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PCR for Detection of *Shigella* spp. in Mayonnaise

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The use of PCR to amplify a specific *virA* gene fragment serves as a highly specific and sensitive method to detect virulent bacteria of the genus *Shigella* and enteroinvasive *Escherichia coli*. Amplification of a 215-bp DNA band was obtained by using isolated genomic DNA of *Shigella*, individual cells of *Shigella dysenteriae*, and mayonnaise contaminated with *S. dysenteriae*. Moreover, a multiplex PCR with specific (*virA*) and bacterium-restricted (16S ribosomal DNA) primers generated an amplification product of approximately 755 bp for all bacteria tested and an additional 215-bp product for *Shigella* and enteroinvasive *E. coli*.

Shigella constitutes a genus of gram-negative, nonmotile, non-spore-forming rod-shaped bacteria with four species or serotypes, namely, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* (12). Virulent *Shigella* organisms cause the human illness known as bacillary dysentery, as do enteroinvasive *Escherichia coli* (EIEC) strains. Bacillary dysentery (shigellosis) causes mild diarrhea, fever, abdominal cramps, and severe fluid loss (25). All of the virulent strains mentioned above harbor a 120- to 230-kb plasmid named the virulence plasmid (7), which was first described for *S. flexneri* 2a (16). It was established that the loss of the virulence plasmid results in avirulent strains (21) and that the genes implicated in virulent functions are localized not only in the virulence plasmid but also in the chromosome (a complete review of chromosome and plasmid virulence genes is presented in reference 6). The *virA* gene has been identified in the virulence plasmid of *S. flexneri* 2a, and it has been implicated in invasion and intercellular spreading (23).

By means of human transmission, *Shigella* can contaminate several kinds of foods, including raw vegetables, milk, poultry, and some dairy products (24). Therefore, as with other pathogenic microorganisms, it is important that the presence of *Shigella* be detected in foods. Traditionally, the detection test of food-borne microorganisms (hazard test) is made by plating a food homogenate on highly selective media, although in the case of some bacteria a preenrichment step is required. After several days of incubation, the presence or absence of the microorganism or the number of colonies is determined (9). This plating technique, based on the phenotype of the bacteria, is labor-intensive and can take several weeks to obtain results (11). On the other hand, rapid, highly sensitive, and specific techniques based on genetic characteristics have been developed recently. DNA probe hybridization and PCR are the best known of these techniques and are used as hazard tests for the detection and identification of food-borne microorganisms (2, 10, 18, 19).

In this paper, we describe the highly sensitive and specific detection of virulent *Shigella* organisms and EIEC by PCR combined with DNA hybridization. The *virA* gene is the target chosen for the PCR. The applicability of this PCR method for detection of these organisms in mayonnaise is demonstrated.

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MATERIALS AND METHODS

Genomic DNA isolation from bacterial strains. The bacterial strains used in this work are listed in Table 1. Bacteria were grown overnight in a liquid medium (5 g of tryptone per liter, 2.5 g of yeast extract per liter, 1 g of glucose per liter), sedimented, and lysed with detergent to release DNA, which was extracted with phenol-chloroform (25:24, vol/vol) and precipitated with ethanol (1). The contaminating RNA was degraded by suspending the DNA sample in TER (10 mM Tris-HCl [pH 8], 0.1 mM EDTA [pH 8], 1 μg of RNase A per ml).

Genomic DNA isolation from yeast strains. The yeast strains used in this work are listed in Table 1. Yeasts were grown overnight in a liquid medium (20 g of peptone per liter, 10 g of yeast extract per liter, 20 g of glucose per liter), precipitated, and lysed with 50 μg Zymolase 20T per ml plus detergent to release DNA, which was extracted with phenol-chloroform (25:24, vol/vol) and precipitated with ethanol (15). Finally, the DNA was suspended in TER.

Food sample preparation. A 5-g sample of commercial mayonnaise (Ybarra, Seville, Spain) was diluted to 50 ml with buffered peptone water (Merck) and mixed to complete homogenization. Diluted mayonnaise was also prepared and externally contaminated with *S. dysenteriae* serovar 1 at $(88 \pm 11) \times 10^4$ cells per ml of mayonnaise. Samples (10 μl) of mayonnaise prepared in these two ways were used in the PCR.

Determination of CFU. Bacteria were grown on plate count agar (Oxoid) to achieve isolated colonies. One colony was suspended in 1 ml of buffered peptone water, and 10-fold serial dilutions were made. Aliquots (10 μl) of selected dilutions were made up to 1 ml with buffered peptone water and spread on plate count agar. After incubation, the colonies were counted. Aliquots (10 μl) of selected dilutions were used in the PCR.

DNA amplifications (PCR). Amplifications were made in a 50-μl reaction mixture which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate, 25 pmol of each primer, and 2.5 U of *Taq* polymerase (Boehringer), with either isolated DNA, a bacterial dilution, or a diluted mayonnaise sample. Temperature conditions were as follows: denaturation for 45 s at 94°C, hybridization for 30 s at 65°C, and polymerization for 30 s at 72°C. Thirty-five cycles were carried out as mentioned above. The sequences of primers used to amplify *virA* and 16S ribosomal DNA (rDNA) gene sequences are shown in Table 2.

Agarose gel electrophoresis. Aliquots (10 or 25 μl) of the amplification reaction solutions were run on a 1% (wt/vol) agarose gel (SeaKem; FMC) stained with 0.8 mg of ethidium bromide (Amresco) per ml. The DNA was observed by irradiating the gel with UV light at 264 nm. When a negative amplification was obtained, a new PCR was done and 25 μl of the resultant solution was run on an agarose gel to confirm the first result.

Cloning and sequencing of the *virA* fragment. A 50-ng sample of the amplified *virA* fragment of *S. dysenteriae* serovar 1 was ligated at 16°C in a 10-μl reaction mixture containing 30 mM Tris-HCl of (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiotreithol, 1 mM ATP, 50 ng of pGEM-T, and 1 U of T4 ligase (Promega). The transformation of the ligated DNA was carried out in XL1-Blue MRF′ bacterial strain (Stratagene), as previously described (14). DNA was sequenced by the chain termination method, modified to use universal primers labeled with digoxigenin (Dig) and *Taq* polymerase (20). A good separation of DNA bands was achieved with MWG-Biotech's direct-blotting electrophoresis system (3).

DNA-DNA hybridization. Amplification reaction solution volumes of 10 or 25 μl were run on an agarose gel and transferred to a nylon membrane (Hybond; Amersham) with a vacuum blotter (model 785; Bio-Rad) at a pressure of 1.72×10^4 Pa applied over 90 min in 0.5 M NaOH–0.6 M NaCl. The membrane was hybridized (22) against a Dig probe at 65°C in 5× SSC (1× SSC is 15 mM sodium citrate and 150 mM NaCl)–0.1% (wt/vol) sodium dodecyl sulfate (SDS)–1% (wt/vol) blocking reagent (Boehringer). The cloned and sequenced *virA* fragment of *S. dysenteriae* serovar 1 was used as a probe in the hybridization. This probe

TABLE 1. PCR results and origin of the DNA samples

Species	Source[e]	PCR results		
		virA (215 bp)	*virA* plus 16S rDNA	
			215 bp	755 bp
Shigella boydii serovar 10	CECT 583	+	+	+
Shigella dysenteriae serovar 1	CECT 584	+	+	+
Shigella flexneri serovar 2a	CECT 585	+	+	+
Shigella sonnei serovar a	CECT 542	+	+	+
Shigella sonnei	CECT 457	+	+	+
Shigella boydii 238[a]	Clinical sample	+	+	+
Shigella dysenteriae 193[a]	Clinical sample	+	+	+
Shigella dysenteriae 300[a]	Clinical sample	+	+	+
Shigella flexneri 295[a]	Clinical sample	+	+	+
Shigella flexneri 299[a]	Clinical sample	+	+	+
Shigella sonnei 296[a]	Clinical sample	+	+	+
Shigella sonnei 298[a]	Clinical sample	+	+	+
Escherichia coli serovar O1	CECT 515	−	−	+
EIEC 41[a]	Clinical sample	+	+	+
EIEC 42[a]	Clinical sample	+	+	+
EIEC 120[a]	Clinical sample	+	+	+
EIEC 121[a]	Clinical sample	+	+	+
EPEC E2348/69[b]	Clinical sample	−	−	+
EPEC B171[b]	Clinical sample	−	−	+
Bacillus sp.	CECT 450	−	−	+
Enterobacter aerogenes	CECT 684	−	−	+
Enterococcus faecalis	CECT 481	−	−	+
Lactobacillus cellobiosus	CECT 562	−	−	+
Lactobacillus sake	CECT 906	−	−	+
Micrococcus luteus	CECT 241	−	−	+
Mycobacterium phlei	CECT 3009	−	−	+
Proteus vulgaris	CECT 484	−	−	+
Pseudomonas fluorescens	CECT 378	−	−	+
Salmonella dublin[c]	Clinical sample	−	−	+
Salmonella enteritidis[c]	Clinical sample	−	−	+
Salmonella montevideo[c]	Clinical sample	−	−	+
Salmonella panama[c]	Clinical sample	−	−	+
Salmonella typhimurium[c]	Clinical sample	−	−	+
Serratia marcescens	CECT 159	−	−	+
Staphylococcus aureus	CECT 240	−	−	+
Yersinia enterocolitica serovar O:3 biovar 4[d]	Clinical sample	−	−	+
Yersinia pestis EV76[f]	Clinical sample	−	−	+
Cryptococcus sp.	Lab stock	−	−	−
Rhodotorula sp.	Lab stock	−	−	−
Saccharomyces cerevisiae	Lab stock	−	−	−
Human	Boehringer	−	−	−

[a] Strain kindly donated by G. Prats, Hospital Universitario Sant Pau, Barcelona, Spain.
[b] Strain kindly donated by M. Donnenberg, University of Maryland School of Medicine, Baltimore. EPEC, enteropathogenic *E. coli.*
[c] Strain kindly donated by J. C. Palomares, Hospital Universitario Virgen Macarena, Seville, Spain.
[d] Strain kindly donated by G. Kapperud, National Institute of Public Health, Oslo, Norway.
[e] CECT, Spanish type culture collection.
[f] Strain kindly donated by E. Carniel, Institut Pasteur, Paris, France.

was labeled by PCR as described above, except that deoxynucleoside triphosphate was replaced by a Dig labeling mix (Boehringer) containing Dig-dUTP (17). After hybridization, the membrane was washed (high-stringency conditions) twice at 65°C over a period of 15 min in 2× SSC–0.1% (wt/vol) SDS and then twice at room temperature over a period of 15 min in 0.1× SSC–0.1% (wt/vol) SDS. The Dig probe was detected by color by using an anti-Dig antibody coupled to alkaline phosphatase (Boehringer), as described elsewhere (13).

Nucleotide sequence accession number. The *virA* sequence of *S. dysenteriae* serovar 1 will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. AF010147.

RESULTS

Specificity of PCR with *virA* primers. We have designed two specific primers flanking a 215-bp region of the *S. flexneri virA* gene (accession no. D26468). These primers amplified not only isolated DNA from *S. flexneri* but also those isolated from all the other *Shigella* and EIEC strains tested (Table 1). All amplification-generated products were of the expected size (approximately 215 bp) on agarose gel electrophoresis. Isolated DNA from microorganisms other than *Shigella* and EIEC produced no amplification product (Table 1), even though there was sufficient DNA (100 ng) to detect a single-copy sequence of *virA*.

TABLE 2. Sequences of primers used in PCR

Primer	Sequence
virA	
Forward	5′-CTG CAT TCT GGC AAT CTC TTC ACA TC-3′
Reverse	5′-TGA TGA GCT AAC TTC GTA AGC CCT CC-3′
16S rDNA	
Forward	5′-AGA CTG CTA CGG GAG GCA GCA GT-3′
Reverse	5′-GTT GCG CTC GTT GCG GGA CTT AA-3′

Sensitivity of PCR with *virA* primers. When *S. dysenteriae* serovar 1 was used, as little as 25 fg of isolated DNA (Fig. 1A), 10 μl of a bacterial dilution containing between 1 and 10 CFU, i.e. 100 to 1,000 cells/ml (data not shown), and deliberately contaminated mayonnaise (data not shown) gave positive amplifications, as revealed on an agarose gel.

The amplification product of *S. dysenteriae* serovar 1 was cloned in a plasmid vector. A representative clone was sequenced in both directions. This sequence (accession no. AF010147) was 215 nucleotides long and identical to that of *virA* from *S. flexneri*. This representative clone was labeled with Dig and used as a highly specific probe to hybridize against the amplification products obtained with different amounts of DNA from *S. dysenteriae* serovar 1. Positive hybridization was observed for all lanes in which template DNA (1 to 100 fg) had been added to the PCR (Fig. 1B); i.e., the sensitivity was 1 fg of template DNA.

Multiplex PCR. The 16S rDNA primers were designed by using conserved regions of 16S rDNA of bacteria and the

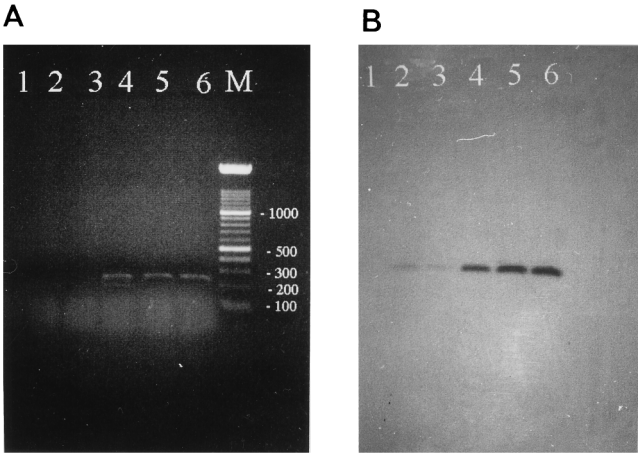

FIG. 1. PCR with *virA* primers and different amounts of DNA of *S. dysenteriae* serovar 1. (A) Twenty-five microliters of the amplification reaction solutions containing different amounts of DNA subjected to agarose gel electrophoresis; (B) same solutions as those in panel A after filter DNA hybridization against the *virA* Dig-labeled probe. Lane M contains a 100-bp marker, with numbers indicating the size in base pairs. Lanes 1 to 6 contain 0, 1, 5, 25, 50, and 100 fg of template DNA, respectively.

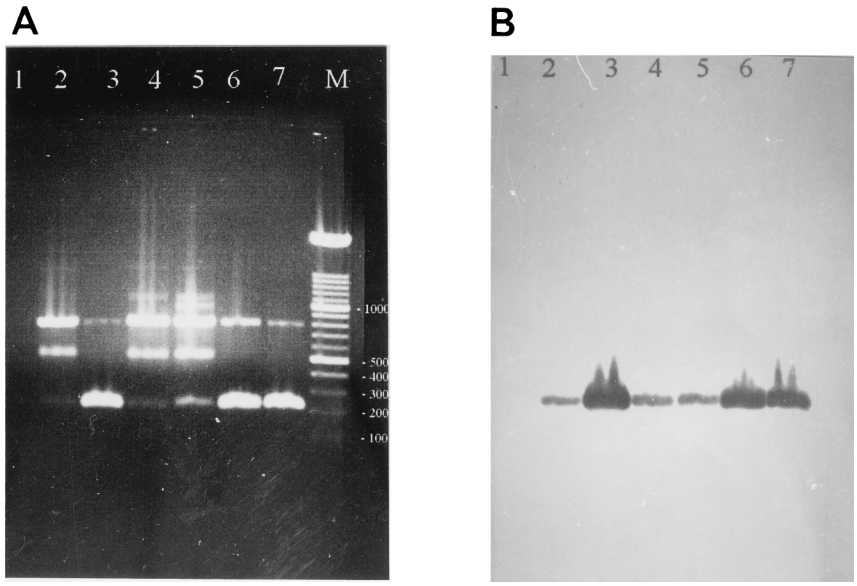

FIG. 2. Multiplex PCR with DNA from *Shigella* spp. or EIEC. (A) Ten microliters of amplification reaction solutions with 100 ng of DNA from various bacteria after agarose gel electrophoresis; (B) the same solutions as those in panel A after filter DNA hybridization against the *virA* Dig-labeled probe. Lane M, 100-bp marker, with numbers indicating the size in base pairs. Lanes 1 to 7, control without DNA, *S. boydii* serovar 10, *S. dysenteriae* serovar 1, *S. flexneri* serovar 2a, *S. sonnei* serovar a, EIEC 41, and EIEC 121, respectively.

corresponding sequence of *E. coli* (accession no. J01695). They should produce an amplification product of around 755 bp whenever bacteria are present. PCR experiments using both *virA* and 16S rDNA primers (multiplex PCR) produced in all bacteria tested one DNA band of approximately 755 bp (Table 1) and an additional DNA band of approximately 215 bp in *Shigella* and EIEC (Table 1 and Fig. 2A). On the other hand, the few samples of eukaryotic DNA that were tested did not produce either the 755-bp DNA band or the 215-bp DNA band (Table 1). Moreover, the 215-bp product could be differentiated from that of 755 bp not only by gel electrophoresis but also by hybridization against the *virA* probe (Fig. 2B).

DISCUSSION

Epidemiological studies on *Shigella* have established that 10 cells are sufficient to be an infective dose (24). This amount of bacteria could easily be present in contaminated food. The results presented in this work showed that PCR with *virA* primers could be a useful hazard test because its sensitivity, similar to that reported elsewhere (4, 5), would allow 1 fg of DNA or 1 to 10 cells in 10 μl of sample to be detected. Moreover, the high annealing temperature between primers and target and the nature of the target itself gave the desirable specificity for strains containing the virulence plasmid, *Shigella* and EIEC.

Legislation in many countries requires the absence of *Shigella* in 25-g amounts of foods (9), such as mayonnaise. Since it is not possible to directly carry out PCR on 25 g of mayonnaise, an enrichment step is necessary. Despite this additional step, the PCR is faster and also more sensitive than conventional methods. A positive result with the *virA* primers does not conclusively demonstrate that a virulent organism is present in the sample. Given that the genes implicated in virulence are located on both the chromosome and the plasmid and that *virA* is on a plasmid, which could be transferred to other bacteria, it is theoretically possible that other bacteria can carry *virA* and be nonpathogenic.

The applicability of PCR with the *virA* primers for detection of *Shigella* and EIEC in mayonnaise was demonstrated since a positive amplification was obtained with mayonnaise diluted with peptone water that had been deliberately contaminated with *Shigella*. The absence of a PCR product when an enrichment medium is used as the source of template DNA could be due to either (i) no contamination (absence of target DNA) or (ii) a failure in the reaction due to the presence of inhibitors and/or the unavailability of DNA, e.g., no bacterial lysis. Failure to detect contamination by PCR could be confirmed by using multiplex PCR to verify the absence of the 755-bp product both in a food sample and, as a positive control, in a food sample contaminated with exogenous bacteria.

Cost factors are likely to be considered when selecting the method for detection of *Shigella* in foods, and PCR seems to be more expensive than the conventional method (if confirmation of positive results is excluded). However, it should be kept in mind that the latter method does not include an enrichment medium intended for *Shigella* (8). Also, the conventional method is time-consuming, requiring food to be stored for a long period. Therefore, a comparison of the costs associated with storage and those of the PCR itself should be made.

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