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Comparison of DNA extraction methods for pathogenic *Yersinia enterocolitica* detection from meat food by nested PCR

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Abstract

The objective of this work was to compare three different methods of DNA extraction from meat food, and to determine whether these methods removed inhibitors of nested PCR for pathogenic *Yersinia enterocolitica* detection. The amplification of the *yadA* gene from the DNA obtained from a pure *Y. enterocolitica* culture could be carried out with all the protocols. DNA amplification from the food samples was observed with two of the three tested protocols, which gave highly sensitive amplifications (detection limit 1 CFU/ml). These protocols detected a lower limit of 0.6 fg/μl of DNA extracted from *Y. enterocolitica* pure culture. We concluded that these protocols were able to eliminate satisfactorily the PCR inhibitors present in the foods. The nested PCR tested could be used satisfactorily in the investigation of pathogenic *Y. enterocolitica* in foods in the presence of a high background of microflora.

Keywords: DNA extraction protocols; Comparison; Nested PCR; *yadA* gene; *Yersinia enterocolitica*; Meat food

1. Introduction

Yersinia enterocolitica is a human pathogen that causes a great variety of intestinal and extraintestinal symptoms, among them enterocolitis, mesenteric lymphadenitis and septicemia. Besides, this bacterium may induce immunological postinfectious sequelae including erythema nodosum, arthritis and glomerulonephritis (Bottone, 1997, 1999).

Y. enterocolitica includes a wide range of phenotypic variants, a group of which can produce diseases in humans. The bioserotypes most frequently associated with human yersiniosis are 4/O:3, 2/O:9, 1B/O:8 and 2/O:5,27. The 4/O:3 bioserotype is globally distributed, and it is most frequently found in pigs (Bottone, 1999). Since healthy pigs have been demonstrated to be the principal reservoirs of this disease (Nesbakken, 1985), pork is considered an important infection source. However, the prevalence of pathogenic *Y. enterocolitica* in pork meat, except for tongue and offal, seems to be very low (Kapperud, 1991).

Virulent and avirulent strains should be distinguished since not all bioserotypes are pathogenic. The pathogenic strains of *Y. enterocolitica* exhibit a virulence plasmid called pYV, of 70–75 kb, which encodes virulence factors of great importance in the clinical picture. Among these virulence factors are the *Yersinia* outer proteins (Yops), which play a major role in yersinial virulence; and an adhesine called *Yersinia* adhesine A (YadA), which promotes adhesion to the intestine epithelial cells and among the bacteria themselves, producing autoagglutination and interference with the bactericidal action of serum (Aepfelbacher et al., 1999; Bottone, 1997). There are considerable difficulties associated with the determination of *Y. enterocolitica* in foods by molecular methods. Likewise, most of the culture methods require very long enrichments, and there is no simple procedure to recover pathogenic serotypes (de Boer, 1992). The

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difficulties associated with the isolation of pathogenic *Y. enterocolitica* strains can be caused by the low amount of these microorganisms in the sample and by the high amount of other organisms present in the accompanying flora (Fredricksson-Ahoma & Korkeala, 2003).

The polymerase chain reaction (PCR) has become a widely used technique for the detection of infectious microorganisms. The PCR capacity to detect microorganisms depends on the purity of the template used as target and on the presence of a sufficient number of target molecules. Some samples, such as foods, can exhibit PCR inhibitors (Powell, Gooding, Garret, Lund, & McKee, 1994; Wilson, 1997). Therefore, additional steps must be performed to limit the effect of these inhibitors. An important number of methods have been examined for the preparation of inhibitor-free templates, including those that require filtration (Waage, Vardund, Lund, & Kapperud, 1999) or flotation (Bhaduri, Wesley, & Bush, 2005). However, these methods are sometimes difficult and laborious. A great number of PCR assays have been developed for the identification of different *Y. enterocolitica* virulence markers, such as the *ail* (Bhaduri & Cottrell, 1998; Lamberts & Danielsson-Tham, 2005; Wannet, Reessink, Brunings, & Maas, 2001) and the *yst* chromosomal genes (Ibrahim, Liesack, Griffiths, & Robins-Browne, 1997; Vishnubhatla et al., 2001). Similarly, genes found on the virulence plasmid such as the *yadA* gene have been used (Boyapalle, Wesley, Hurd, & Reddy, 2001; Fredricksson-Ahoma, Hielm, & Korkeala, 1999; Kapperud, Vardnund, Skjerve, Hornes, & Michaelsen, 1993).

The aim of this work was to compare different techniques of DNA extraction from pork sausages and minced meat, and to determine whether these techniques removed PCR inhibitors, enabling the detection of *Y. enterocolitica* by nested PCR in meat foods. These two sample types were selected in order to compare a well-seasoned sample with an unprocessed one.

2. Materials and methods

2.1. Bacterial cells and inoculum preparation

Two strains, *Y. enterocolitica* W1024 O:9 pYV (+), provided by Dr. Guy Cornelis, Catholic University of Louvain, Belgium, and *Y. enterocolitica* 1A isolated from pork sausage in our laboratory, were used. These strains were kept in Luria broth supplemented with 20% glycerol (LB; Merck Laboratories, Darmstad, Germany) at −20 °C.

A *Y. enterocolitica* colony obtained from Mac Conkey agar (MC; Merck) was inoculated in 100 ml of tripticase soy broth (TSB; Merck) and incubated for 18 h at 25 °C to obtain a concentration of 1×10^9 CFU/ml. The optical density was measured at 600 nm after which the concentration was diluted to 1×10^4 CFU/ml. Plate counts on MC agar (Merck) were carried out to corroborate the concentration obtained. One milliliter of this concentration was used to perform the DNA extraction when the extraction was done directly from the pure culture or to inoculate the samples.

2.2. Samples inoculation

Twenty-five grams of sample consisting of either pure pork sausage or minced meat, were inoculated with 1 ml of the inoculum prepared as described above, placed in 225 ml of TSB (Merck) and homogenized using a stomacher (IUL Instruments, Germany) for 90 s at 1500 rpm. Then, the samples were statically incubated over night at 25 °C and DNA was extracted.

2.3. DNA extraction

Three DNA extraction methods were compared. The first protocol was a modification of the method developed by Kapperud et al. (1993). One hundred microliter of the enriched sample were centrifuged at 16,000*g* for 10 min at 4 °C in a refrigerated Sigma 3K30 laboratory centrifuge (Sigma, Steinheim, Germany). The pellet was resuspended in 50 μl of PCR 1× buffer (Promega Corporation, Madison, USA) containing 0.2 mg of Proteinase K/ml (Fluka Chemie, Buchs, Switzerland). After being incubated at 37 °C for 1 h, the suspension was boiled for 10 min and then centrifuged at 16,000*g* for 5 min at 4 °C. The supernatant was used to perform the PCR. The second protocol consisted of a method by Fontana, Cocconcelli, and Vignolo (2005), with some modifications. One milliliter of the enriched sample was washed with 200 μl of ammonia hydroxide, 200 μl of absolute ethanol, 400 μl of petrol ether and 20 μl of SDS (10%). The sample was centrifuged at 14,000*g* for 10 min at 4 °C, and the pellet was resuspended in a solution containing 200 μl of 6 M urea, 200 μl of absolute ethanol, 400 μl of petrol ether, 80 μl of SDS (10%) and 13 μl of 3 M sodium acetate. A second centrifugation for 10 min at 14,000*g* at 4 °C was performed, and the pellet was resuspended with 600 μl of TE buffer (Tris–EDTA) pH 8.0, 35 μl of SDS (10%) and 10 μl of DNase-free RNase (10 mg/ml). The tubes were incubated at 37 °C for 30 min before the addition of 10 μl of Proteinase K (20 mg/ml; Sigma Chemical, Steinheim, Germany). This preparation was incubated at 37 °C for 30 min. Finally, 130 μl of 6 M sodium perchlorate and 500 μl of phenol chloroform isoamyl alcohol (25:24:1; pH 6.7) were added for DNA extraction. The tubes were then centrifuged at 12,000*g* for 5 min, the aqueous phase was collected and the nucleic acids were precipitated with absolute alcohol. DNA was dissolved in 50 μl of deionized water (ultra pure water with final ultrafiltration, Milli-Q-UF Equipment, Millipore Intertech). For protocol 3, the Prepman Ultra reagent (Applied Biosystems, Foster City, California, USA) nucleic acid isolation method was performed according to the manufacturer's instructions.

2.4. Determination of DNA integrity

Two microliter of each solution containing the extracted DNA obtained by the techniques described above were visualized in a 0.9% agarose gel, after electrophoresis at 80 V for 40 min. The gels were stained with ethidium

Table 1
Characteristics of the nested PCR for the detection of pathogenic *Y. enterocolitica*

Step	Primer	Sequence (5′–3′)	Cycle profile
1	Yad 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
	Yad 2	TAG TTA TTT GCG ATC CCT AGC AC	
2	Yad 3	GCG TTG TTC TCA TCT CCA TAT GC	Initial denaturation at 95 °C for 3 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s, extension at 72 °C for 90 s
	Yad 4	GGC TTT CAT GAC CAA TGG ATA CAC	

bromide (0.5 μg/ml) and the DNA bands were visualized using a transluminator with ultraviolet light source (UVP UltraViolet Products) and photographed with a Gel Camp Polaroid camera (Sigma).

2.5. PCR

The nested-PCR method of Kapperud et al. (1993) was used with some modifications. Five microliter of the template was used for the first PCR step, and 2 μl of the product obtained was used as template for the second PCR step. Two sets of primers, based on the nucleotide sequence of the *yadA* gene, were used for the amplification by PCR (Table 1) that was performed in a programmable Gem Amp System 2.400, Perkin Elmer thermocycler. The reaction mixture (50 μl) contained 1 U of Taq DNA-polymerase (Promega), reaction buffer (1×, Promega), 200 μM of each dNTP (Promega) and 0.1 μM of each primer. The amplicon size of the first PCR was 747 bp and the product size of the second PCR was 529 bp. These products were determined by electrophoresis in a 1% agarose gel by comparing them with a 100-bp molecular weight marker (Biodynamics SRL, Buenos Aires, Argentina). The bands were visualized by staining with ethidium bromide.

2.6. Determination of the PCR sensitivity

Serial dilutions (1:10) from 1×10^4 to 0 CFU/ml of *Y. enterocolitica* carrying the virulence plasmid were carried out in TSB (Merck). Plate counts on MC (Merck) were performed to corroborate these concentrations. One milliliter of each concentration was inoculated in 25 g of sample. Each sample was placed in 225 ml of TSB (Merck) and homogenized in stomacher for 90 s. After incubating the samples over night at 25 °C, 1 ml of each concentration was taken, and DNA extraction and the subsequent PCR were carried out.

Serial dilutions (1:10) of DNA from a pure culture of *Y. enterocolitica* were also performed in sterile distilled water (concentration 600 ng/μl to 0.66 fg/μl). Every determination was carried out at least three times.

2.7. Processing of the food samples for Y. enterocolitica detection

Samples of pure pork sausages ($n = 14$), pork and beef sausages ($n = 15$) and minced meat ($n = 15$) were purchased from different stores in the city of San Luis and immediately processed or stored at 4 °C for up to 4 h. Twenty-five grams of sample were enriched in 225 ml TSB (Merck), homogenized in stomacher for 90 s, and incubated at 25 °C for 18 h. After that, 100 μl of broth was transferred to 10 ml of modified Rappaport broth (MRB) and incubated at 25 °C for 4 days. The TSB was incubated at 4 °C for 21 days. After enrichment, plate isolations were carried out on cefsulodin–irgasan–novobiocin agar (CIN, Merck) and MC (Merck). The cultures were incubated at 25 °C for 48 h. The typical "bull's eye" colonies in CIN and the small and creamy colonies in MC were reisolated and identified by biochemical assays (Bercovier & Mollaret, 1984).

After enrichment in TSB, 1000 μl of broth was taken to perform the DNA extraction and the subsequent nested PCR. The PrepMan Ultra extraction kit was used, which enables rapid DNA extraction.

3. Results

3.1. Comparison of the effectiveness of the DNA extraction protocols

All the DNA extraction protocols kept intact the *Y. enterocolitica* DNA from the studied sources (Fig. 1).

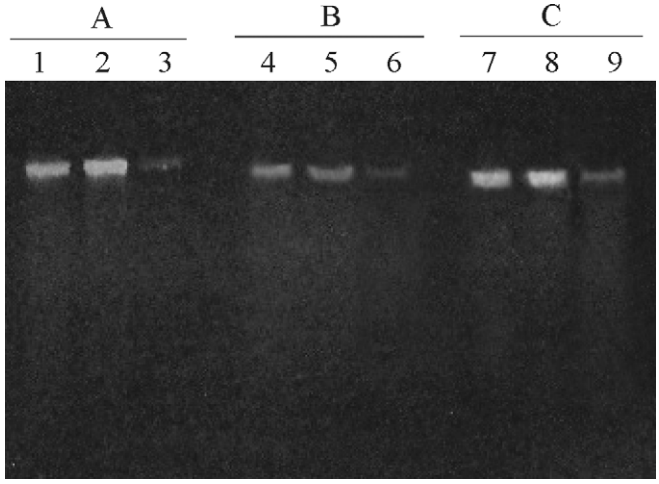

Fig. 1. Electrophoresis agarose gel stained with ethidium bromide of total *Y. enterocolitica* DNA obtained by (A) protocol 1, (B) protocol 2 and (C) protocol 3 (described in Section 2). Lanes 1, 4 and 7 represent bacterium inoculated pure pork sausage and incubated for 18 h at 25 °C; lanes 2, 5 and 8 represent bacterium inoculated minced meat and incubated for 18 h at 25 °C; lanes 3, 6 and 9 represent the inoculum pure culture.

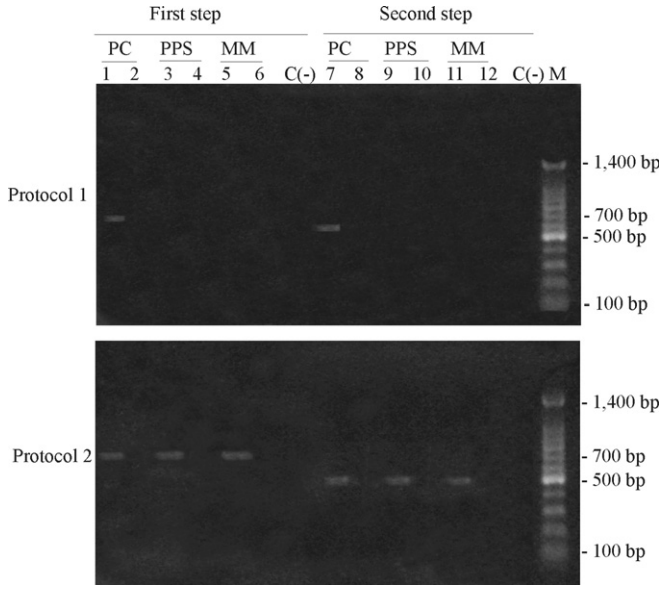

Fig. 2. Electrophoresis agarose gel stained with ethidium bromide of nested-PCR products. Lanes 1, 3, 5, 7, 9, and 11: *Y. enterocolitica* W1024 pYV (+); lanes 2, 4, 6, 8, 10, and 12: *Y. enterocolitica* B1A pYV (−). Lane M: 100 bp ladder; C (−): negative control; PC: inoculum pure culture; PPS: bacterium inoculated pure pork sausage; MM: bacterium inoculated minced meat.

The amplification of the *yadA* gene from the DNA obtained from a pure culture of *Y. enterocolitica* W1024 pYV (+) could be carried out with all the methods. No amplification of the *Y. enterocolitica* 1A strain, which does not contain the virulence plasmid, was observed with any of these methods. The presence of the amplicon from the studied food samples was observed using protocols 2 (Fig. 2) and 3 (data not shown) for DNA extraction, but not with protocol 1 (Fig. 2).

Fig. 3 shows that the *yadA* amplicon was observed even when the sample of pork sausage was contaminated with 1 CFU/ml using protocol 3 and with 10 CFU/ml using protocol 2. The same results were obtained from minced meat (results not shown).

Fig. 4 shows the results obtained with the DNA extracted from pure culture of *Y. enterocolitica*. Both protocols 2 and 3 detected up to the lowest examined concentration (0.6 fg/μl).

As shown by Figs. 3 and 4, the nested approach increases the sensitivity of the assay since the subdetectable levels of the products generated during the first PCR were amplified and were detected during the second PCR. The results obtained with protocols 2 and 3 were reproducible.

3.2. Detection of virulent *Y. enterocolitica* strains in foods

Forty-four meat samples were examined to test the nested-PCR protocol. By culture techniques, one *Y. enterocolitica* strain (0.15%) was isolated from beef and pork sausage, while no isolations were obtained from pork sausage or minced meat. The strain was isolated after a twenty-one-day enrichment in TSB and subsequent selection in CIN agar.

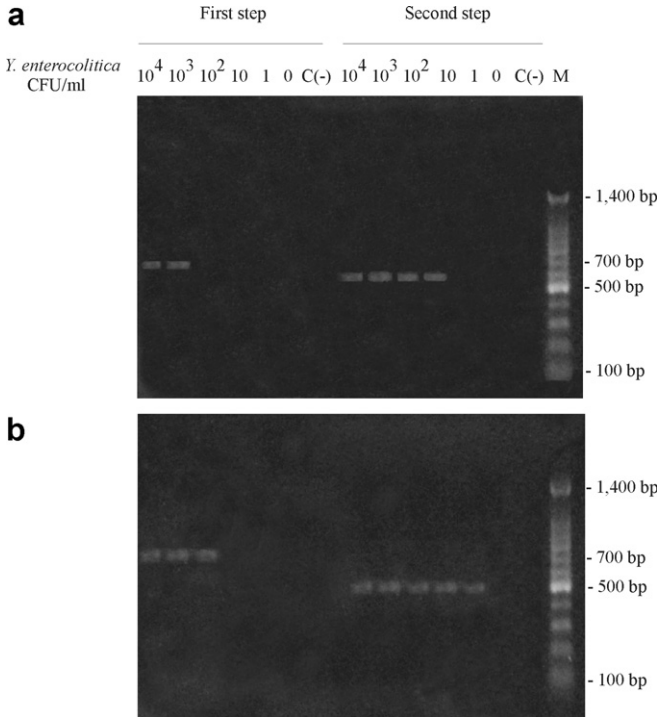

Fig. 3. Electrophoresis agarose gel stained with ethidium bromide of nested-PCR products from pure pork sausage inoculated with *Y. enterocolitica* in different concentrations. (a) DNA extracted by protocol 2; (b) DNA extracted by protocol 3. Lane M: 100 bp ladder; C (−): negative control.

In contrast, *yadA* positive samples were identified in 5 samples (36%) from pure pork sausage, 3 samples (20%) from beef and pork sausage and 8 samples (53%) from minced meat. The sample of beef and pork sausage was identified as positive by both the culture and the molecular methods.

4. Discussion

The presence of pathogenic *Y. enterocolitica* in meat foods is controversial. The results obtained by different researchers often differ according to the work methodology used (Lamberts & Danielsson-Tham, 2005; Logue, Sheridan, Wauters, Mc Dowell, & Blair, 1996). Culture techniques are often laborious and time-consuming. On the other hand, molecular biology techniques are more rapid, but have the disadvantage that DNA amplification may be interfered by PCR inhibitors that are sometimes present in some foods (Bhaduri et al., 2005; Boyapalle et al., 2001; Fredricksson-Ahoma & Korkeala, 2003).

In this work three methods of DNA extraction were studied, among them, a modified version of the method proposed by Kapperud et al. (1993). These authors performed nested PCR from DNA extracted from pork and beef samples and the same procedure has been used successfully by other researchers investigating foods such as minced meat and pig tongues (Fredricksson-Ahoma et al., 1999). In our work, this technique could not eliminate the

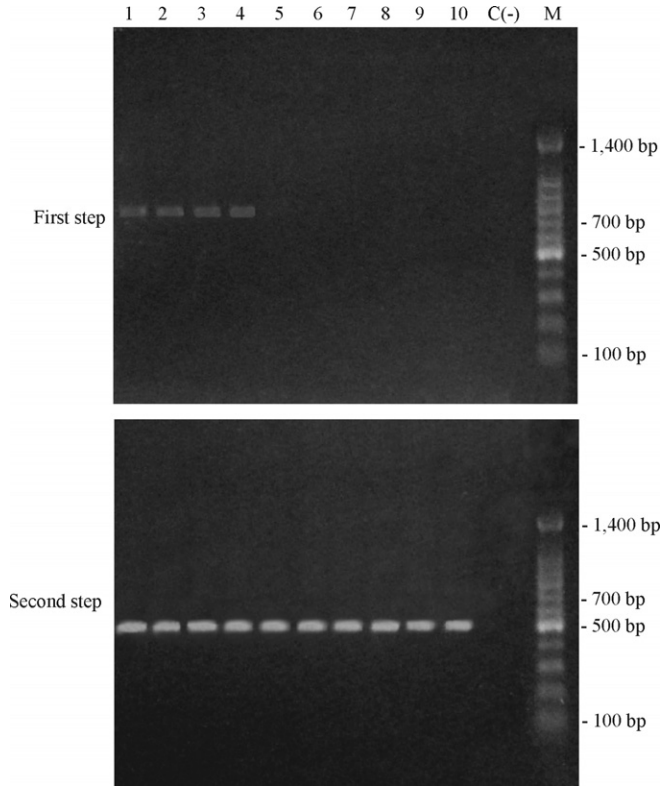

Fig. 4. Electrophoresis agarose gel stained with ethidium bromide of nested-PCR products from *Y. enterocolitica* pure culture. Lanes 1 to 10: different concentrations of DNA (600 ng/μl to 0.6 fg/μl); lane M: 100 bp ladder; C (−): negative control.

PCR inhibitors since DNA extraction from the studied foods could be detected but no amplification band was obtained after the PCR. As regards pork sausages, we may suggest that they exhibit a high amount of inhibitors as compared to the foods studied by the above mentioned authors since grease particles and a great amount of spices and condiments are used in their preparation in order to enhance flavor. The same cannot be claimed for minced meat. In fact, Fredricksson-Ahoma et al. (1999) were able to carry out the *yadA* amplification from *Y. enterocolitica* virulent strains in a study using minced meat samples. Waage et al. (1999) also identified *Y. enterocolitica* in drinking water and wastewater samples using the same procedure; however, the inhibitors in their samples are likely to be different from those present in our samples.

Protocol 2 has been used by Fontana et al. (2005) for the isolation of lactic bacteria in sausages obtained in Argentina. These authors were able to remove PCR inhibitors and carry out the PCR and denaturing gradient gel electrophoresis (DGGE). This method was therefore selected for the study of sausage samples in our work, although lysozyme was not used here since *Y. enterocolitica* is a gram-negative bacterium. The PCR inhibitors were also removed in our work.

Protocol 3 was chosen because it has already been used successfully by Vishnubhatla et al. (2001) for the search of *Y. enterocolitica* in raw meat and tofu. In our work this method could also eliminate the PCR inhibitors.

The study of the sensitivity of the nested PCR in the DNA obtained from pure cultures of *Y. enterocolitica* indicated that the detection limit was higher than 0.6 fg/μl. The sensitivity obtained in this work was higher than that obtained by Boyapalle et al. (2001). These authors used the PrepMan Ultra reagent for DNA extraction and carried out two assays: a multiplex PCR with a sensitivity of 1 ng/μl and a TaqMan assay with a sensitivity of 1 pg/μl. Wannet et al. (2001) carried out a duplex PCR for the *ail* and 16S rRNA genes and obtained a sensitivity of 5 fg of DNA, similar to 3 fg obtained in our work. Protocol 3 permitted to identify up to 1 CFU/ml *Y. enterocolitica* while protocol 2 up to 10 CFU/ml. These results are similar to those obtained by Waage et al. (1999) who detected $\leqslant 1$ CFU/ml of *Y. enterocolitica* in water samples using the same nested-PCR protocol. It can be said that the nested-PCR technique increases the sensitivity of the PCR. Boyapalle et al. (2001) observed a detection limit of 4×10^2 CFU/ml using protocol 3 for DNA extraction and subsequent multiple PCR. Likewise, Vishnubhatla et al. (2001) detected up to 3.2×10^2 CFU/ml of *Y. enterocolitica* in minced pork samples using TaqMan assay for the *yst* gene. One possible explanation for the increased sensitivity observed in our nested-PCR protocol is that any inhibitory substance present in the food would be diluted during the second step of the PCR since only 1:50 of the first step PCR product is transferred to the second step reaction mix.

In this work, only one strain was isolated when the search for *Y. enterocolitica* was carried out by culture methods. However, when *yadA* nested PCR was used, the positive samples were 53% minced meat, 36% pure pork sausage and 20% pork and beef sausage. Similar results have been obtained by other authors (Boyapalle et al., 2001; Fredricksson-Ahoma et al., 1999; Vishnubhatla et al., 2001).

When a direct detection of the searched pathogen is carried out, false-positive results can be observed due to the presence of dead cells in the samples studied. This could lead to an erroneous conclusion in relation to the potential risk for the consumer. Therefore, an enrichment step was included in this work in order to promote the growth of the target organism and dilute any dead bacterium or exogenous DNA present in the sample.

The differences observed between the results obtained by PCR and by the culture methods may be due to the lower sensitivity of the selective culture media. Vishnubhatla et al. (2001) observed that when the concentration of microorganisms was lower than 10^6 CFU/ml in minced pork inoculated with *Y. enterocolitica*, the results obtained were false negatives. It is also important to emphasize that the PCR is a rapid method for the detection of virulent strains. The possible existence of a small number of damaged or stressed cells could be the cause of long incubation times needed so that these cells may reach the detection limit of the culture methods.

The results of this work allow us to conclude that protocols 2 and 3 were able to eliminate satisfactorily the PCR inhibitors present in the studied foods. Although, protocol 2 requires longer times and is more laborious than protocol 3, its lower cost makes it useful when searching for *Y. enterocolitica* in meat foods. The protocol of nested PCR used in this work could be successfully used in the investigation of pathogenic *Y. enterocolitica* strains from foods with a great level of background microflora, such as pork sausages and pork and beef sausages. By using either of these protocols, the analysis can be carried out in less than 48 h, a period shorter than the one needed for the isolation of this microorganism by traditional culture techniques. The *yadA* nested-PCR method allows detect a low number of pathogenic *Y. enterocolitica* strains in samples of meat foods without the requirement of biotypification or serotypification.

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