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High Prevalence of *yadA*-Positive *Yersinia enterocolitica* in Pig Tongues and Minced Meat at the Retail Level in Finland

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ABSTRACT

Polymerase chain reaction (PCR) was used to determine the prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tongues and minced meat at the retail level in Finland and to confirm the *yadA*-positive *Y. enterocolitica* isolates recovered from the same samples using the conventional culture method. A total of 51 pig tongues purchased at 12 retail outlets and 255 minced meat samples purchased at 40 retail outlets in the Helsinki area were studied. The prevalence of *Y. enterocolitica* carrying the *yadA* gene was 92% in pig tongues and 25% in minced meat using PCR and 78% in tongues and 2% in minced meat with the culture method. The prevalence of *yadA*-positive tongues was higher (98%) when both PCR- and culture-positive results were included because *Y. enterocolitica* carrying the *yadA* gene could also be isolated in three PCR-negative tongue samples. In the minced meat samples, all PCR-negative samples were also culture-negative. With the culture method, 66 of 80 *yadA*-positive isolates in 38 tongues and all *yadA*-positive isolates (4) in four minced meat samples were recovered after selective enrichment. A total of 92 isolates of *Y. enterocolitica* bioserotype 4/O:3 in tongues and 5 isolates in minced meat were found, of which 13% in tongues and 20% in minced meat did not carry the *yadA* gene.

The epidemiology of *Yersinia enterocolitica* is still mostly unknown, even though this organism is recognized as a significant foodborne pathogen causing yersiniosis (1, 13). Healthy pigs have been found to carry human pathogenic *Y. enterocolitica* in the oral cavity (22), and there is indirect evidence that food, particularly pork products, is considered to be an important source of infection (26, 33). *Y. enterocolitica* is widespread and is often present in food, although pathogenic strains are very seldom isolated from foods (7).

In Finland, yersiniosis is the fourth most commonly reported bacterial enteric infection, with about 800 diagnosed cases (16 cases per 100,000 inhabitants) (2) and some 100 *Yersinia*-triggered arthritis cases annually (21). Approximately 70% of the cases in Finland are caused by *Y. enterocolitica* serotype O:3 (17). However, no pathogenic *Y. enterocolitica* was found in Finland when 104 samples of pork and minced pork were studied using the conventional culture method (3). The true prevalence of pathogenic *Y. enterocolitica* in pork products may well be higher because conventional isolation methods may underestimate the occurrence of pathogenic strains (24).

There are considerable difficulties associated with detecting *Y. enterocolitica* in foods. Most methods require time-consuming enrichment steps, and there is no single procedure available to recover all pathogenic serotypes (6). Since not all *Y. enterocolitica* strains are pathogenic, it is necessary to distinguish between pathogenic and nonpathogenic types. This can be difficult and unreliable using phenotypic tests (13). Polymerase chain reaction (PCR) has the

advantage of providing a rapid, specific, and sensitive assay to detect pathogenic *Y. enterocolitica* in natural samples (11, 15, 29, 34) and to confirm the virulence of isolates (4, 5, 18). Pathogenic *Y. enterocolitica* carries the 42 to 48 Md virulence plasmid (pYV), which is essential for the virulence (27). The *yadA* gene (32), located on the virulence plasmid of all pathogenic serotypes, encodes for the protein YadA, a polymeric protein associated with the outer membrane, which is very important in the pathogenesis of *Y. enterocolitica* (14, 19, 20). The *yadA* gene has been used as a PCR target by Kapperud et al. (15).

This study was conducted to determine the prevalence of pathogenic *Y. enterocolitica* in pig tongues and minced meat at the retail level using both the PCR method targeting the *yadA* gene and the conventional culture method.

MATERIALS AND METHODS

Samples and sample preparation. A total of 51 pig tongues and 255 minced meat samples containing minced pork were purchased at 40 retail outlets in the Helsinki area in 1996. The samples were analyzed immediately after purchase. The pig tongues were examined using both swabs and tissue samples. The upper surface of the tongue was swabbed thoroughly with a sterile cotton wool swab and placed in a tube containing 10 ml trypticase soya broth (TSB, Difco, Detroit, Mich.) Tissue samples of the tongue surface were obtained by cutting pieces from the same area that had been swabbed. A 25-g sample of the tongue tissue or minced meat sample was homogenized in 225 ml TSB for 1 min in a stomacher blender. The homogenates were enriched at 22°C for 16 to 18 h and, after overnight enrichment, were studied using both the PCR and culture methods.

Detection of *yadA*-positive *Y. enterocolitica* with PCR in tongues and minced meat. The modified method of Kapperud et

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TABLE 1. The primers and the cycle profiles used in the nested PCR (15)

Step	Primer	Sequence (5'-3')	Cycle profile
1	1	TAA-GAT-CAG-TGT-CTC-TGC-GGC-A	Initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 60 s, extension at 72°C for 90 s
	2	TAG-TTA-TTT-GCG-ATC-CCT-AGC-AC	
2	3	GCG-TTG-TTC-TCA-TCT-CCA-TAT-GC	Initial denaturation at 95°C for 3 min followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 60 s, extension at 72°C for 90 s
	4	GGC-TTT-CAT-GAC-CAA-TGG-ATA-CAC	

al. (15) was used to detect pathogenic *Y. enterocolitica* using nested PCR. One hundred milliliters from the enriched samples was centrifuged at full speed ($16,000 \times g$) for 10 min. The pellets were resuspended in 50 μ l of $1 \times$ PCR buffer (Finnzymes, Espoo, Finland) containing 0.2 mg of proteinase K (Finnzymes) per ml. After incubation at 37°C for 1 h the suspension was boiled for 10 min and then centrifuged at full speed ($16,000 \times g$) for 5 min. Twenty-five milliliters of the supernatant was used as a template in the first PCR step and 2 μ l of the first PCR product was used as template in the second PCR step. Two sets of Pharmacia Biotech (Vantaa, Finland) oligonucleotide primers, based on the nucleotide sequences of the *yadA* gene, were used for PCR amplification, which was performed in a 16-well PTC-150 thermal cycler (MJ Research, Watertown, Mass.) (Table 1). The sample volume was 50 μ l and contained 1 U of Dynazyme DNA-polymerase (Finnzymes), 200 μ M of each dNTP, and 0.1 μ M of each primer. The size of the amplified second PCR product (about 530 bp) was determined in 0.7% agarose gel by comparison to the DNA molecular weight marker VI (Boehringer Mannheim, Mannheim, Germany) (Fig. 1).

Isolation of *yadA*-positive *Y. enterocolitica* with the culture method. *Y. enterocolitica* was isolated with the culture method, which includes overnight enrichment, selective enrichment (25), and cold enrichment (25). Every sample was at first enriched in TSB at 22°C for 16 to 18 h and then further at 4°C for 4 days, after which 100 μ l of this enrichment was inoculated into 10 ml

of selective enrichment broth, modified Rappaport broth (MRB), incubated at 25°C for 4 days. The TSB homogenates were stored at 4°C for 21 days for cold enrichment. Subculture on selective cefsulodin-irgasan-novobiocin (CIN) agar plate (Yersinia Selective Agar Base, Oxoid, Basingstone, UK) was done after every enrichment step according to the method of the Nordic Committee on Food Analysis (25). One to four suspect colonies of typical "bull's eye" appearance on the CIN agar plates were streaked onto blood agar plates to create a pure culture. One colony from a blood agar was inoculated onto a urea agar slant (Difco) and incubated for 1 day at 30°C. Isolates showing urea hydrolysis were further identified using the API 20E system (BioMérieux, Marcy l'Etoile, France) and incubated at 25°C for 18 to 20 h. *Y. enterocolitica* isolates were biotyped according to the revised scheme of Wauters et al. (36) and serotyped by slide agglutination using O:3, O:5, and O:9 antisera (Denka Seiken, Tokyo, Japan). The *yadA* gene was detected in the pure culture using PCR (15). Four small (<2 mm) colonies were boiled in 100 μ l of water for 10 min, and 2 μ l of this boiled bacteria suspension was used as a template in the PCR (15).

RESULTS

The prevalence of *yadA*-positive *Y. enterocolitica* in retail pig tongues and minced meat is shown in Table 2. The prevalence was higher with PCR as compared to the culture method, especially when minced meat was studied. The highest prevalence (98%) was obtained in tongues when both PCR and culture-positive results were included because *yadA*-positive *Y. enterocolitica* could be isolated from three PCR-negative tongues. Using the culture method, *yadA*-positive *Y. enterocolitica* could be isolated from 40 (78%) tongues when positive results from both swabs and tissue samples were combined. *YadA*-positive isolates were recovered from 19 swabs and 35 tissue samples.

MW 1 2 3 NC 4 5 6 NC PC MW

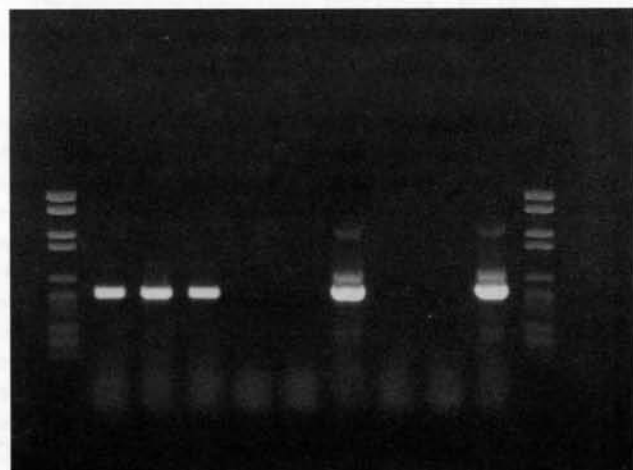


FIGURE 1. Gel electrophoresis patterns of PCR products using nested PCR (15). Lanes 1 to 6, results obtained by examination of six retail tongues; MW, molecular weight marker; NC, negative control; and PC, positive control.

TABLE 2. Prevalence of *yadA*-positive *Yersinia enterocolitica* in pig tongues and minced meat at the retail level

Origin of samples	Number of samples	Number of PCR-positive samples		Number of culture-positive ^a samples		Number of both PCR- and culture-positive ^a samples	
		n	%	n	%	n	%
Pig tongues	51	47	92	40	78	50	98
Minced meat	255	63	25	4	2	63	25

^a *YadA*-positive *Y. enterocolitica*.

TABLE 3. Number of *Yersinia enterocolitica* isolates from pig tongues and minced meat after different enrichment steps

Origin (no. of isolates)	Biotype	Overnight enrichment ^a	Selective enrichment ^b	Cold enrichment ^c
Tongues (94)	4	6 (3) ^d	66 (38)	8 (5)
	4 ^e	0	9 (7)	3 (3)
	1A	0	1 (1)	1 (1)
Minced meat (9)	4	0	4 (4)	0
	4 ^e	0	0	1 (1)
	1A	0	3 (3)	1 (1)

^a Overnight enrichment in TSB at 22°C for 16 to 18 h.

^b Selective enrichment in MRB at 25°C for 4 days.

^c Cold enrichment in TSB at 4°C for 21 days.

^d Number of samples.

^e *YadA*-negative isolates.

YadA-positive *Y. enterocolitica* could only be isolated from four (2%) minced meat samples.

Figure 1 shows the results of six tongue samples studied with PCR. The PCR-positive samples, i.e., samples 1, 2, 3, and 5, and the positive control (PC) gave a strongly predominant band at about 530 bp. The PCR-negative samples, i.e., samples 4 and 6, and the negative control (NC) gave no visible band on the gel. The PCR-negative samples were in these cases also culture-negative.

Using the culture method, selective enrichment was the most productive. Thirty-eight of 40 culture-positive tongues could be found after selective enrichment. Only one new positive tongue was obtained after overnight enrichment and another one after cold enrichment. Table 3 shows that 66 *yadA*-positive isolates were found in 38 tongues after selective enrichment, while only six pathogenic isolates were found in 3 tongues after overnight enrichment and eight pathogenic isolates were found in 5 tongues after cold enrichment. *YadA*-positive isolates could be recovered from minced meat only after the selective enrichment step.

Most of *Y. enterocolitica* isolates (92 of 94) in pig tongues belonged to bioserotype 4/O:3, and 80 (87%) of these were *yadA*-positive (Table 4). Only two isolates out of 94 belonged to nonpathogenic biotype 1A when tongues were studied. Almost half of the *Y. enterocolitica* isolates

(4 of 9) in minced meat samples belonged to the nonpathogenic biotype 1A. All isolates belonging to bioserotype 4/O:3 did not carry the *yadA* gene, but they were all negative in pyrazinamidase, esculin, and salicin tests.

DISCUSSION

The prevalence of pathogenic *Y. enterocolitica* in pig tongues and minced meat was high at the retail level, which reinforces the assumption that these products can be possible transmission links between the swine reservoir and human infection. The high contamination rate of pathogenic *Y. enterocolitica* in pig tongues may be due to the slaughtering process, where the tonsils are removed together with the tongue. It is difficult to avoid the spread of pathogenic bacteria from the tonsils to the tongue when they hang together on a hook in the slaughterhouse. Cross-contamination from tongue to tongue may occur in the slaughterhouse and later in retail outlets. More *yadA*-positive samples were detected using PCR, especially when minced meat was studied, which shows that the prevalence is underestimated when the culture method is used.

PCR has only recently gained interest as a detection method in food hygiene and control. The PCR method targeting the *yadA* gene located on the virulence plasmid (pYV) (15) was used because it has been shown that pYV is essential for the full virulence of *Y. enterocolitica* (27). Because of the instability of the virulence plasmid at 37°C, the samples were not exposed to temperatures above 30°C in order to avoid the possibility of losing the plasmid. To increase the sensitivity and to restrict the detection to culturable organisms, only enriched samples were used in this study. Three of the tongue samples in the present study were PCR-negative and culture-positive, but it is known that many components of natural samples can influence the effectiveness of PCR and cause false-negative results (28). There is not yet any easy method for overcoming the problem of inhibitory substances in food. In this study, proteinase-K treatment was used to decrease the inhibition. Yet, despite the possible inhibitory substances in the samples and possible false-negative results, more *yadA*-positive samples were obtained with PCR compared to the conventional culture method, which shows that the culture method is less sensitive.

TABLE 4. Virulence-associated properties of *Yersinia enterocolitica* isolates recovered from pig tongues and minced meat

Origin (no. of samples)	Number of isolates	Biotype	Serotype	<i>yadA</i> ^a	PYZ ^b	Esculin	Salicin
Tongues (51)	80 (40) ^c	4	O:3	+	—	—	—
	12 (9)	4	O:3	—	—	—	—
	2 (2)	1A	NT ^d	—	+	+	+
Minced meat (255)	4 (4)	4	O:3	+	—	—	—
	1 (1)	4	O:3	—	—	—	—
	4 (4)	1A	NT	—	+	+	+

^a The *yadA* gene located on the virulence plasmid.

^b PYZ, pyrazinamidase activity.

^c Number of samples.

^d Not tested.

Pathogenic *Y. enterocolitica* harboring the *yadA* gene could be recovered from 78% of 51 tongues with the culture method. Sixty-nine percent of the tongues were positive when tissue samples were used and only 37% were positive when swab samples were used, which shows that the tissue sampling was clearly more productive than swabbing. All *yadA*-positive isolates belonged to bioserotype 4/O:3. Pig tongues have been studied in many countries, but only in Belgium has the recovery rate of *Y. enterocolitica* serotype O:3 from pig tongues been this high (35). Wauters et al. (35) found serotype O:3 in 28 (97%) of 29 pig tongues. In Canada, Schiemann (30) recovered *Y. enterocolitica* serotype O:3 from 11 (30%) of 37 retail tongues. Shiozawa et al. (31) found *Y. enterocolitica* bioserotype 4/O:3 in 11 (22%) of 50 pig tongues at the retail level in Japan. In the Netherlands, de Boer and Nouws (8) isolated *Y. enterocolitica* serogroup O:3 from 6 (15%) of 40 tongues from four abattoirs. In Germany, *Y. enterocolitica* isolates from 14 (27%) of 52 tongues belonged to bioserotype 4/O:3 (16). De Giusti et al. (9) studied 86 tongues at the retail level in Italy and found only four (5%) samples positive for bioserotype 4/O:3.

Pathogenic *Y. enterocolitica* harboring the *yadA* gene could be recovered from 2% of 255 minced meat samples with the culture method. The isolation rate in minced meat samples was comparable to other studies. In Norway, *Y. enterocolitica* O:3 was recovered from 1 (1%) of 127 raw pork samples (23). In Italy, no *Yersinia* spp. were detected in 90 retail pork products with any of the four different culture methods used (9). Logue et al. (20) examined 340 samples of Irish meat and meat products and found pathogenic serotypes in both raw (9%) and cooked meats (2%). Fukushima et al. (12) recovered pathogenic serotypes from 37 (3%) pork samples out of 1,278. Wauters et al. (35) found 24% of minced meat samples to be positive for serogroup O:3. The explanation of this high isolation percentage in Belgium may be the use of head meat and tonsillar tissue in minced meat (33), which is forbidden in Finland.

Conventional culture methods are time consuming and, at present, no single isolation procedure is available for the recovery of all pathogenic bioserotypes. Most (83%) of *yadA*-positive *Y. enterocolitica* isolates were isolated after selective enrichment when tongues were studied. Some isolates were also found after overnight enrichment and after cold enrichment. Only two isolates of nonpathogenic biotype 1A were found in tongues, which shows that bioserotype 4/O:3 dominated in pig tongues. The isolation of *Y. enterocolitica* in minced meat was difficult because of the rich background flora. The CIN agar plates were overgrown with atypical colonies and also with urea-negative colonies of bull's eye appearance. The few pathogenic isolates carrying the *yadA* gene could be recovered only after selective enrichment. The failure to detect *yadA*-positive *Y. enterocolitica* in minced meat when cold enrichment was used is presumably due to the inability of a few virulent strains to grow sufficiently in the presence of other bacteria (35).

No isolation procedure can clearly differentiate pathogenic from nonpathogenic variants. In the present study, the

pathogenicity of isolates was confirmed with PCR targeting the *yadA* gene located on the virulence plasmid (15). All pathogenic isolates belonged to bioserotype 4/O:3, which is the most common bioserotype associated with disease in humans in most countries (13). However, all isolates belonging to bioserotype 4/O:3 did not harbor the *yadA* gene in the present study. Pyrazinamidase activity, esculin hydrolysis, and salicin fermentation have been used to identify pathogenic serotypes of *Y. enterocolitica* (10). These tests correlated well with bioserotype 4/O:3, but they did not correlate with the presence of the *yadA* located on the virulence plasmid. PCR targeting of the *yadA* gene seems to be a rapid and specific assay to confirm the full virulence of pathogenic serotypes.

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