y. enterocolitica*, serotype O:3 (Weynants *et al*. 1996; Wannet *et al*. 2001; Lambertz & Danielson-Tham 2005). In the present study, the *ail* and *rfbC* genes were found in all isolates and pathogenic reference strain of *Y. enterocolitica* CCM 5671.

While the presence of the *ystA* gene is reported in the studies on pathogenic strains of *Y. enterocoli-*

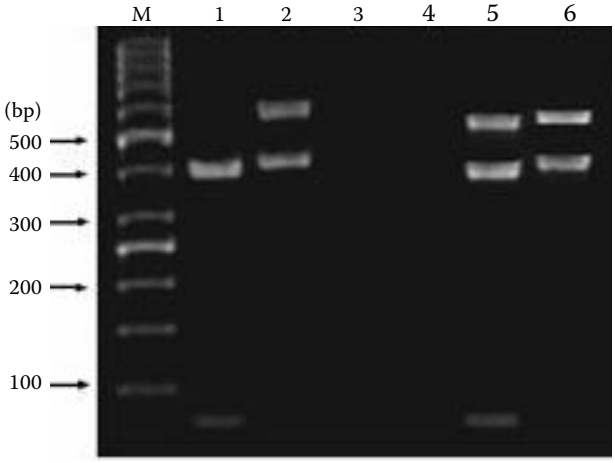
Table 2. Primers used for the amplification of the *ail, yadA*, *ystA, rfbC* and *virF* genes

Genes	Primers	Primers sequence (Reference)	Amplicon length (bp)
ail	A1, A2	Wannet *et al.* (2001)	425
yadA	P1, P2	Lantz *et al.* (1998)	600
rfbC	rfbC1, rfbC2	Weynants *et al.* (1996)	405
ystA	ystA1, ystA2	Thoerner *et al.* (2003)	79
virF	virF1, virF2	Thoerner *et al.* (2003)	561

Table 3. Evaluation appearance of *Y. enterocolitica* O:3

Source	No. of samples	No. of isolates *Y. enterocolitica* O:3 (%)
Pigs	1388	46 (3.3)
tongue	296	15
tonsils	296	17
carcass halves	402	4
surface	394	10
Cattle	633	3 (0.5)
tongue	180	1
carcass halves	273	2
surface	180	0
Poultry	902	2 (0.2)
surface after wash	400	2
surface after full-dressed	266	0
intestines	236	0
Slaughter surroundings	825	2 (0.2)
pigs	358	1
cattle	245	0
poultry	222	1
	Σ 3748	Σ 53 (1.4)

tica, it is only rarely mentioned in connection with non-pathogenic *Y. enterocolitica* strains (biotype 1A) (Tennant *et al*. 2005). In contrast with the studies of pathogenic strains of *Y. enterocolitica* O:3 by Thoerner *et al*. (2003) and Platt-Samoraj *et al*. (2006) where the *ystA* gene was identified in 100% and 99%, respectively, in our study the *ystA* gene was found in only 83% of isolates. A study of reference strains showed the



M: molecular mass marker (GeneRuler™ 50 bp DNA Ladder), 1 and 2: *Y. enterocolitica* O:3 CCM 5671 (*ystA, rfbC, ail, yadA*), 3: *Y. rohdei* CCM 4075^T, 4: *Campylobacter jejuni* subsp. *jejuni* ATCCC 33560, 5 and 6: *Y. enterocolitica* O:3 (*ystA, virF, rfbC, ail, yadA*)

Figure 1. Agarose gel electrophoresis of PCR products amplified with PCR methods for the *ail* (425 bp), *yadA* (600 bp), *rfbC* (405 bp), *ystA* (79 bp) and *virF* (561 bp) genes from *Yersinia enterocolitica*

presence of the gene in pathogenic strain of *Y. enterocolitica* CCM 5671.

The *yadA* and *virF* genes were identified in 79% and 49% of isolates of *Y. enterocolitica* O:3, respectively, the *yadA* gene was found only in pathogenic reference strain of *Y. enterocolitica* CCM 5671, and the *virF* gene was not identified in any of the reference strains. Lantz *et al*. (1998) and Lambertz & Danielsson-Tham (2005) found the *virF* and *yadA* genes in 92% and 66% of isolates of *Y. enterocolitica* O:3, respectively, but failed to find them in any other pathogenic or non-pathogenic *Yersinia* spp. strains. Both genes (*yadA* and *virF*)

Table 4. Occurrence of virulence genes (*ail, yadA, rfbC, ystA, virF*) in *Yersinia* spp.

Species	Serotype	No. positive/total				
		ail	*rfbC*	*ystA*	*yadA*	*virF*
Pathogenic *Y. enterocolitica*						
Yersinia enterocolitica CCM 5671	O:3	1/1	1/1	1/1	1/1	0/1
Yersinia enterocolitica	O:3	53/53	53/53	44/53	42/53	26/53
Nonpathogenic *Yersinia* spp.						
Yersinia rohdei CCM 4075^T		0/1	0/1	0/1	0/1	0/1
Campylobacter jejuni subsp. *jejuni* ATCCC 33560	23	0/1	0/1	0/1	0/1	0/1

are contained on the virulent plasmid pYV, and the fact that the passaging of strains may lead to the loss of the plasmid may explain the less frequent detection of pathogenic strains of *Y. enterocolitica*. In their comparative study on the prevalence of the *ail* and *yadA* genes in *Y. enterocolitica* strains and non-pathogenic *Yersinia* spp. strains, Blais & Phillippe (1995) found the *ail* gene in 100% of pathogenic *Y. enterocolitica* strains, the *yadA* gene in only 86% of pathogenic *Y. enterocolitica* strains, but they found neither of the genes in non-pathogenic strains of *Yersinia* spp.

Biochemical and serological methods were able to identify 52 isolates of *Y. enterocolitica* O:3. In addition to biochemical identification, Enterotest24 was also performed, and on the basis of the results obtained, 52 isolates were identified as *Y. enterocolitica*. The list of the results of biochemical tests is presented in Table 5.

Table 5. Results of biochemical tests

Test	No. of isolates *Y. enterocolitica* (%) positive or negative
Species identification	
Urease	+ (92%), – (8%)
Indole	+ (9%), – (91%)
Glucose	+ (100%), –
Gas formation from glucose	+ (15%) weak positive, – (85%)
Lactose	+, – (100%)
Hydrogen sulphide	+ (6%) weak positive, – (94%)
Oxidase	+, – (100%)
Lysine decarboxylase	+, – (100%)
Ornithine decarboxylase	+ (96%), – (4%)
Sucrose	+ (100%), –
Trehalose	+ (100%), –
Rhamnose	+, – (100%)
Xylose	+ (60%), – (40%)
Simmons citrate	+ (2%), – (98%)
Pathogenicity determination	
Eesculin hydrolysis	+ (8%), – (92%)
Pyrazinamidase activity	+ (6%), – (94%)
Salicin	+ (6%), – (94%)

PCR methods were able to identify 53 isolates of *Y. enterocolitica*. PCR methods based on the identification of *ail* and *rfbC* genes identified 100% of *Y. enterocolitica* O:3 isolates. PCR methods based on the identification of *ystA, yadA*, and *virF* genes identified 83%, 79% and 49% of *Y. enterocolitica* isolates, respectively.

Differences in the rate of identification of *Y. enterocolitica* pathogenic strains using traditional cultivation and biochemical methods and PCR methods were also found by Johannessen *et al*. (2000), who reported even much greater differences in the detection of pathogenic strains between individual methods than those found in our study. When they used cultivation and biochemical methods, the authors were able to detect 6 pathogenic strains of *Y. enterocolitica*, but when they used PCR methods, they successfully identified up to 50 pathogenic strains.

The specificity of identification of pathogenic strains of *Y. enterocolitica* O:3 by PCR methods based on the identification of *ail* and *rfbC* genes is better than that of biochemical and serological methods. It is not recommendable to use other PCR methods (detection of *ystA, yadA* or *virF* genes) for the detection of pathogenic strains because those methods are not very specific for *Y. enterocolitica* pathogenicity determinations. The use of PCR methods (detection of *ail* and *rfbC* genes) provides for a sufficiently specific identification of pathogenic *Y. enterocolitica* O:3 strains with optimum time consumption in comparison with biochemical and serological methods.

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