

Detection of Pathogenic *Yersinia enterocolitica* in Foods and Water by Immunomagnetic Separation, Nested Polymerase Chain Reactions, and Colorimetric Detection of Amplified DNA

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A two-step polymerase chain reaction (PCR) procedure with two nested pairs of primers specific for the *yadA* gene of *Yersinia enterocolitica* was developed. The PCR assay identified all common pathogenic serogroups (O:3, O:5,27, O:8, O:9, O:13, and O:21) from three continents and differentiated pathogenic *Y. enterocolitica* from *Y. pseudotuberculosis* and from a variety of nonpathogenic yersiniae representing 25 serogroups and four species. The performance of the method was evaluated with seeded food and water samples. We compared two procedures for sample preparation prior to PCR: one was based on immunomagnetic separation of the target bacteria from the sample, using magnetic particles coated with immunoglobulin antibodies to *Y. enterocolitica* serogroup O:3, and the other method consisted of a series of centrifugation steps combined with proteinase treatment. Regardless of the method used, the PCR assay was capable of detecting 10 to 30 CFU/g of meat in 10⁶-fold excess of indigenous bacteria. When the samples were enriched overnight in a nonselective medium, the sensitivity was increased to approximately 2 CFU/g, except for samples with an extremely high background flora (>10⁷ CFU/g). We compared gel electrophoretic detection of PCR products with a colorimetric detection method designated DIANA (detection of immobilized amplified nucleic acids), which enabled easy visualization of amplified fragments in a microtiter plate format with an optical density reader. DIANA and gel electrophoresis showed complete concordance in their discrimination between positive and negative samples. The combination of immunomagnetic separation, nested PCR, and DIANA makes possible the development of a fully automated analytic process which requires a minimum of laboratory manipulations.

Yersinia enterocolitica is the etiological agent of a range of clinical entities in humans, although acute noncomplicated enteritis is by far the most frequent manifestation (3, 18). *Y. enterocolitica* encompasses a spectrum of phenotypic and genotypic variants, of which only a few have been conclusively associated with human or animal disease (18). There is strong indirect evidence that food, especially pork products, and water are important sources of human infection (10, 31). However, there are considerable difficulties associated with the isolation of pathogenic *Y. enterocolitica* from these sources. Most methods require time-consuming enrichments, and the development of isolation procedures which clearly differentiate pathogenic from nonpathogenic yersiniae has proven to be problematic (10). The polymerase chain reaction (PCR) provides a way of overcoming these difficulties. Although several reports have described the use of PCR for the detection of pathogenic *Y. enterocolitica* (4, 5, 8, 14, 20, 36, 40), until now no reports have described the application of the PCR to food microbiology, and none of the applications published thus far appears to be suited for routine screening of large numbers of samples.

We have developed a two-step PCR for detection of pathogenic *Y. enterocolitica* based on the use of two pairs of oligonucleotide primers in a nested configuration. As a target for PCR, we selected the *yadA* gene (previously termed *yopA*) located on a virulence-associated plasmid shared by *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (30).

The *yadA* gene encodes a subunit of the outer membrane protein YadA (Yop1), which is a virulence determinant in *Y. enterocolitica* (11, 30). To facilitate concentration and purification of the target bacteria from crude samples, we used the principle of immunomagnetic separation (IMS), which utilizes small, uniform, paramagnetic particles coated with antibodies specific to surface antigens (27, 35). The IMS technology has been applied successfully to detect several pathogens in foods and in clinical specimens and to concentrate bacteria from environmental samples (2, 6, 15, 16, 19, 27, 28, 32, 33). Finally, the amplified PCR products were visualized by using a colorimetric detection procedure designated DIANA (detection of immobilized amplified nucleic acids), which eliminates the need for gel electrophoretic analysis (37).

The purposes of the present report were (i) to determine the sensitivity and specificity of the two-step PCR and IMS for detection of pathogenic *Y. enterocolitica* in foods and water, (ii) to compare IMS with an alternative sample preparation procedure based on differential centrifugation, and (iii) to compare the relative performance of colorimetric and electrophoretic detection of PCR products.

MATERIALS AND METHODS

Bacterial strains. A total of 55 *Yersinia* isolates with known plasmid profiles were selected for this study (Table 1). Nineteen *Y. enterocolitica* strains harbored the 40- to 50-MDa virulence-associated plasmid. Each of these strains was also represented by a plasmid-cured mutant which was

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TABLE 1. *Yersinia* strains examined

Species	Serogroup	Biovar ^a	Phagevar	Country(ies)	Source(s)	No. of strains	Plasmid ^b
<i>Y. enterocolitica</i>	O:3	4	VIII	Norway	Human, pig	2	+
	O:3	4	IXb	Canada	Human, pork	2	+
	O:3	4	II	Japan	Human, pork	2	+
	O:5,27	2	Xz	Canada, Japan	Pork, dog	2	+
	O:8	1B	Xz	United States, Canada	Human, pig	6	+
	O:9	2	X3	Belgium, Canada, Japan	Human, pig	3	+
	O:13	1B	Xo	Canada	Monkey	1	+
	O:21	1B	Xz	Canada	Human	1	+
	Miscellaneous	1A		Norway	Meat, rodents	13	-
	<i>Y. frederiksenii</i>	Miscellaneous		Norway, Germany	Meat, fish, sewage	5	-
<i>Y. kristensenii</i>	Miscellaneous		Norway	Pig, meat products	4	-	
<i>Y. intermedia</i>	Miscellaneous		Norway, Germany	Pig, pork, water	8	-	
<i>Y. pseudotuberculosis</i>	Ib, IIa, IIb, III, IV, V		France	Human	6	+	

^a According to the revised biogrouping scheme of Wauters et al. (39).

^b +, Strains harboring the 40- to 50-MDa virulence plasmid; -, strains lacking the plasmid.

derived from the plasmid-bearing parent by repeated subcultivation on magnesium oxalate agar (22, 29). The strains have been characterized previously with respect to restriction enzyme cleavage patterns of plasmid and chromosomal DNAs and virulence-associated properties (12, 22). All strains were maintained at -70°C in heat-inactivated horse serum with 17% glycerol. Strain 29C-43P+ (serogroup O:3, biovar 4, phagevar VIII), which harbored the virulence plasmid, was used to inoculate food and water samples.

Cultivation and enumeration of bacteria. Bacteria used for examination of primer specificity were grown with shaking at room temperature overnight in tryptic soy broth containing 0.6% yeast extract (TSB; BBL, Cockeysville, Md.), and 1 loopful was transferred to 100 μl of distilled water which was subsequently boiled for 10 min to lyse bacteria. Aliquots of 2 μl were then examined in PCR. Bacteria used for inoculation of food and water and for examination of IMS specificity were grown in TSB as described above. The bacterial density was adjusted turbidimetrically to approximately 10^8 bacteria per ml (optical density at 660 nm [OD₆₆₀], 0.2), and the appropriate concentrations were subsequently achieved by serial 10-fold dilutions in sterile saline. The exact number of yersiniae in the inocula was estimated by plating 100- μl aliquots onto four agar plates.

Food and water samples. The food and water samples examined are described in Table 2. Food products were purchased from local stores, brought to the laboratory at ambient temperature, kept frozen at -20°C , and thawed overnight at 4°C before examination. Standard plate count procedures were used to enumerate total and fecal coliforms

and to determine total aerobic counts (23, 24). Isolation of *Y. enterocolitica* was attempted by using a method recommended by the Nordic Committee on Food Analysis (25), as described previously (21). *Y. enterocolitica* was identified by established criteria (1). Water samples were collected from surface sources in sterile 500-ml glass flasks, brought to the laboratory at ambient temperature, and kept at 4°C before examination within 18 h after collection. A standard plate count procedure was used to enumerate heterotrophic organisms (26). Total and fecal coliform counts were determined by standard membrane filtration techniques (26).

Coating of IMP. Monodisperse polystyrene superparamagnetic particles, 2.8 μm in diameter, with covalently linked sheep anti-rabbit immunoglobulin G (IgG) (Dynabeads M-280; Dynal A/S, Oslo, Norway) were coated with IgG antibodies to *Y. enterocolitica* serogroup O:3. Specific O antisera against a clinical isolate of *Y. enterocolitica* O:3 were prepared by immunizing rabbits with a boiled suspension of bacterial cells, as detailed by Wauters (38). Antisera from two rabbits were pooled, and the immunoglobulins were precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation; the precipitate was incubated at room temperature for 20 min before centrifugation at $10,000 \times g$ for 10 min. The precipitate was dissolved in distilled water and subjected to gel filtration on a Sephadex G-150 column (2.5 by 95 cm). The protein peaks corresponding to IgM (void volume) and IgG were collected and separately passed through a column (30 ml) with Sepharose-protein A (Pharmacia, Uppsala, Sweden). The IgM fraction was eluted in the fall-through fraction (phosphate-buffered saline [PBS] eluate), and the IgG fraction was eluted by 0.2 M acetate buffer, pH 4.0. The IgG fraction containing the *Yersinia*-specific antibodies was neutralized, dialyzed against PBS (pH 7.3), and subsequently employed to coat the magnetic particles, using the procedure recommended by the supplier. The amount of antibody required to achieve optimal binding of target bacteria was determined in a series of pilot experiments; the highest number of bound bacteria was obtained with 20 μg of IgG per mg of immunomagnetic particles (IMP). The IMP were stored and washed before use as described by the manufacturer.

Preparation of food samples prior to PCR. Two different procedures for food preparation were compared (Fig. 1). In both cases, a 10-g sample of each food item was initially inoculated with bacterial suspension and homogenized for 1

TABLE 2. Food and water samples examined

Sample no.	Source	Plate count (CFU/g)		
		Total	Coliforms	Fecal coliforms
1	Minced beef	5.7×10^6	2.2×10^3	<10
2	Pork chops	8.5×10^7	1.0×10^3	<10
3	Minced pork	6.0×10^6	10	<10
4	Minced beef	1.2×10^8	$>2.0 \times 10^6$	2.0×10^4
5	Pork chops	8.6×10^4	<10	<10
6	Minced beef	5.0×10^7	1.2×10^6	<10
7	Minced pork	8.0×10^5	9.0×10^3	<10
	Surface water	11/ml	60/100 ml	22/100 ml
	Surface water	500/ml	25/100 ml	25/100 ml

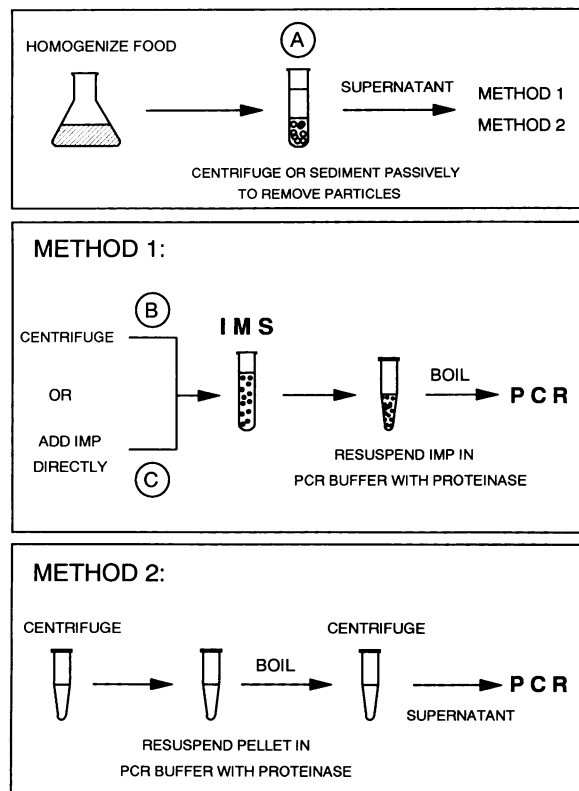


FIG. 1. Procedures for preparation of food samples prior to PCR.

min in 90 ml of PBS (pH 7.4) containing 0.25% Triton X-100, using a Colworth 400 stomacher. Alternatively, the inoculated samples were homogenized in TSB and incubated with shaking at room temperature overnight for enrichment. Coarse particles were subsequently removed by low-speed centrifugation at $50 \times g$ for 5 min or by passive sedimentation at 4°C for 1 to 2 h (step A, Fig. 1). Different volumes of the supernatant were then processed as outlined below. The volumes selected for further examination were optimized in a pilot study. All experiments were done in duplicate.

Method 1. IMS. Five milliliters of supernatant from non-enriched samples was centrifuged at $2,500 \times g$ for 20 min; from enriched samples, 0.5 ml was sedimented at $14,900 \times g$ (full speed) for 10 min in an Eppendorf microcentrifuge (step B, Fig. 1). The pellets were resuspended in 1 ml of PBS and

incubated at room temperature for 30 min with 1.2×10^7 IMP coated with *Y. enterocolitica* IgG. In a separate experiment, IMP were added directly to the supernatant (step C, Fig. 1). To avoid sedimentation of IMP, the incubations were carried out under slight agitation, which was achieved by using a roller drum operated at 10 rpm. IMP with bound bacteria were then harvested with a magnetic particle concentrator (MPC-6; Dynal), washed gently one time in 2 ml of PBS, and resuspended in $50 \mu\text{l}$ of PCR buffer (see below) with 0.2 mg of proteinase K per ml. After incubation at 37°C for 1 h, the bacteria were lysed by boiling for 10 min. PCR reagents were finally added to a total volume of $100 \mu\text{l}$, and the entire suspension, including the IMP, was assayed by PCR.

Method 2. Differential centrifugation. One milliliter of the supernatant from nonenriched samples or $100 \mu\text{l}$ from enriched samples was sedimented at full speed for 10 min in an Eppendorf microcentrifuge. The pellets were resuspended in $50 \mu\text{l}$ of PCR buffer (see below) containing 0.2 mg of proteinase K per ml. After incubation at 37°C for 1 h, the bacteria were boiled for 10 min, and the suspension was centrifuged for 5 min. The pellet was discarded, and PCR reagents were added to the supernatant to a final volume of $100 \mu\text{l}$; this was followed by analysis of the entire solution in PCR.

Preparation of water samples prior to PCR. From each water sample, 200 ml was inoculated with bacterial suspension, blended thoroughly, and passed through a $0.45\text{-}\mu\text{m}$ membrane filter (Gelman Sciences Inc., Ann Arbor, Mich.). Filters were incubated with agitation in 10 ml of TSB at room temperature for 3 h or overnight. Five milliliters of 3-h enrichments or 0.5 ml of overnight enrichments was examined by method 1. In addition, 1 ml of 3-h enrichments or 0.1 ml of overnight enrichments was processed by method 2.

Selection and synthesis of primers. Four primers were selected on the basis of published sequence analysis of the *yadA* gene (Table 3) (34). The primers were produced with an automatic DNA synthesizer (ABI 381A; Applied Biosystems International, Foster City, Calif.). Primer 3 was synthesized with a free amino group at the 5' end by using the reagent Aminolink 2 (Applied Biosystems), and a biotin residue was attached to the amino group by reaction with Biotin X-NHS Ester as described by the manufacturer (Clontech, Palo Alto, Calif.). Primer 4 was synthesized with a noncomplementary *lac* operator sequence consisting of 21 nucleotides at the 5' end.

Nested PCR. The reaction mixture contained $1 \times$ PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl_2 , 0.001% [wt/vol] gelatin), 200 μM (each) deoxynucle-

TABLE 3. Primers used in the nested PCR for detection of pathogenic *Y. enterocolitica*

Primer ^a	Position ^b	Sequence (5'-3')	Product length (bp) ^c
1	279-300	TAA-GAT-CAG-TGT-CTC-TGC-GGC-A	700-750
2	1003-1025	TAG-TTA-TTT-GCG-ATC-CCT-AGC-AC	
3	309-331	Biotin-GCG-TTG-TTC-TCA-TCT-CCA-TAT-GC ^d	520-530
4	815-838	AAT-TGT-TAT-CCG-CTC-ACA-ATT-GGC-TTT-CAT-GAC-CAA-TGG-ATA-CAC ^e	

^a Primers 1 and 2 were used in the first PCR step (PCR-1). Primers 3 and 4 were used in the second PCR step (PCR-2). Primers 2 and 4 are complementary sequences to the specified gene locations.

^b Position of primers within the nucleotide sequence of the *yadA* gene from *Y. enterocolitica* O:3 (34).

^c Sizes of amplified products vary slightly depending on the serogroup (Fig. 2).

^d Primer 3 was biotinylated at the 5' end.

^e Primer 4 was synthesized with a *lac* operator sequence at the 5' end (in italics).

oside triphosphate, 0.1 μ M (each) primer, and 1 U of *Taq* DNA polymerase per 50 μ l (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction mixture was overlaid with a drop of mineral oil and incubated in a programmable DNA thermal cycler (Perkin-Elmer Cetus). The first PCR step (PCR-1) was done in a total volume of 100 μ l. The samples were initially denatured at 95°C for 3 min and then subjected to 40 cycles, each consisting of 30 s at 95°C, 60 s at 58°C, and 90 s at 72°C; the synthesis was completed at 72°C for 10 min. The second PCR step (PCR-2) was carried out in a total volume of 50 μ l. A 2- μ l sample of the first PCR product was used as the template. The cycle profile consisted of denaturation at 95°C for 3 min followed by 20 cycles, each consisting of 30 s at 95°C, 60 s at 62°C, and 90 s at 72°C; samples were finally held at 72°C for 10 min. A successful PCR yielded specific DNA with biotin and *lac* operator nucleotides incorporated into the amplified PCR-2 fragments.

Electrophoretic detection of PCR products. Samples (10 μ l) of the PCR-1 and PCR-2 products were loaded on horizontal, submarine 0.7% agarose gels and subjected to electrophoresis for 2 h at 120 V in Tris-borate buffer (17). Gels were stained with ethidium bromide and photographed under UV-light transillumination. *Hae*III-digested DNA from phage ϕ X174 RF (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was included as a molecular weight standard on each gel.

Colorimetric detection of PCR products. Colorimetric detection of amplified DNA sequences was based on the DIANA principle, using a commercial kit as recommended by the supplier (DIANA Basic Reagents; Dynal). Samples (40 μ l) of PCR-2 products were mixed with 40 μ l (100 μ g) of washed, streptavidin-coated magnetic particles (Dynabeads M280 Streptavidin; Dynal). During incubation at room temperature for 15 min with gentle agitation, the biotinylated PCR product was selectively captured and immobilized on the magnetic particles, which were subsequently harvested with a magnetic particle concentrator. Particles with immobilized DNA were then washed once in 100 μ l of a solution containing 25 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, 10 mM MgCl₂, 10 mM dithiothreitol, and 0.05% Tween 20 (DIANA buffer). The particles were resuspended in 50 μ l of the same buffer, and a fusion protein consisting of *lac*I repressor and β -galactosidase was added (50 μ l of a 0.5-mg/ml solution). The mixture was incubated at room temperature for 20 min to promote binding between the *lac* repressor protein and the *lac* operator sequence. The particles were subsequently separated magnetically, washed four times with 150 μ l of DIANA buffer, and resuspended in 50 μ l of buffer. The enzyme conjugate was visualized by adding the chromogenic substrate *ortho*-nitrophenyl- β -D-galactoside (50 μ l of a 1.25-mg/ml solution). After incubation at room temperature for 20 min, 75 μ l was transferred to a 96-well microtiter plate, and the reaction was stopped by adding 75 μ l of 1 M Na₂CO₃. The OD of the color reaction was read in an automatic photometer (MR 580 MicroELISA Auto Reader; Dynatech Instruments, Inc., Santa Monica, Calif.) at a wavelength of 405 nm, with 490 nm as the reference wavelength (dual mode). Zero adjustment was done against wells containing everything except the PCR product. The cutoff value for discrimination between positive and negative results was based on data from testing of 20 uninoculated samples (mean OD plus two standard deviations). Positive and negative controls were included on each plate.

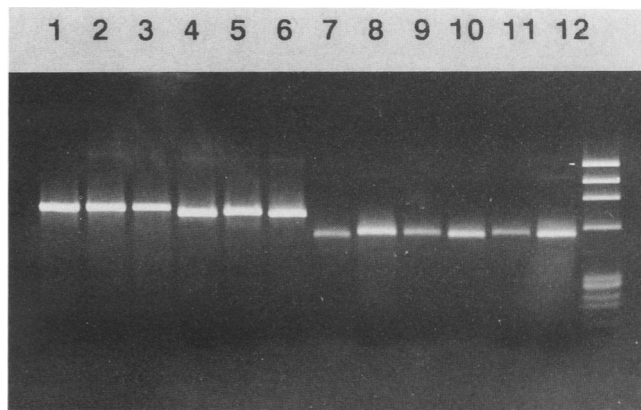


FIG. 2. Gel electrophoresis patterns of PCR products obtained by examination of lysed bacterial pure cultures in two separate PCRs, using primers 1 and 2 (lanes 1 to 6) and primers 3 and 4 (lanes 7 to 12), respectively (Table 3). The following serogroups are shown (strains in parentheses): O:3 (lanes 1 and 7) (29C-43P+), O:9 (lanes 2 and 8) (YE099P+), O:5,27 (lanes 3 and 9) (YE873P+), O:8 (lanes 4 and 10) (8081P+), O:13 (lanes 5 and 11) (YE886P+), and O:21 (lanes 6 and 12) (YE737P+). The size markers in the rightmost lane are from an *Hae*III digest of phage ϕ X174 RF. The following fragments are shown (top to bottom): 1,353, 1,078, 872, 603, and 310 bp.

RESULTS

Specificity of PCR primers. All 55 *Yersinia* strains (Table 1) were grown in pure culture, lysed, and used as the template in two separate PCRs operated at 40 (primers 1 and 2) or 30 (primers 3 and 4) cycles, respectively. The amplified sequences were analyzed by gel electrophoresis. Regardless of the primer set used, all 19 *Y. enterocolitica* strains harboring the virulence plasmid yielded one single DNA fragment of the expected size, whereas their isogenic plasmid-cured derivatives did not produce any visible amplification products. Both primer sets correctly identified plasmid-bearing variants of *Y. enterocolitica* belonging to serogroups O:3, O:5,27, O:8, O:9, O:13, and O:21, isolated from human patients, animals, and foods in Europe, North America, and Japan (Table 1). The strains concerned harbored plasmids with 11 different restriction endonuclease cleavage patterns (12, 22). The PCR products exhibited small size variations depending on the serogroup affiliation of the test strain (Fig. 2). No detectable PCR products were observed when DNAs from six plasmid-bearing strains of *Y. pseudotuberculosis* were used as the template.

To ascertain whether the primers were able to differentiate pathogenic yersiniae from nonpathogens, we tested both primer sets against 30 environmental *Yersinia* strains belonging to 25 serogroups and four species (Table 1). No amplification was detectable with 13 *Y. enterocolitica* strains belonging to biovar 1A (12 serogroups) or with 17 strains of *Y. frederiksenii*, *Y. kristensenii*, and *Y. intermedia*, all of which lacked the virulence plasmid.

Specificity of IMS. Overnight cultures of 24 *Yersinia* strains were adjusted to 200 to 500 CFU/ml in PBS, and 1-ml samples were mixed with 1.2×10^7 IMP before incubation at room temperature for 30 min. The IMP were harvested with a magnet, washed once in PBS, and spread plated onto lactose agar. Bound bacteria were enumerated by counting the number of colonies. The percentage of bound bacteria, relative to the number initially present, was significantly

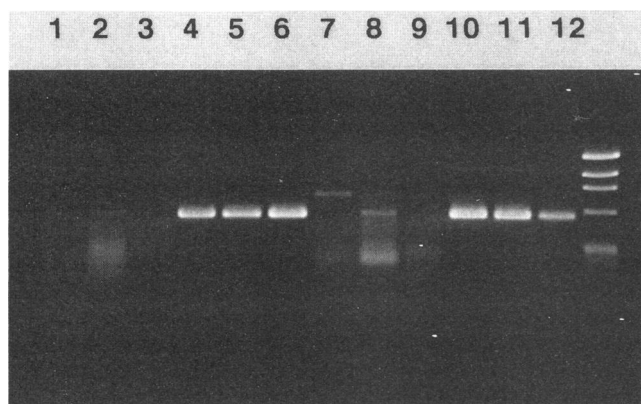


FIG. 3. Gel electrophoresis patterns of PCR products obtained by examination of seeded food products by nested PCR combined with method 1 (Fig. 1). Lanes 1 to 6, three food items (samples 1, 2, and 4, Table 2) seeded with 10 to 30 *Y. enterocolitica* cells per g and examined without prior enrichment; lanes 7 to 12, the same foods (samples 5, 6, and 7, Table 2) seeded with approximately 2 CFU/g and examined after overnight enrichment in TSB. Lanes 1 to 3 and 7 to 9 show results obtained in PCR-1 with the outer primer set. Lanes 4 to 6 and 10 to 12 show DNA fragments generated during PCR-2 with the first PCR product as template and the inner primer set. Size markers are in the rightmost lane (see legend to Fig. 2).

higher for serogroup O:3 than for other serogroups. Among seven strains of serogroup O:3 tested, IMS was capable of isolating, on average, 23% (median, 22%; range, 20 to 28%). In comparison, the bacterial yield averaged only 0.5% (median, 0%; range, 0 to 2%) when 17 strains belonging to 13 other serogroups from five species were examined. The spectrum of serogroups tested included O:4, O:4,33, O:5,27, O:7,8, O:8, O:9, O:12, O:12,25, O:13, O:14, O:17, O:21, and *Y. pseudotuberculosis* I1b. It is notable that IMS recovered 28% of an environmental O:3 strain (8018) that belonged to the nonpathogenic biovar 1A.

Examination of seeded foods. Seven meat products (Table 2) were inoculated and homogenized, and duplicate samples from each food item were prepared for PCR by both methods 1 and 2 (Fig. 1). Regardless of the method used for sample preparation, the nested PCR was capable of detecting 10 to 30 CFU/g of the original food (1 to 3 CFU per PCR reaction mixture) against a large background of indigenous bacteria (10^4 to 10^8 CFU/g) without prior enrichment (Fig. 3). When the samples were enriched overnight in TSB, the sensitivity was increased to approximately 2 CFU/g, except for two samples (samples 4 and 6, Table 2) which required an inoculum of approximately 20 CFU/g to produce a positive PCR result. These two samples, however, differed from the others by exhibiting both an extremely high total aerobic plate count and a coliform count exceeding 10^6 CFU/g (Table 2). Uninoculated control samples were invariably negative. Two food products which produced positive PCR results without inoculation were excluded from the study; both of these yielded *Y. enterocolitica* O:3 by a conventional isolation method.

Although methods 1 and 2 gave the same sensitivity, the IMS included in method 1 has the advantage of being easily adaptable for automation. To achieve this, a minimum of laboratory manipulations prior to IMS, especially centrifugation steps, would be required. The sensitivity of the PCR assay was not affected when passive sedimentation was

substituted for the initial centrifugation step (step A, Fig. 1). Furthermore, we examined whether method 1 could be completed without prior centrifugation and resuspension in PBS (step B, Fig. 1). Duplicate samples from two food items (samples 5 and 6, Table 2) were inoculated with 10 to 30 CFU/g, homogenized, and subjected to IMS by adding 1.2×10^7 IMP directly to 1 and 5 ml of supernatant from step A (alternative C, Fig. 1). One food item (item 6) was also inoculated with approximately 2 CFU/g and enriched overnight in TSB, and IMS was then conducted in a mixture of 0.5 ml of supernatant plus 0.5 ml of PBS. All samples yielded positive results in the PCR assay, indicating that step B as well as step A may be omitted without reduction of sensitivity. For sample 6, the sensitivity was actually increased from 20 to 2 CFU/g (see above) after enrichment.

Examination of seeded water. Two surface water specimens (Table 2) were inoculated, filtered, and enriched in TSB for 3 h or overnight, and duplicate samples from each specimen were prepared for PCR by both methods 1 and 2. Regardless of sample preparation procedure, the nested PCR was capable of detecting 10 to 30 CFU per 100 ml of water after 3 h of enrichment despite a large background flora. No further improvement of the sensitivity was achieved when the enrichment proceeded overnight; negative PCR results were obtained when the water specimens were inoculated with 1 to 3 CFU/100 ml. Control samples not inoculated with *Y. enterocolitica* were negative.

Electrophoretic versus colorimetric detection of PCR products (DIANA). The PCR amplification products from four food and one water specimen were analyzed by gel electrophoresis as well as by the DIANA procedure. All samples positive by gel electrophoresis were also positive by DIANA and vice versa. The intensity of the color reaction obtained by DIANA was significantly higher for positive samples than for their negative counterparts. When all results were pooled, the zero-adjusted OD ranged from 0.12 to 0.37 (mean, 0.20; median, 0.20; $n = 29$) for positive samples, while that for the negative samples ranged between 0.00 and 0.04 (mean, 0.03; median, 0.03; $n = 24$). The color reaction was sufficiently distinct to enable visual differentiation between positive and negative results.

DISCUSSION

A two-step PCR amplification procedure with two nested pairs of primers specific for the *yadA* gene of *Y. enterocolitica* was developed. We selected a plasmid-encoded virulence determinant as the target for PCR because the plasmid concerned is a necessary prerequisite for the bacteria to induce disease (30). Although the presence of plasmid-cured cells in a food product may be useful information regarding the hygienic status of the production process, such products do not represent a health hazard. We cannot exclude the possibility that the plasmid may be lost occasionally under the nonselective, competitive conditions which prevail during the overnight enrichment step included in our study. However, this is not likely to be a common event since no false-negative results were obtained after overnight enrichment of seven food samples seeded with low numbers of plasmid-bearing *Y. enterocolitica* cells.

Several conventional isolation procedures for *Y. enterocolitica* are currently in use. However, the problem with all selective media described thus far is that they provide inadequate differentiation between pathogenic *Y. enterocolitica* and related microbes (10). Colony hybridization with a synthetic oligonucleotide probe has indicated that the use

of conventional isolation procedures may lead to underestimation of pathogenic *Y. enterocolitica* in pork products (21). The PCR assay was capable of differentiating pathogenic *Y. enterocolitica* from a broad spectrum of nonpathogenic yersiniae. This is an important characteristic as such environmental yersiniae are common in food and water.

Important advantages of the two-step PCR detection procedure with two nested primer pairs are its high specificity and sensitivity (9, 37). A large number of cycles are run in the first round of PCR to obtain many templates for use in the second PCR step. The number of cycles required in the second PCR is consequently decreased, resulting in negligible levels of nonspecific fragments. If nonspecific amplification occurs during the first PCR, it is corrected in the second step since the inner primer set will function only when the correct fragment has been amplified (6, 13). The presence of a single or strongly predominant DNA fragment of the predicted size in electrophoretic analysis of primary and secondary PCR products indicates specific amplification of the target DNA. Additional evidence of specific amplification, using Southern blot hybridization with an internal DNA probe, is not needed since the secondary PCR yields information equivalent to that provided by hybridization. Kaneko et al. (9) demonstrated that the sensitivity and specificity achieved by a two-step PCR amplification with two successive sets of primers and electrophoretic detection of PCR products were comparable to the results obtained by one round of PCR followed by Southern blot hybridization.

Theoretically, PCR can detect a single copy of target DNA. Consequently, the sensitivity of a PCR assay is limited by the efficiency with which the target sequence is recovered from the sample to be analyzed. Although different methods for purification of nucleic acids from biological samples have been described, they are rather time-consuming and may reduce the sensitivity of the test. We describe here two relatively rapid procedures for sample preparation prior to PCR which do not require isolation of DNA. When combined with the nested PCR assay, both procedures allowed sensitive detection of pathogenic *Y. enterocolitica* in foods and water despite a large background of indigenous bacteria. It is notable, though, that our methods were evaluated with samples inoculated with actively growing *Yersinia* cells. Further multiplication of the inoculum may have occurred during the sample preparation. A slightly lower sensitivity would therefore not be unexpected when naturally contaminated samples which are likely to contain stressed or sublethally weakened yersiniae are examined.

Method 1 was based on IMS of the target bacterium from the sample. In addition to concentrating the pathogenic strain, IMS also provided a purer specimen suitable for PCR. *Taq* polymerase is very susceptible to inhibition by compounds that may be present in the sample. IMS provides an efficient way of removing PCR inhibitors; the presence of IMP did not adversely affect PCR amplification. The IMS employed in our work was specially designed to detect the pathogenic serogroup O:3, the dominant causal agent of human *Y. enterocolitica* infection in most parts of the world (18). Similar IMS protocols may be developed for other pathogenic serogroups. The production of polyvalent IMP coated with a mixture of antibodies to several serogroups may also be attainable.

The DIANA procedure is designed for colorimetric detection of PCR-amplified DNA sequences (37). This solid-phase system eliminates the need for electrophoresis, restriction mapping, or hybridization assays for identification of PCR products and enables easy detection of PCR products in a

microtiter plate format, using an OD reader. Positive samples can be analyzed further by direct solid-phase DNA sequencing (7). When there is a need for screening large numbers of samples, the combination of IMS, nested PCR, and DIANA makes possible the development of a fully automated analytic process which requires a minimum of laboratory manipulations. Technological developments, now in progress, may enable automatic completion of the entire procedure including IMS, PCR, and DIANA, with the appropriate washing steps, incubations, and OD measurements, all performed in a single programmable apparatus.

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