The Comparison of the Methods for the Identification of Pathogenic Serotypes and Biotypes of *Yersinia enterocolitica*: Microbiological Methods and PCR

Miroslava VÁZLEROVÁ and Iva STEINHAUSEROVÁ

Department of Meat Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic

Abstract

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In this study, pathogenic strains of *Y. enterocolitica* were identified by microbiological and PCR methods. The samples were collected from pigs, cattle, poultry, and slaughter houses. Three common techniques were used to isolate *Y. enterocolitica* from the samples – ITC, PSB, and direct on the CIN. Primers A1/A2, Y1/Y2, and *rfbC* 1/*rfbC* 2 were used for the specific detection of the pathogenic strains of *Y. enterocolitica.* Traditional microbiological methods were found to be insufficient for the specific identification of the *Y. enterocolitica* pathogen. In comparison with PCR which was able to detect 149 strains, the biochemical test could detect only 138 species. These results show that the use of biochemical methods of cultivation did not allow the identification of all *Y. enterocolitica* pathogens. In total, 149 strains of pathogenic *Y. enterocolitica* were examined of which 120 were from pigs, 19 from poultry, 8 were cattle strains, and 2 came from the environments of slaughterhouses.

Keywords: *Yersinia enterocolitica*; pathogenic serotype; PCR detection; cultivation; DNA; identification; serotype; biotype

Yersinia enterocolitica belongs to the *Enterobacteriaceae* family. The highly heterogenic species can be divided into several biotypes and serotypes. From the 60 known serotypes of *Y. enterocolitica*, 11 are capable of causing disease in humans. The differentiating between the forms of serotypes is based on the observation of various thermostable O antigens in the cell wall. The serotypes O:3 O:3, O:5,27, O:8, and O:9 are the most frequent causative agents of illness in humans. Other less common serotypes include O:13 and O:21. The occurrence of the individual serotypes is based on the geographic location. The serotypes O:3 and O:9 are commonly located in Europe and Japan whereas the pathogens of the serotypes O:5, 27 are spread throughout the globe (Wannet *et al*. 2001).

For a long time, *Y. enterocolitica* was considered a rare microorganism. However, during the last two decades it was isolated from animals, vegetables,

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the environments, water, and humans all over the world (Bottone 1999). Yersinia is most common to the farm animals such as pigs, chickens, cattle, and occasionally sheep (Zamora *et al*. 1997; Siriken 2004; McNally *et al*. 2004). Due to its capability to grow in low climates, Yersinia is commonly isolated from cooled or frozen consumable goods (i.e. meat, milk, ice-cream, vegetables, and chickens). There have only been a few reports on the isolation of *Y. enterocolitica* from animals and foods in the Czech Republic.

Yersinia is found all around the world, the highest numbers of the pathogen having been notified in Scandinavia (Johannessen *et al*. 2000; Nesbakken *et al*. 2003). Yersinia commonly appears in the household microflora and animals. 75% of patients suffering from Yersinia are under 15 years of age. People can be infected by the contact with animals or direct consumption of consumable goods prepared using meat from infected animals (such as chicken, pork, beef). The meat is usually raw or undercooked during the preparation.

Sufficient and specific identification of the pathogen *Y. enterocolitica* is a necessity and therefore we focused our study on the analysis and comparison of the well-established methods for the identification of the pathogen. Two various methods for the identification were chosen for comparison and optimalisation: the standard microbiological method, and PCR. The use of the classic methods of cultivation is time consuming in comparison to the modern PCR methods. *Y. enterocolitica* was extracted from the samples using 3 different isolation methods. The individual methods were compared with one another and tested using the samples taken from the tonsils and tongue of pig. The individual methods were monitored for their effectiveness in the cultivation of the pathogen, time consumption, and financial demand.

MATERIALS AND METHODS

The samples were collected from various slaughterhouses in the Czech Republic from January 2004–March 2005. A total of 5140 samples were collected: 2982 pig, 990 cattle, 929 chicken samples, and 239 from slaughter houses. The samples were collected in a sterile environment and transported in medium (Oxoid) to the laboratory and immediately analysed. The analysis of *Y. enterocolitica* had two phases. 1000 samples from pig (500 tonsils and 500 tongue) were used for the optimalisation of the cell culture procedure. 3 types of culture media were analysed: CIN agar, ITC medium, PSB medium, and ITC were chosen to analyse further 3617 samples.

Culture methods. The following 3 methods were used for the isolation of the pathogen (ČSN ISO 10273: Mikrobiologie – Všeobecné pokyny pro průkaz suspektních patogenních kmenů *Yersinia enterocolitica*).

(1) Inoculation by directly plating the sample on CIN agar (Oxoid) at 30°C for 24 h.
(2) Selective enrichment ITC was used with irgasan, tikarcilin, and potassium chloride (Merck). The samples on swabs were cultivated in ITC at 25°C for further 48 h, plated onto CIN agar, and incubated at 30°C for 24 h.
(3) The samples were kept in cold enrichment PSB (Oxoid) and left at 4°C for three weeks, then plated onto CIN agar.

The method capable of growing the largest amounts of pathogen was the cultivation in ITC. The colonies were then grown on CIN agar, and the cells with a typical dark red centre known as the "red bull's eye"(Sinha *et al*. 2000) were transferred to blood agar and used for biochemical testing and PCR.

Biochemical tests and biotyping. Following the examination of pure cultures, the colonies were collected and put through several common tests including oxidase and urease production, deamination of tryptophane, the behaviour in Kligler agar, glucose fermentation, hydrogen sulphide production, gas formation from glucose, and lactose fermentation. The colonies that were urease and glucose positive, capable of the deamination of tryptophane and of lactose fermentation, oxidase negative, and did not produce hydrogen sulphide and gas from glucose, were selected for further testing. Biochemical confirmation of the presence of lysine, ornithine, sucrose, rhamnose, and citrate was carried out and the positive colonies underwent further testing for pathogens salicin, eskulin, and pyrazimidase. Pure cultures were commercially tested using Enterotest (Pliva-Lachema). These individual biochemical tests allowed the samples to be divided into various biotypes (Bottone 1997, 1999). Table 1 shows the reactions of the individual biotypes to various tests.

Serotyping. Selected strains with typical characters were serotyped using the commercial antisera O:3, O:5, O:8, and O:9 (Itest Plus, s. r. o). The individual colonies were isolated and suspended

Table 1. Biochemical tests used for biotyping *Y. enterocolitica*

Test	1A	1B	2	3	4	5
Salicin (acid production in 24 h)	+	–	–	–	–	–
Eskulin hydrolysis (24 h)	+/–	–	–	–	–	–
Xyloza (acid production)	+	+	+	+	–	v
Trehaloza (acid production)	+	+	+	+	+	–
Indol production	+	+	v	–	–	–
Ornithine decarboxylase	+	+	+	+	+	+
Inositol (acid production)	+	+	+	+	+	+
Sorbose (acid production)	+	+	+	+	+	0
Pyrazimidase activity	+	–	–	–	–	–
Lipase activity	+	+	–	–	–	–

in physiological solution. A drop of specific antisera for the individual pathogenic serotype O:3, O:5,27, O:8, and O:9 was then added to the suspensions.

***PCR identification of** Y. enterocolitica.* The bacterial strains biochemically identified as *Yersinia* sp. and cultivated on CIN medium were tested using the PCR method. Primers Y1/Y2 were used for the specific PCR identification and differentiation of strains *Yersinia* sp. Primers A1/A2 were used for the specific detection of pathogenic strains of *Y. enterocolitica* (Wannet *et al*. 2001). Primers *rfbC* 1/*rfbC* 2 were used for the identification of pathogenic strains of the serotype O:3 (Weynants *et al*. 1996).

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 TE buffer (Tris-HCl, EDTA, pH 8), frozen, 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) and once with chloroform. DNA concentrated by ethanol precipitation was dissolved in 40 µl TE buffer and stored at –20°C.

The multiplex PCR method, using the following pair of specific primers, was used for the identification of specific pathogens in culture. Primer A1 (5'-TTAATGTGTACGCTGGGAGTG-3') and A2 (5'-GGAGTATTCATATGAAGCGTC-3'). On the basis of this sequence, a PCR product of 425 bp was expected. To amplify specifically the *Y. enterocolitica* 16S rRNA gene, a second set of primers, Y1 (5'-AATACCGCATAACGTCTTCG-3') and Y2 (5'-CTTCTTCTGCGAGTAACGTC-3'), was used, resulting in a PCR product of 330 bp (Wannet *et al*. 2001). PCR using primers rfbC1 5'-CGCATCTGGGACACTAATTCG-3' and rfbC2 5'-CCACGAATTCCATCAAAACCACC-3' (Weynants *et al*. 1996) were used for the specific detection of the serotype O:3 *Y. enterocolitica*.

PCR conditions 1. Precautions were taken to use sterile reagents and conditions wherever possible. The reaction mixture contained 1 µl of DNA, 4.2 µl H_2O, 6.3 µl of PCR-Mix (PP MASTER MIX, Praha) to which 0.5 µl primer was added. Amplification was performed in a Mastercycler Personal instrument (Germany). The cycling conditions started with a denaturation step at 94°C for 5 min, which was followed by 36 subsequent cycles consisting of heat denaturation at 94°C for 45 s, primer annealing at 62°C for 45 s, and extension at 72°C for 45 s. The final extension was performed at 72°C for 7 min to complete the synthesis of all strands. PCR products were detected using agarose gel electrophoresis at 150 V for 30 min. PCR amplicons were separated on 1.5% (w/v) agarose gel (Top-Bio) and visualised by staining with ethidium bromide and excitation under UV light. The GeneRuler^{TM} 50 bp DNA Ladder Plus (MBI Fermentas) was used as a molecular marker.

PCR conditions 2. Serospecificity is associated with the O chain of the lipopolysaccharide, a major component of the outer membrane of the

gram-negative bacteria and an immunodominant antigen (Al-Hendy *et al*. 1992). The genes responsible for the biosynthesis of the O side chain of *Y. enterocolitica* O:3 are located in the *rfb* cluster, which was recently cloned and sequenced. A PCR method was developed for the detection of *Y. enterocolitica* serotype O:3 by amplification of a 405-bp fragment of the *rfbC* gene using *rfbC* primers 5'-CGCATCTGGGACACTAATTCG-3' and 5'-CCACGAATTCCATCAAAACCACC-3' (Weynants *et al*. 1996).

Precautions were taken to use sterile reagents and conditions wherever possible. The reaction mixture contained 1 µl of DNA, 4.2 µl H_2O, 6.3 µl of PCR-Mix (PP MASTER MIX, Praha) to which 0.5 µl primer was added.

Amplification was performed in a Mastercycler Personal instrument (Germany) using the following step: DNA denaturing at 95°C for 10 min prior to amplification, 30 cycles of denaturation at 95°C for 0.5 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. After PCR amplification, 15 ml of PCR products was loaded on to horizontal submarine 1.5% agarose gels and subjected to electrophoresis in TAE buffer (40mM Tris-HCl, 20mM acetic acid, 1mM EDTA). The gels were stained with ethidium bromide.

RESULTS AND DISCUSSION

Three common techniques were used to isolate *Y. enterocolitica* from the samples. In order to find the optimal technique, 500 liver and tonsil samples taken from pigs were tested using selected isolation methods. The first method consisted of inoculation on the elective medium CIN. The second method used inoculation on CIN agar following a cold climate growth in PSB media (4°C for 3 days). The third one used cultivation in ITC medium (at 25°C for 48 h) and inoculation on CIN agar. The individual methods were monitored for their effectiveness in the cultivation of the pathogen, time consumption, and financial demand.

The third method proved to be most effective – culture in ITC medium and inoculation on CIN. From 500 of the collected samples of tongue and tonsil, only 70 and 90% of the positive culture analysed using the third method were grown using the first and second methods, respectively. ITC was chosen as the most optimal method from the three, with the greatest ability to capture and isolate the pathogen in the optimal amount of time and under minimal financial demand. This method was used for testing further 3617 samples. In total, 149 strains of pathogenic *Y. enterocolitica* were examined of which 120 came from pigs, 19 from poultry, 8 from cattle strains, and 2 from the environments of the slaughterhouses. Table 2 summarises the capabilities of the individual methods.

The comparison of two various methods of isolation of *Y. enterocolitica* carried out using PCR and the classic biochemical identification is shown in Table 3.

These results show that the use of biochemical methods of cultivation did not allow the identification of all *Y. enterocolitica* pathogens. In comparison with PCR which was able to detect 149 species, the biochemical test was only able to detect 138 of them. The greatest difference was seen in the samples collected from pigs and chickens. 111 positive species were identified in the pig samples with the help of microbiological methods whereas 120 were identified using PCR. In the case of the chicken samples, 17 positive species were identified with the help of microbiological methods, whereas 19 were identified using PCR.

The results suggest that pigs are the main reservoir for the pathogen. A German study detected as

Table 2. Turge methods used for isolation of *Y. enterocolitica* from tonsils and tongue samples from pigs – direct on CIN agar (24 h, 30°C), cold enrichment PSB (3 weeks, 5°C) and selective enrichment ITC (48 h, 25°C)

Used method	Tonsils samples		Tongue samples	
	total number	number of positive (%)	total number	number of positive (%)
Direct on CIN	500	20 (4)	500	19 (3, 8)
Cold enrichment PSB	500	27 (5, 4)	500	25 (5)
Selective enrichment ITC	500	30 (6)	500	29 (5, 8)

Table 3. A comparison of two various methods of isolation of *Y. enterocolitica* carried out using PCR and the classic biochemical identification

Source	Number of samples	PCR positive	Biochemical test positive	Prevalence (%)
Pigs	2982	120	111	4.1
Cattle	990	8	8	0.8
Poutry	929	19	17	2.0
Slaughter	239	2	2	1.3
Total	4617	149	138	3.2

much as 65.4% of their samples as positive (Gultler *et al*. 2005). In total, 149 strains of pathogenic *Y. enterocolitica* were examined of which 120 came from pigs, 19 from poultry, 8 from cattle strains, and 2 from the environment of slaughterhouses.

The most frequently detected serotype was *Y. enterocolitica* O:3. A higher frequencies of other serotypes were found with the strains *Y. enetrocolitica* O:5,27, and O:9. The serotype *Y. enterocolitica* O:8 was not found. The most frequently detected serotype in pig strains was *Y. enterocolitica* O:3 and less common serotypes O:5,27 and O:9. In poultry, *Y. enterocolitica* O:5,27 was most frequently detected.

Serotype/biotype O:3/4 were most commonly found in the samples (126 occurrences). Gultler *et al*. (2005) reported similar results. Other common strains were O:9/2 and O:5,27/2, with a total of 11 and 6 occurrences, respectively. The serotype O:3 was found to be most common in pigs and cattle; it was found in up to 88% samples. This serotype was detected in 26% of the hicken samples. On the other hand, the second most frequent serotype of pig and cattle strains *Y. enterocolitica* O:5,27 was also most frequently found in the poultry strains. Other pig serotypes such as O:9 were not detected in poultry and cattle.

Three classic microbiological methods were tested on the isolation of *Y. enterocoliticaI* pathogen from the samples. The tests were aimed to determine the optimal technique that can be used for the detection of *Y. enterocolitica* from the collected samples. The methods used include cultivation in ITC, PSB, and direct inoculation in CIN agar. The study was also aimed to determine the true sensitivity of the selective enrichment of ITC with irgasan, tikarcilin and potassium chlorate. The results show that the detection of *Y. enterocolitica* using swabs is more effective than the cold enrichment technique or direct plating on CIN agar (Wannet *et al*. 2001; ČSN ISO 10273). The cultivation in PSB led to partial growth of microflora and samples needed to be cultured several times in live media to allow us to isolate the pathogen of interest. This was very time consuming. The samples that were directly inoculated in CIN were not selective to *Y. enterocolitica*, hence other species were raised on the agar leading to an insufficient amount of the pathogen of interest. The selective enrichment technique using irgasan, tikarcilin and potassium chloride, although not specific for *Yersinia* spp. and a number of other genera including *Citrobacter*, *Serratia*, *Enterobacter* and *Proteus*, was able to (poorly) grow the pathogen of interest under these conditions (Hilbert *et al*. 2003). Selective enrichment ITC offers the distinct advantage of reducing the detection time required.

The multiplex PCR developed to identify pathogenic strains of *Y. enterocolitica* was used to compare the detection using biochemical tests (ČSN ISO 10273). The results showed that all positive samples were not detected using the biochemical identification methods. For instance, the identification of the pig samples using classic methods detected only 92.5% of the pathogens detected using PCR (Table 3). Johannessen *et al*. (2000) also studied the problems associated with the identification of *Y. enterocolitica* serotypes using classic microbiological method in comparison to PCR. Johannessen identified 50 positive samples using PCR as opposed to only 6 using classic methods.

Traditional microbiological methods have been found to be insufficient for the specific identification of the *Y. enterocolitica* pathogen. Future research focusing on the identification using molecular methods or combining the two methods is necessary. The use of PCR has shown to be a sufficient standard of specific identification and is not time consuming.

References

Al-Hendy A., Toivanen P., Skurnik M. (1992): Lipopolysaccharide O side chain of *Yersinia enterocolitica* O:3 is an essential virulence factor in an orally infected murine model. Infection and Immunity, **60**: 870–875.

Bottone J.E. (1997): *Yersinia enterocolitica*: The charisma continues. Clinical Microbiology Reviews, **10**: 257–276.

Bottone J.E. (1999): *Yersinia enterocolitica*: Overview and epidemiologic correlates. Microbes and Infection, **1**: 323–333.

Gultler M., Alter T., Kasimir S., Linnebur M., Fehlhaber K. (2005): Prevalence of *Yersinia enterocolitica* in fattening pigs. Journal of Food Protection, **68**: 850–854.

Hilbert F., Mayrhofer S., Smulders F.J.M. (2003): Rapid urease screening of *Yersinia* on CIN agar plates. International Journal of Food Microbiology, **84**: 111–115.

Johannessen S.G., Kapperud G., Kruse H. (2000): Occurrence of pathogenic *Yersinia enterocolitica* in Norwegian pork products determined by a PCR metod and a traditional culturing metod. International Journal of Food Microbiology, **54**: 75–78.

McNally M.C.A., Cheasty T., Fearnley C., Dalziel R.W., Paiba G.A., Manning G., Newell D.G. (2004): Comparison of the biotypes of *Yersinia enterocolitica* isolated from pigs, cattle and sheep at slaughter and from humans with yersiniosis in Great Britain during 1999–2000. Letters in Applied Microbiology, **39**: 103–108.

Nesbakken T., Eckner K., Hoidal K.H., Rotterud O. (2003): Occurrence of *Yersinia enterocolitica* and *Camphylobacter* spp. in slaughter pigs and consequences for meat inspection, slaughtering and dressing procedures. International Journal of Food Microbiology, **80**: 231–240.

Sambrook J., Fritsch E.F., Maniatis T. (1989): Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sinha I., Chodhary I., Virdi J.S. (2000): Isolation of *Yersinia enterocolitica* and *Yersinia intermedia* from wastewaters and their biochemical and serological charackteristic. Current Science, **79**: 510–513.

Siriken B. (2004): The presence of *Yersinia enterocolitica* and other *Yersinia species* in ground beef in Aydin, Turkey. Turkish Journal of Veterinary & Animal Sciences, **28**: 489–495.

Wannet J.B.W., Reessink M., Brunings H.A. (2001): Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive Duplex PCR assay. Journal of Clinical Microbiology, **39**: 4483–4486.

Weynants V., Jadot V., Denoel A.P., Tibor A., Letesson J.J. (1996): Detection of *Yersinia enterocolitica* serogroup O:3 by a PCR metod. Journal of Clinical Microbiology, **5**: 1224–1227.

Zamora J., Reinhardt G., Polette M., Macias P., English J. (1997): Isolation of *Yersinia enterocolitica* and *Yersinia kristensenii* from ovine faeces. Archivos de Medicina Veterinaria, **29**: 301–305.

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Corresponding author:

Mgr. Miroslava Vázlerová, Veterinární a farmaceutická univerzita Brno, Fakulta veterinární hygieny a ekologie, Ústav hygieny a technologie masa, Palackého 1–3, 612 42 Brno, Česká republika
tel.: + 420 541 562 740, e-mail: vazlerovam@centrumi.cz