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Detection of pathogenic *Yersinia enterocolitica* using the multiplex polymerase chain reaction

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SUMMARY

A multiplex polymerase chain reaction (PCR) was developed to detect the presence of the *ail*, *yst*, and *virF* genes of *Yersinia enterocolitica* simultaneously, quickly and accurately. The amplified fragment sizes were 356 base-pairs (bp) for the *ail* gene, 134 bp for the *yst* gene, and 231 bp for the *virF* gene. The specificity of the amplified products was confirmed by hybridization with digoxigenin-labelled oligonucleotide probes. Amplification was successful whether the template was derived from a single colony of bacteria, aliquots of boiled bacterial suspensions, from DNA extracted from pure or mixed cultures or from stool specimens. Amplification of the *virF* gene was also achieved from strains of *Y. pseudotuberculosis* carrying the 70 kb plasmid but not with preparations from other related *Yersinia* species or from other members of the family *Enterobacteriaceae*. The detection limit we established was 5–10 colony forming units per millilitre (cfu/ml) and 1·0 pg of DNA.

INTRODUCTION

Yersinia enterocolitica is an enteric pathogen responsible for a variety of disorders in humans and animals [1, 2]. Of the more than 50 serotypes described, only a few have been implicated in disease, namely O:1, 2, 3, O:2, 3, O:3, O:4, O:5, 27, O:8, O:9, O:13a, 13b, O:18, O:20 and O:21 [1, 3]. Virulence of pathogenic isolates has been associated both with the presence of a 70 kb plasmid, designated pYV, and chromosomally encoded genes involved in invasion [4–6]. *Y. enterocolitica* also produces a heat-stable enterotoxin, (YST), which is encoded by the chromosomal *yst* gene (7). The biological properties of YST are similar to those of the methanol-soluble heat-stable enterotoxin (STa) of enterotoxigenic *Escherichia coli* (ETEC) [8]. Recently the gene, *yst*, for the toxin was cloned and sequenced [7]. The role of YST in disease is not clearcut, although many isolates of *Y. enterocolitica* produce the toxin. In the animal model the use of yst^+ and yst^- isogenic strains of *Y. enterocolitica* suggest that the toxin is an important cause of diarrhoea [9].

Most phenotypic markers for clearly differentiating pathogenic from non-pathogenic yersiniae are time-consuming and sometimes inconsistent [10]. We report here the development of a multiplex polymerase chain reaction (PCR), based assay which can detect the presence of three virulence-associated mechanisms of pathogenic isolates of *Y. enterocolitica*, and which can be used both with pure cultures and clinical specimens. Amplification of the *virF* gene from strains of *Y. pseudotuberculosis* carrying the 70 kb plasmid was also achieved. (Presented in part at the 94th General Meeting of the American Society for Microbiology, Las Vegas, Nevada, 1994.)

MATERIALS AND METHODS

Bacterial strains

A total of 200 isolates of *Y. enterocolitica* and related species from human and animal sources submitted to

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Table 1. Y. enterocolitica *and related species examined for the* ail, yst *and* virF *genes in a multiplex PCR*

Species	Serotype	Source	Total number of strains tested
Y. enterocolitica (pathogenic)	1, 2, 3	Human*	10
		Porcine*	15
	3	Human	30
	4	Human	20
	5, 27	Human	20
		Porcine	9
		Monkey	2
	8	Human	8
	9	Human	3
		Rat	1
	13a, 13b	Human	3
		Monkey	2
	18	Human	2
	20	Human	2
	21	Human	2
Y. enterocolitica (non-pathogenic)	5	Human	6
	6, 30	Human	5
	6, 31	Human	5
	7, 8	Human	5
	10, 34	Human	2
	41, 43	Human	4
Y. bercovieri	ND	Human	4
Y. frederiksenii	ND	Human	6
Y. intermedia	ND	Human	4
Y. kristensenii	ND	Human	8
Y. mollaretii	ND	Human	3
Y. rohdei	ND	Human	3
Y. ruckeri	I, II, III, V	Fish	4
Y. pseudotuberculosis	IIa	Bovine	4
	III	Deer	3
	III	Human	5

* Two human isolates and all porcine isolates belonging to serotype O:1, 2, 3 were positive with the pyrazinamidase test.
ND, not determined.

the National Yersinia Reference Service at the Central Public Health Laboratory, Toronto, Ontario, Canada was investigated (Table 1). The strains were chosen to represent all known pathogenic serobiotypes of *Y. enterocolitica* and related organisms. The term 'pathogenic' refers to the O-antigen groups well recognized as being associated with human disease by virtue of their occurrence in outbreak-and sporadic-cases, possession of chromosomally encoded ability to invade tissue culture cells, a heat-stable enterotoxin, and a virulence plasmid which may aid bacterial survival within host tissue. Pathogenic strains of *Y. enterocolitica* are also distinguished from non-pathogenic strains by *in vitro* virulence tests in animals. All 100 human pathogenic serobiotypes were selected from clinical isolates, the clinical status of animals from which the 29 remaining pathogenic isolates were selected, is not known. A selected number of bacterial strains from species other than *Y. enterocolitica* were also examined (Table 2); these are all clinical isolates from specimens submitted to our laboratory.

Preparation of template for PCR

(*a*) Single colony inoculation. Inoculations from single

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Table 2. *Non-yersinia bacterial strains tested for* ail, yst *and* virF *genes in the multiplex PCR procedure*

Bacterial species	Total number of strains tested
Acinetobacter calcoaceticus	1
Aeromonas caviae	1
Aeromonas hydrophila	4
Aeromonas sobria	1
Campylobacter coli	3
Campylobacter jejuni	5
Citrobacter diversus	1
Enterobacter aerogenes	1
Enterobacter cloacae	1
Klebsiella pneumoniae	3
Klebsiella oxytoca	1
Morganella morganii	1
Proteus mirabilis	2
Proteus vulgaris	2
Providencia stuartii	1
Pseudomonas aeruginosa	3
Salmonella enteritidis	2
Salmonella hadar	2
Salmonella heidelberg	2
Salmonella infantis	2
Salmonella litchfield	1
Salmonella poona	2
Salmonella typhi	2
Salmonella typhimurium	2
Salmonella paratyphi A	2
Salmonella paratyphi B	1
Serratia marcescens	1
Serratia liquefaciens	1
Vibrio cholerae 01	1
Vibrio cholerae non-01	2
Shigella boydii	5
Shigella dysenteriae, type 1	2
Shigella dysenteriae, other types	5
Shigella flexneri	5
Shigella sonnei	5
Escherichia coli (non-pathogenic)	4
ETEC (LT)	2
ETEC STa, STb	5
ETEC STa	5
ETEC LT, ST	5
VTEC	3

ETEC (LT), heat-labile enterotoxigenic *Escherichia coli*; ETEC (STa), methanol-soluble heat-stable enterotoxigenic *E. coli*; ETEC (STb), methanol-insoluble heat-stable enterotoxigenic *E. coli*; VTEC, verotoxigenic *E. coli*.

colonies were done essentially as described [11]. *Yersinia* sp. isolates were grown overnight at 28 °C on Brain Heart Infusion Agar (BHIA, BBL-Becton Dickinson Microbiology Systems, USA) containing 5% sheep red blood cells. (*b*) Boiled bacterial suspension. A single colony of *Yersinia* sp. organisms was inoculated into 2·0 ml of Brain Heart Infusion broth (BHIB) and grown at 28 °C in a shaker incubator. Aliquots, 1·2 ml of the culture were centrifuged, washed × 1 with 0·85% NaCl and sterile distilled water. The bacteria, resuspended in 100 μl distilled water, were boiled for 10 min, placed on ice, and after recentrifugation 5 μl of the supernatant used as template in the PCR mixture. (*c*) DNA extraction from bacterial isolates. Total DNA extraction from *Yersinia* spp. was accomplished by growing the bacteria in BHIB overnight at 28 °C. Cells were harvested by centrifugation and washed once in Tris-EDTA (TE) buffer, pH 8·0. The pellet was resuspended in 250 μl TE buffer and lysozyme (Sigma Chemical Co) added to a final concentration of 0·5 mg/ml. The suspension was incubated at 37 °C for 5 min, then lysis buffer containing 0·01 M Tris-HCl, [pH 8·0], 0·05 M NaCl, 0·005 M EDTA, 1% sodium dodecyl sulfate (SDS) and 50 μg/ml proteinase K (Bethesda Research Laboratories) was added and incubation continued at 65 °C for 2 h. Extraction with phenol-chloroform was carried out and the DNA precipitated with two volumes of ice-cold ethanol. The DNA pellet was dried and redissolved in TE buffer. (*d*) Extraction of DNA from stools. Normal stool (100 mg) from healthy donors was suspended in 0·5 ml phosphate buffered saline (PBS) pH 7·2 to which 10^5–10^6 cfu of *Y. enterocolitica* was added. Insoluble particulate matter was removed using low-speed centrifugation (750 g for 5 min) and the supernatant centrifuged at 13000 rpm for 3 min to pellet the bacteria. The pellet was resuspended in 100 μl of 50 mM Tris-HCl pH 8·0, 50 mM EDTA, 25% sucrose, 300 μg/ml lysozyme and incubated at 37 °C for 60 min. Specimens were recentrifuged and the pellet resuspended in 500 μl of 50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl containing 500 μg/ml proteinase K (BRL, Canada) and 1% sarkosyl and incubated at 56 °C for 60 min. After centrifugation the supernatant was either (i) applied directly to the NACS Prepac ion-exchange column system (BRL) and treated according to the manufacturer's instructions or (ii) extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and the DNA precipitated with two volumes of absolute ethanol in the presence of 0·3 M sodium acetate, pH 5·2. After centrifugation (13000 rpm for 5 min), the DNA pellet was dried, resuspended in TE and used for PCR. Two hundred to 500 ng (20 μl) of DNA was used routinely. Normal unseeded stool controls were processed in the same

Table 3. *Oligonucleotide primers and probes used in this study*

Primer/probe	Oligonucleotide sequence (5′-3′)	Location within gene	Size of amplified product (bp)
Primer			
Ail-a	TGGTTATGCGCAAAGCCATGT	580–600	
Ail-b	TGGAAGTGGGTTGAATTGCA	915–934	356
Yst-a	GTCTTCATTTGGAGGATTCGGC	152–173	
Yst-b	AATCACTACTGACTTCGGCTGG	264–285	134
VirF-a	GCTTTTGCTTGCCTTTAGCTCG	142–149	
VirF-b	AGAATACGTCGCTCGCTTATCC	212–219	231
Probe			
Ail	GTCATTCACGCTTCATATGAATAC	—	—
Yst	GCAGTTCAGTGATGCATTATCGAC	—	—
VirF	TTAGGCAACCGCCCAGAAGAACGG	—	—

way as the seeded samples, and diarrheic stools from symptomatic patients from which *Y. enterocolitica* was cultured were treated in a similar manner.

PCR primers and probes

The three sets of oligonucleotide primers and probes (Table 3) were selected according to published sequences of the *Y. enterocolitica ail* gene [12], *yst* gene [7] and *virF* gene [13] using the computer programmes Primer Detective (Clontech Laboratories, Inc.) and OLIGSCAN [14]. The primers and probes were synthesized at Allelix Corporation, Mississauga, Ontario, Canada.

Amplification of target sequences

PCR amplifications of bacterial DNA were routinely carried out in a 50 μl reaction mixture containing 10 mM KCl, 10 mM Tris hydrochloride (pH 8·3), 1·5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP and dTTP, 10 pmol each primer and 2·5 U of the Stoffel fragment of *Taq* DNA polymerase (Perkin Elmer Cetus). The samples were overlaid with 50 μl of mineral oil to prevent evaporation and subjected to 25 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer Cetus). The parameters for the amplification cycles were as follows: denaturation for 1 min at 94 °C, annealing of primers for 1 min at 55 °C, and primer extension for 2 min at 72 °C. After the last cycle, the samples were kept at 72 °C for an additional 7 min to ensure that the polymerization of every fragment was complete. A negative control with each of the reaction components except template DNA, and a positive control (1 ng of purified genomic DNA from *Y. enterocolitica* O:13a, 13b) were included with each test run. Ten microlite aliquots of the PCR-amplified product were analysed by agarose gel electrophoresis (3% NuSieve GTG agarose-FMC, Rockland, Maine) and stained with ethidium bromide before being photographed with a polaroid camera. Molecular size markers included in all gels were the 123-bp DNA ladder (GIBCO/BRL, GIBCO Canada Inc., Burlington, Ontario).

Southern blotting and hybridization

PCR reaction products were transferred onto Zeta-probe membranes (Bio-Rad), using a hybridot manifold (GIBCO/BRL), and hybridized with oligonucleotide probes specific to the internal fragments of the three pairs of primers (Table 3). The membranes were baked at 80 °C for 2 h. Prehybridization was carried out at 37 °C for 1 h in 5 ml of hybridization buffer: 5 × SSC (1 × SSC contains 0·15 M NaCl and 0·015 M sodium citrate), 0·02% SDS, 0·1% N-lauroylsarkosine, and 1% blocking reagent (Boehringer Mannheim, Canada). Hybridization was done overnight at 37 °C with 10 pmol of labelled oligoprobe per millilitre of buffer. Oligonucleotide probes were non-isotopically labelled by the random primed labelling technique using digoxigenin-dUTP according to the protocol provided by Boehringer Mannheim Canada. The membranes were washed three times (5 min each) in cold 5 × SSC followed by two 20-min washings at 4 °C in 5 × SSC and two 15-min washings at 55 °C in 3 M tetramethylammonium chloride wash. Immunological detection was carried out according to standard procedures. Membranes were incubated for 30 min in 1:5000 alkaline phosphatase conjugate for 3 min and in substrate solution for 4–6 h (Boehringer Mannheim Canada).

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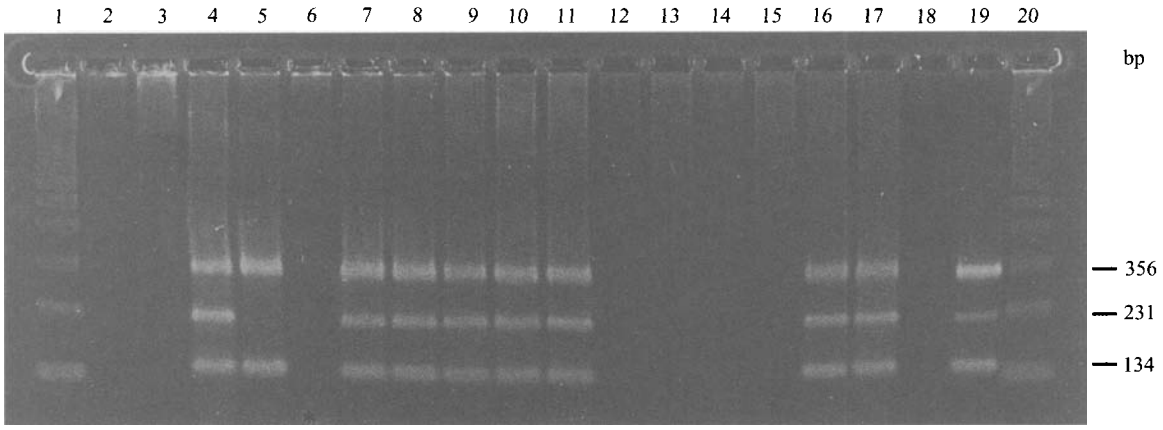

Fig. 1. Agarose gel electrophoresis of nucleic acid amplification products from *Yersinia* spp. using *ail*, *virF* and *yst* primers. Lanes 1 and 20, 123 bp DNA ladder (Bethesda Research Laboratories); Lane 2, *Y. ruckeri*; Lane 3, *Y. rohdei*; Lane 4, *Y. enterocolitica* O:1, 2, 3, human isolate (pyrazinamidase-positive); Lane 5, *Y. enterocolitica* O:3, human isolate; Lane 6, *Y. enterocolitica* O:5, lanes 7–10, *Y. enterocolitica* O:3, O:5, 27, O:5, 27, O:3, human isolates; Lane 11, *Y. enterocolitica* O:5, 27, porcine isolate; Lane 12, *Y. bercovieri*; Lane 13, *Y. frederiksenii*; Lane 14, *Y. intermedia*; Lane 15, *Y. mollaretii*; Lanes 16, 17, *Y. enterocolitica* O:3, human isolates; Lane 18, reagent control (no DNA); Lane 19, positive control, *Y. enterocolitica* O:13a, 13b.

RESULTS

Bacterial strains

The results of the multiplex PCR for *Y. enterocolitica* strains indicate that all human and animal isolates examined, belonging to 10 pathogenic serobiotypes, gave a positive reaction for the *ail* gene, the *yst* gene, and the *virF* gene, yielding fragments of 356, 134 and 231 base pairs (bp) respectively (Figs 1, 2*a*). Isolates which had lost the 70 kb virulence plasmid after storage gave negative results with primers designed for the *virF* gene. The limit of detection was established to be 5–10 cfu/ml of bacterial cells and 1·0 pg of total DNA. PCR products were not obtained with any of the three primers using template DNA from non-pathogenic *Yersinia* spp. (Fig. 1) except for one isolate, serotype O:5 which showed a fragment of approximately 50 bp (Fig. 1, Lane 6). Other members of the family *Enterobacteriaceae* examined in this study (Table 1) were also negative for the target genes. Isolates of *Y. pseudotuberculosis* carrying the virulence plasmid gave a positive reaction for the *virF* gene (data not shown).

In order to verify the authenticity of the product obtained after amplification, Southern hybridization was carried out using oligonucleotide probes specific to the internal regions of the fragments used for PCR amplification (Table 2). The amplified product for each reaction could be readily detected as illustrated in Fig. 2*b*. Three isolates which did not show the *virF* PCR product after electrophoresis showed a faint signal when the oligoprobe was used during the dot–blot hybridization (Fig. 2*a*, *b* – lanes 2, 5 and 6). None of the non-pathogenic *Yersinia* spp. or non-*Yersinia* strains showed positive results with the probes.

Detection of *Y. enterocolitica* in stool specimens

Normal stool samples seeded and unseeded with *Y. enterocolitica* bacteria, and diarrhoeic stools from symptomatic patients were examined using the multiplex PCR assay. The assay was also performed directly on specimens not subjected to DNA purification and on preparations following DNA purification with and without additional treatment using the NACS prepac column. DNA amplification was not observed from stool specimens which were not subjected to DNA purification steps and inconsistent results were obtained from samples which were purified with phenol-chloroform. Following purification of the total DNA using the NACS Prepac columns (BRL) all samples were amplified successfully. Simultaneous amplification of the three fragments was observed from all positive samples but not from negative ones.

DISCUSSION

We have shown that amplification with the multiplex PCR is specific and sensitive, differentiating *Y. enterocolitica* from a broad spectrum of non-pathogenic yersiniae and non-*Yersinia* strains. For the present

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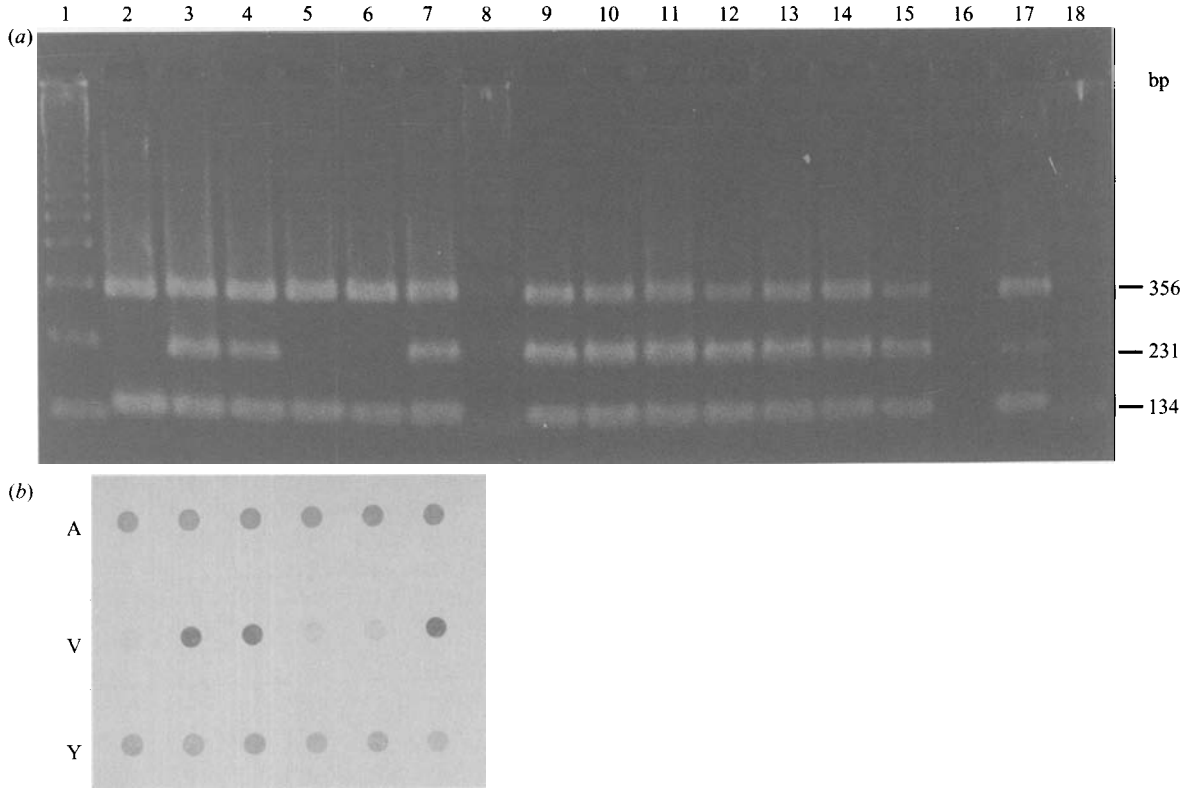

Fig. 2(*a*) Agarose gel electrophoresis of multiplex PCR products from *Y. enterocolitica* isolates with *ail*, *virF* and *yst* primers. Lanes 1, 8, 18, 123 bp DNA ladder (Bethesda Research Laboratories); Lanes 2, 3, *Y. enterocolitica* O:4, O:8 human isolates; Lane 4, O:13a, 13b, isolate from monkey; Lanes 5, 6, O:18, O:20, reference strains; Lanes 7, 9, 10, O:21, O:3, O:3, human isolates; Lane 11, O:5, 27, isolate from monkey; Lane 12, O:1, 2, 3, human isolate (pyz-negative); Lane 13, O:1, 2, 3, human isolate (pyz-positive); Lane 14, O:9, human isolate; Lane 15, O:1, 2, 3, human isolate (pyz-negative); Lane 16, reagent control (no DNA); Lane 17, positive control, O:13a, 13b. (b) Dot blot hybridization of PCR-amplified products from isolates of *Y. enterocolitica* (Lanes 2–7, Fig. 2*a*). A hybridot manifold (Bethesda Research Laboratories) was used to blot the samples onto Zeta-probe membrane (Bio-Rad laboratories). Oligonucleotide probes were labelled with digoxigenin (Boehringer Mannheim Canada). (A) *ail* (V) *virF* (Y) *yst*.

study both *ail* and *yst* were chosen because they are chromosomal genes and reportedly present in all pathogenic strains of *Y. enterocolitica* [5, 7]. The *ail* gene has also been shown to promote invasion of mammalian cells in tissue culture assays [6], and YST is important in causing yersinia-associated diarrhoea [9]. The *virF* gene, a key regulatory gene on the virulence plasmid [13] was included for completeness as one of the important virulence mechanisms, although the 70 kb plasmid may be lost during the culture or storage of strains of *Y. enterocolitica*. The first set of primers that we selected from the published sequence of the *ail* gene [16] were 5′-CCATCTTTC CGCATCAACGAA-3′ (nucleotides 817–832) and 5′-GGTGCCAACTTTTATGCTATCG-3′ (nucleotides 873–994). These were, however, unsuccessful in amplifying *Y. enterocolitica* of serotypes O:1, 2, 3, O:3, O:5, 27 and O:9, when using more stringent annealing temperatures (55 °C) during the PCR procedure. When annealing temperatures were changed to conditions of a more moderate stringency (37 °C), PCR amplification products could be demonstrated on agarose gels from some isolates of each of the four serovars. These results suggest possible differences, variation or mismatch among strains in that region of the *ail* gene. It is possible that the differences seen with the *ail* gene amplification using these primers under less stringent conditions, could be utilized to differentiate *ail* gene variants of *Y. enterocolitica*. Whether this procedure can be used as a molecular epidemiological typing scheme is being investigated. When a second set of primers (Table 3) was chosen, amplification was achieved consistently with all pathogenic serotypes. Recent studies by Beer and Miller [15] have shown that the two groups of *ail* gene variants from the American and non-American serotypes are associated with differences in ability to invade tissue culture cells and in virulence in humans.

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The PCR assay was easy to perform involving one reaction tube per sample, and required much less time compared with the traditional methods. Bioassays are time consuming and can take several days to obtain clear-cut results. The method described in this study is also practical for simultaneous screening of a large number of samples for three important virulence factors of *Y. enterocolitica* and for the *virF* homologous sequences of *Y. pseudotuberculosis*, quickly and accurately. The assay can provide clear-cut results on the same day that specimens arrive at the laboratory, speed and accuracy are of a definite advantage in the case of outbreaks or for epidemiological investigations. The sensitivity of the assay was also demonstrated; we were able to detect as few as 5–10 cfu/ml of bacterial cells from the majority of pathogenic serobiotypes of *Y. enterocolitica*. When extracted total DNA was used, the limit of detection was 1·0 pg of nucleic acid. These levels are more sensitive by comparison to other methods for detection of *Y. enterocolitica* [16, 17].

None of the isolates of *Y. kristensenii* yielded amplified products with the *ail*, *virF* or *yst* primers. Since serotyping on those strains was not determined, it is not known whether any of the *Y. kristensenii* isolates belonged to serotype 11 or 12 which were shown in previous studies [7, 16] to contain *yst*-homologous DNA. Amplification products were not produced by any of the enterotoxigenic *E. coli* strains or other toxin-producing strains listed in Table 2. The antigenic properties and mode of action of YST are similar to those of the heat-stable enterotoxin, STa, of *E. coli* [7, 18] but no studies so far have shown hybridization of *Y. enterocolitica* with probes derived from ETEC [19] or amplification of ETEC with primers derived from *yst* [16].

It is noteworthy that strains belonging to serogroup O:1, 2, 3 which gave a positive reaction in the pyrazinamidase (pyz) test generated similar PCR products as did those which were pyz-negative. In the original article describing the pyz-test, Kandolo and Wauters [20] found this test to differentiate pathogenic from non-pathogenic serotypes accurately. Recent studies have found that some isolates of serogroup O:1, 2, 3 can be pyz-positive and suggests that the pyz-test may not be a reliable indicator of pathogenicity among isolates of this serogroup [10, 21]. We have not yet been able to explain the presence of the 50 bp amplification products in one isolate of *Y. enterocolitica* serotype O:5, biotype 1A. This product was found after several repeat examinations of the isolate. The strain, isolated from faeces, contains a plasmid of approximately 65 kb in size but was negative with the CRMOX assay as is expected for strains belonging to a non-pathogenic serobiotype. In a recent report [22] isolates belonging to serotype O:5 were recovered from the stools of patients with mild gastroenteritis. In that study no other recognizable causes for the diarrhoea were found suggesting a link between the isolates and the infection. The possibility that strains belong to serotypes other than the well-defined serobiotypes [1] and appear to be involved in disease needs further investigation. Further studies are being conducted on the isolates in our laboratory.

The specificity of the amplified products was verified by probing the PCR generated products with non-radioactive labelled internal oligoprobes. Small concentrations of the probe (10 pmol), produced comparable results in terms of specificity and sensitivity to other published reports [17]. The use of non-radioactive probes has the added advantage in that the unlabelled probe can remain stable over several months which is convenient for work in clinical laboratories. During this study we found three isolates which did not show the *VirF* PCR products after ethidium bromide staining but demonstrated a faint signal when dot blots were hybridized with DIG-labelled oligonucleotide specific probes (Fig. 2*b*). We believe these results to be legitimate and not a matter of non-specific hybridization. Signals were not observed in slots to which negative controls of reaction components, distilled water, non-homologous species or other plasmid-free *Y. enterocolitica* were applied. The difference between a positive and a negative reaction was always distinct. Other investigators [23, 24] have reported dot blots especially after radioactive labelling to be more sensitive than ethidium bromide staining of gels.

This study also demonstrates that the multiplex PCR method can be used to detect specific gene sequences in stool specimens. A number of studies have used the PCR assay for detection of various enteropathogens from stool specimens and environmental sources [25–27] but, due to inhibitors present in the faecal material, the yield in some instances has been low and results inconsistent [26, 28]. The absence of PCR amplification products in normal unseeded stool and the presence of these products in seeded stools and stools from symptomatic patients confirms the specificity of the assay to detect strains of *Y. enterocolitica* and plasmid-containing *Y. pseudotuberculosis*. In our experience, in order to obtain

consistent results, stools should be treated with proteinase K or purified on NACS-prepac columns.

In conclusion, the findings reported here describe a rapid, sensitive, specific and reliable method for the detection of three virulence determinants of *Y. enterocolitica* and one from *Y. pseudotuberculosis* from pure cultures and from clinical samples. The simultaneous detection of more than one virulence determinant is also important for epidemiological studies.

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