University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Public Health Resources

Public Health Resources

1990

Detection of *Shigella* in Feces Using DNA Amplification

Gad Frankel

Division of Geographic Medicine

Lee Riley

Howard Hughes Medical Institute

Jorge A. Giron

Stanford University

Janice Valmassoi

Stanford University

Adam Friedmann

Hebrew University

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.unl.edu/publichealthresources>

Part of the [Public Health Commons](#)

Frankel, Gad; Riley, Lee; Giron, Jorge A.; Valmassoi, Janice; Friedmann, Adam; Strockbine, Nancy; Falkow, Stanley; and Schoolnik, Gary K., "Detection of *Shigella* in Feces Using DNA Amplification" (1990). *Public Health Resources*. 227.

<https://digitalcommons.unl.edu/publichealthresources/227>

This Article is brought to you for free and open access by the Public Health Resources at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Public Health Resources by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Gad Frankel, Lee Riley, Jorge A. Giron, Janice Valmassoi, Adam Friedmann, Nancy Strockbine, Stanley Falkow, and Gary K. Schoolnik

Detection of *Shigella* in Feces Using DNA Amplification

Gad Frankel, Lee Riley, Jorge A. Giron,
Janice Valmassoi, Adam Friedmann,
Nancy Strockbine, Stanley Falkow,
and Gary K. Schoolnik

From the Departments of Microbiology and Immunology and of
Medicine, Division of Geographic Medicine, and Howard Hughes
Medical Institute, Stanford University, Stanford, California; Department
of Genetics, Hebrew University, Jerusalem, Israel; and Division of
Bacterial Diseases, Centers for Disease Control, Atlanta, Georgia

A rapid diagnostic method employing a polymerase chain reaction procedure (PCR) was used to identify *Shigella* and enteroinvasive *Escherichia coli*. This procedure amplified a region of the invasive-associated locus (*ial*) from a crude DNA extract of feces. A synthetic 21-base oligonucleotide corresponding to the *ial* gene sequence was shown to specifically hybridize only with enteroinvasive *E. coli* (EIEC) strains and *Shigella* species. Upon PCR amplification, a 320-base pair fragment was generated in DNA extracted from feces reconstituted with EIEC or *Shigella flexneri* but not in DNA from 70 normal stools lacking these organisms and could be readily detected by the *ial* probe. For identifying *Shigella* and EIEC, the PCR assay was 10⁵- and 10²-fold more sensitive than standard biochemical tests and the macrocolony hybridization assay, respectively. These findings demonstrate a novel methodology for rapid, sensitive, and culture-independent diagnosis of diarrhea caused by these pathogens and underscores the utility of PCR in the diagnostic laboratory.

Shigella species are responsible for a substantial proportion of cases of bacillary dysentery, resulting in endemic disease and sporadic epidemics in developing countries [1]. *Shigellae* produce diarrhea by penetration and destruction of colonic epithelia [2]; this capacity is mediated in part by a large 120–140 MDa plasmid, which carries the genetic determinants that encode epithelial cell invasion by each of the four species of *Shigella* and by the closely related enteroinvasive *Escherichia coli* strain (EIEC) [3].

Shigellae are ordinarily identified in stool specimens by in vitro cultivation of the organism and by several biochemical tests and an agglutination assay [4]. Recently, DNA probes have been developed corresponding to the large "invasion" plasmid [5, 6] and used to identify shigellae and EIEC by nucleic acid hybridization. While these probes negate the need for biochemical tests and agglutination assays, they are too insensitive to be used directly with stool specimens. Instead, feces must be plated onto solid media, incubated until visible colonies arise, and the colonies then analyzed by DNA hybridization. Further, spontaneous loss of the invasion plasmid or deletion of plasmid-encoded genes mediating epithelial cell invasion can occur during in vitro growth and storage of *Shigella* isolates, even at frequencies as high as 50% per generation per cell for some *Shigella sonnei* strains [7]. As a result, DNA probes derived from the invasion plasmid may

not detect *Shigella* strains in stool specimens that have been cultured in vitro.

We identified DNA sequences within an invasion-associated locus (*ial*) of the invasion plasmid that are specific for shigellae and EIEC. These were shown to hybridize with the four *Shigella* species and with all tested EIEC. Analysis of these sequences led to development of a test for identifying shigellae directly in stool through the amplification of *ial*-specific DNA using the polymerase chain reaction (PCR), a procedure that does not entail in vitro cultivation of the specimen.

Materials and Methods

Bacterial strains, colony hybridization, and plasmid profiles. Strains used are listed in table 1. Plasmids were extracted [8] from 106 *Shigella* and 14 EIEC isolates and analyzed by electrophoresis using 0.8% agarose gels. For the colony hybridization assay, 50 μ l of broth culture were filtered through nylon membranes (Biotrans; ICN, Irvine, CA) and the membranes tested with the *ial* probe as described below.

Oligonucleotides: probes and primers. The *ial* probe and primers were derived from the nucleotide sequence of a 2.5-kilobase pair (kbp) *Hind*III fragment of an EIEC invasion plasmid [6]; synthesized using solid-phase phosphoramidite chemistry [9]; and purified by high-pressure liquid chromatography (HPLC). The *ial* probe was prepared according to the method of Jablonski et al. [10] and coupled to alkaline phosphatase (AP) using a single modified base containing a 12-carbon linker with a terminal primary amine.

DNA preparation and amplification. To evaluate the performance of the diagnostic test in feces, normal stool (200 mg) was suspended in 1 ml of phosphate-buffered saline (PBS; pH 7.4) to which was added 10⁵ colony-forming units (cfu) of *Shigella flexneri*. Insoluble particulate matter was removed by low-speed centrifugation and the bacteria in the supernatant obtained by centrifugation for 1 min in an Eppendorf microcentrifuge. The resulting pellet was resuspended

Received 26 April 1989; revised 5 December 1989.

Supported by the Howard Hughes Medical Institute.

Reprints and correspondence: Dr. Gary K. Schoolnik, Dept. of Microbiology and Immunology, Beckman Center B-239, Stanford University, Stanford, CA 94305.

Table 1. Specificity of *ial* alkaline phosphatase probe for different enteropathogens and for nonpathogenic *Escherichia coli*.

Microbial species or type examined	Total tested	Probe-positive	Probe-negative
Nonpathogenic <i>E. coli</i>	36	0	36
EPEC	8	0	8
ETEC	12	0	12
EHEC	1	0	1
<i>Campylobacter jejuni</i>	1	0	1
<i>Salmonella</i> (typhi, adelaide, greenside)	3	0	3
<i>Yersinia</i> (enterocolitica, pseudotuberculosis)	2	0	2
<i>E. coli</i> HB101 (pBR325)*	1	0	1
<i>E. coli</i> HB101 (<i>ial</i>)†	1	1	0
EIEC			
Large plasmid‡	14	14	0
<i>Shigella dysenteriae</i>			
Large plasmid‡	17	15	2
Altered plasmid profile§	11	1	10
<i>Shigella boydii</i>			
Large plasmid‡	16	12	4
Altered plasmid profile§	19	0	19
<i>Shigella flexneri</i>			
Large plasmid‡	5	5	0
Altered plasmid profile§	19	0	19
<i>Shigella sonnei</i>			
Large plasmid‡	8	8	0
Altered plasmid profile§	11	0	11

NOTE. EPEC = enteropathogenic *E. coli*, ETEC = enterotoxigenic *E. coli*, EHEC = enterohemorrhagic *E. coli*, EIEC = enteroinvasive *E. coli*. No EIEC isolates were found without plasmids or with plasmids <140 MDa.

*† HB101 containing vector plasmid pBR325 and clone of *ial* locus, respectively.

‡ ≥140 MDa.

§ <140 MDa.

in 75 μ l of 50 mM Tris-HCl buffer, pH 8.0, containing 20% (wt/vol) sucrose, 50 mM EDTA, and 100 μ g lysozyme (Sigma, St. Louis); the reactants were incubated at 37°C for 30 min. Then, 300 μ l of 50 mM NaCl containing 1% (wt/vol) sodium dodecylsulphate (SDS) and 800 μ g of proteinase K (Boehringer, Indianapolis) were added and the incubation was continued an additional 60 min. The extracted DNA was precipitated by adding absolute ethanol, harvested by centrifugation, and then resuspended in 100–300 μ l of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. DNA was also extracted, as described above, from Luria broth cultures of EIEC and of an HB101 strain of *E. coli* containing a recombinant plasmid with the *ial* locus. DNA was amplified by PCR with 1–5 μ l of the extracted DNA using 0.2 μ g of each of the primers [11, 12]. PCR was performed for 26 1-min cycles at 94°C (denaturation), 2 min at 43°C (annealing of primers to single-stranded DNA), and 3 min at 72°C (DNA polymerase-mediated primer extension). The amplified product (10 μ l) was analyzed by electrophoresis using 1.5% agarose gels. *Taq* polymerase was purchased from Perkin-Elmer-Cetus (Norwalk, CT).

Detection of PCR-amplified products with the alkaline-phosphatase-labeled oligonucleotide *ial* probe. PCR-amplified reactants (10 μ l) were analyzed by slot blot hybridization with the *Shigella ial* probe. Before hybridization, the DNA was incubated in 0.3 M NaOH for 5 min at 70°C and applied to a nylon membrane. Before hybridiza-

tion the membrane was incubated in 5× SSPE, containing 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml yeast tRNA (BRL, Gaithersburg, MD) for 15 min at 42°C. Then 4 ng of the AP probe or 10⁶ cpm/ml of the ³²P-labeled probe (*ial* RA probe) was added and hybridization allowed to proceed for 15 min. The membrane was then washed four times at room temperature: 2× SSC, 0.5% SDS for 10 min; 1× SSC, 0.1% SDS for 10 min; 1× SSC, 0.5% Triton X-100 for 5 min; 1× SSC for 1 min. The membranes were either exposed to x-ray film (for the *ial* RA probe) or incubated with a substrate for the AP probe consisting of a mixture of nitroblue tetrazolium and 3-bromo-4-chloro-3-indolyl phosphate for 4 h at room temperature.

Sensitivity of PCR, biochemical testing, and "macrocolony" hybridization to detect shigellae in feces. To compare the sensitivity of the PCR assay described above with the *ial* probe using cultivated organisms and with the standard biochemical tests for detecting *Shigella* species, three normal stool specimens were reconstituted with serial dilutions of *S. flexneri*. Each dilution was plated, in duplicate, onto MacConkey agar, and four lactose-negative colonies were selected and tested by the API test (Analytab Products, Plainview, NY) for gram-negative bacteria. Macrocolonies were obtained by applying the stool specimens to a nylon membrane with Dacron-tipped swabs. The membrane was placed on the surface of nutrient agar and inoculated at 37°C for 12 h; the resulting macrocolonies were tested with the *ial* AP probe. For PCR the DNA was extracted as described above, *ial* locus DNA amplified, and the products tested by hybridization with the *ial* AP probe. The sensitivity of each assay was defined as the lowest concentration of *S. flexneri* (in cfu) that yielded a positive result for each of the three assays. Seventy normal stools and four stool specimens containing, respectively, each of the other three *Shigella* species and EIEC were also tested by the PCR procedure.

Results

Selection, preparation, and specificity of the *ial* locus oligonucleotide probe. The *ial* locus lies within a 2.5-kb *Hind*III fragment of the large invasion plasmid that directs epithelial cell penetration by *Shigella* species and EIEC. The sequence of a 700 base-pair (bp) segment of this fragment was determined (data not shown) and compared with all other DNA sequences in the gene bank [13], leading to the identification of three unique regions (figure 1A). One unique sequence was prepared as the 21 base oligonucleotide 3' GTG TCC ATA AGA TTA TCT ACC 5'; this was used in the DNA hybridization assays described below either as a ³²P-labeled probe (the *ial* RA probe) or as an AP conjugate (the *ial* AP probe). The *ial* probe was tested by a colony blot hybridization assay and shown to recognize strains of EIEC and each of the four *Shigella* species (*S. dysenteriae*, *S. boydii*, *S. flexneri*, and *S. sonnei*). The probe did not hybridize with *Campylobacter*, *Salmonella*, and *Yersinia* species or nonpathogenic *E. coli* (table 1). The *ial* AP probe was then evaluated and found to be as specific and sensitive as the *ial* RA probe (data not shown).

A collection of 106 *Shigella* isolates and 14 EIEC isolates collected from patients between 1949 and 1986 was tested

Figure 1. A. Sequences within *ial* locus of oligonucleotide primers used for polymerase chain reaction (PCR) and of *ial* probe. Polarity of primers is indicated. B. *ial*-specific amplification of *Shigella* and enteroinvasive *Escherichia coli* (EIEC) DNA by PCR. Amplified product of each reaction was analyzed by electrophoresis through 1.5% agarose gels: amplification of cloned *ial*, lane b; amplification of EIEC DNA, lane c; amplification of *Shigella flexneri* DNA (from reconstituted stool), lane d; amplification of DNA extracted from normal stool not containing *ial*-specific DNA, lane e; and molecular weight markers, lane a. C. Detection of PCR-amplified products by *ial* AP probe. Reactants from each of the PCR assays in B were analyzed by slot blot hybridization with *ial* AP probe. Amplified products of cloned *ial*, lane 1; broth culture of EIEC, lane 2; DNA extracted from stool containing *S. flexneri*, lane 3; and DNA extracted from normal stool not containing *ial*-specific DNA, lane 4.

with the *ial* AP probe (table 1). Each of the 14 EIEC isolates, but only 41 of the shigellae hybridized with the probe. As expected, a positive hybridization result correlated strongly with the presence of a 140-MDa plasmid; loss of the large plasmid during in vitro growth or storage resulting in a negative hybridization test in 59 of the 106 isolates (table 1). In contrast, only 6 of 46 *Shigella* isolates with plasmids of ≥ 140 MDa did not hybridize with the probe; in these strains small deletions involving the *ial* locus had apparently occurred during or after their isolation from feces.

Use of PCR to detect *ial*-specific sequences. To prevent falsely-negative identification of *Shigella* species due to plasmid loss or deletion of the *ial* locus during in vitro growth, we developed a diagnostic method that uses DNA extracted

directly from feces and therefore does not entail propagation of organisms on laboratory media. The *ial* locus sequence was examined and two additional unique regions were identified that flank the 5' and 3' ends of the *ial* probe described above. As with the *ial* probe, each of the two flanking sequences was prepared as a synthetic oligonucleotide (figure 1A) and shown to hybridize specifically to the *ial* locus of the four *Shigella* species and EIEC (data not shown). When these flanking sequence oligonucleotides were used as primers for PCR, the intervening *ial* sequence was specifically amplified by using DNA extracted from L broth cultures of EIEC or from feces to which *S. flexneri* had been added. In either case, a 320-bp fragment was amplified (figure 1B); this fragment, which corresponded with the region between the two primers, was readily detected using the *ial* AP probe (figure 1C). The entire procedure could be completed in <7 h. In addition to the 320-bp fragment, a high molecular weight band appeared inconsistently and did not hybridize with the *ial* probe.

Detection of *Shigella* in feces by PCR, macrocolony hybridization, and biochemical assays. The sensitivity of the described test was assessed by determining its capacity to detect the *ial* locus in feces from three normal individuals to which known numbers of colony-forming units of *S. flexneri* had been added. Each stool sample, for each dilution of *S. flexneri*, was processed in duplicate. Stool specimens containing as few as 10 *S. flexneri* cfu yielded a positive hybridization result after 26 cycles of amplification. This was compared with the sensitivity of a macrocolony hybridization assay using the *ial* AP probe. The same stool specimens used to determine the sensitivity of the PCR assay were spotted onto nylon membranes lying on the surface of nutrient agar, giving rise 12 h later to visible macrocolonies. When these were analyzed by DNA hybridization with the *ial* AP probe, as few as 1000 cfu of *S. flexneri* could be identified. Finally, the same stool specimens were evaluated using standard biochemical tests for detecting *Shigella* species, resulting in identification of as few as 10^6 cfu of *S. flexneri*. No lactose-negative colonies were present in the normal stools before reconstitution with *Shigella*. Thus, the PCR assay was 10^5 - and 10^2 -fold more sensitive than the biochemical tests and the macrocolony procedure, respectively. Also, EIEC and the other three *Shigella* species were detected in reconstituted stool specimens after PCR and hybridization with the *ial* probe. In contrast, 70 normal stools, not containing *Shigella* or EIEC, were examined by the PCR procedure and none showed a positive result.

Discussion

Invasion of colonic cells by shigellae and EIEC is essential for producing the dysenteric syndrome. This process is mediated in part by a 180- to 210-kbp plasmid that has been termed the virulence or invasion plasmid. Loss of this plasmid, which

can occur spontaneously during in vitro growth, results in the simultaneous loss of virulence (as determined by the inability of the strain to cause keratoconjunctivitis in the guinea pig) and the strain's ability to penetrate cultured epithelial cells. Transfer of the invasion plasmid to an avirulent *E. coli* K12 strain confers epithelial cell invasiveness, but not virulence, indicating that virulence requires chromosomal loci in addition to the plasmid loci that encode invasiveness in vitro.

To date, no single locus within the invasion plasmid, when transferred to a noninvasive *E. coli* recipient, has been shown to encode epithelial cell penetration. Tn5 mutagenesis of the invasion plasmid, however, has led to detecting two separate regions that appear to be required for the invasion phenotype in vitro. One such region is located on a 4.6-kbp *HindIII* fragment that was derived from a 39-kbp segment of the invasion plasmid; this segment was found to restore invasion to a plasmid-cured *Shigella* strain and to code for several outer membrane proteins of the bacterium, termed invasion plasmid antigens (Ipa), that are immunogenic in *Shigella*-infected humans [6]. When used as a DNA probe, this region was shown by Venkatesan et al. [14] to hybridize specifically with all tested virulent *Shigella* and EIEC strains. A second region was identified by Small and Falkow [5] within a 15-kbp *ClaI* fragment of an EIEC invasion plasmid. This in turn yielded a 2.5-kbp *HindIII* fragment that hybridized specifically with each of the four *Shigella* species and with EIEC. Further evaluation of this fragment by Gomes et al. [15] indicated that although it hybridized primarily with virulent *Shigella* strains, it also hybridized to several strains that did not cause keratoconjunctivitis, raising the possibility that its use as a diagnostic test could lead to falsely positive results. Further analysis, however, indicated that most of the probe-positive, avirulent *Shigella* strains gave rise to a mixed population of colonies, some of which were virulent. Thus, the 4.6-kbp *HindIII* fragment of Venkatesan et al. [14] and the 2.5-kbp *HindIII* fragment of Small and Falkow [5] appear to hybridize with virulent strains of *Shigella* and EIEC, with a substantial degree of specificity.

In the work reported here, we determined the DNA sequence of a 700-bp segment within the 2.5-kbp *HindIII* fragment of Small and Falkow; we have termed this segment the invasion-associated locus (*ial*). A unique region of *ial* was prepared as a 21-base synthetic oligonucleotide, conjugated to AP and tested by hybridization with EIEC and *Shigella* strains containing the virulence plasmid. All the tested isolates had been stored in vitro, some for as long as 40 years. Because of the recognized tendency for some *Shigella* strains to lose the invasion plasmid, the results of the hybridization tests were correlated with the plasmid profile of each strain. The *ial* AP probe hybridized with all EIEC, *S. flexneri*, and *S. sonnei* strains harboring a plasmid >140 MDa. In contrast, 2 of 17 *S. dysenteriae* strains and 4 of 16 *S. boydii* strains carrying a plasmid >140 MDa did not hybridize with this

probe, indicating that the invasion plasmids of these strains may have sustained small deletions in *ial*. However, the effect of these putative deletions in the 6 probe-negative *Shigella* strains on virulence or invasiveness was not determined.

Examination of the *ial* sequence led to identifying two additional unique sequences flanking the sequence corresponding to the *ial* AP probe; these were used as primers for amplifying the 320-bp intervening sequence by PCR. The amplified product could be detected by gel electrophoresis or DNA hybridization using the *ial* AP probe. Use of this assay with normal stool specimens that had been reconstituted with various concentrations of *S. flexneri* indicated that as few as 10 cfu could be detected by the *ial* AP probe after 26 cycles of amplification and that the entire procedure, including the extraction of DNA from stool, amplification, and the use of the *ial* AP probe to detect the amplified product, could be completed in 7 h. The assay was found to be 10²-fold more sensitive than the macrocolony hybridization assay using the same *ial* AP probe and 10⁵-fold more sensitive than conventional biochemical tests for identifying enteric bacteria. Recently, this amplification assay was evaluated under field conditions in a rural region of Mexico using stool specimens from 71 children with diarrhea. *Shigella* species or EIEC or both were detected by PCR in 10 of the 71 specimens, whereas only 8 of these 10 were detected by a combination of macrocolony hybridization and biochemical tests (unpublished data). Thus, PCR may be substantially more sensitive than previously described tests, particularly when used during the course of epidemiologic investigations in developing countries.

The foregoing discussion provides evidence that the PCR test for detecting *Shigella* species and EIEC is not only highly sensitive but also can provide a result on the day a specimen is submitted for evaluation, a potential advantage during outbreak investigations, especially in refugee camps and following disasters. Because of the essential role of the invasion plasmid for virulence, the test is likely to identify stool specimens containing clinically significant variants of these pathogens. Moreover, since the test does not entail cultivation of the specimen in vitro, falsely negative results due to loss of the invasion plasmid should not occur. The test, however, cannot distinguish EIEC from *Shigella* species or one *Shigella* species from another, a significant disadvantage for epidemiologic studies seeking to establish the precise etiology of enteric infections within a community or the prevalence of a particularly virulent species, such as the Shiga bacillus. The great potential of the PCR test for extreme sensitivity may also lead to identifying many asymptotically infected persons carrying small numbers of these organisms, particularly in developing countries where enteric infections are prevalent. This could lead to difficulties in interpreting epidemiologic data. The number of amplification cycles, however, can be selected to yield a predetermined level of sensitivity, since in principle, PCR is a quantitative technique, the concentra-

tion of amplified DNA being doubled by each amplification cycle.

Acknowledgment

We thank L. Tompkins for helpful discussion; Kaye Wachsmuth, Enteric Diseases Laboratory at the Centers for Disease Control, Atlanta, and Sam Formal, Walter Reed Army Institute of Research, Washington, DC, for providing strains; and Molecular Biosystems, Inc., San Diego, for custom synthesis of the invasion-associated-locus (*ial*) AP probe.

References

1. Mata LJ, Gangarosa EJ, Cáceres A, Perera PR, Mejicanos ML. Epidemic shiga bacillus dysentery in Central America. I. Etiologic investigations in Guatemala, 1969. *J Infect Dis* 1970;122:170-180
2. LaBrec EH, Schneider H, Magnani TJ, Formal SB. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *J Bacteriol* 1964;88:1503-1518
3. Sansonetti PJ, Kopecko DJ, Formal SB. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. *Infect Immun* 1982;35:852-860
4. Edwards PR, Ewing WH. Identification of *Enterobacteriaceae*. 3rd ed. Minneapolis: Burgess Publishing, 1972
5. Small PL, Falkow S. Development of a DNA probe for the virulence plasmid of *Shigella* spp. and enteroinvasive *Escherichia coli*. In: Lieve L, Bonventre PF, Morello JA, Silver SD, Wu WC, eds. Microbiology. Washington, DC: American Society for Microbiology, 1986: 121-124
6. Buysse JM, Stover CK, Oaks EV, Venkatesan M, Kopecko DJ. Molecular cloning of invasion plasmid antigen (*ipa*) genes from *Shigella flexneri*: analysis of *ipa* gene products and genetic mapping. *J Bacteriol* 1987;169:2561-2569
7. Sansonetti PJ, Kopecko DJ, Formal SB. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. *Infect Immun* 1981;34:75-83
8. Brinboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979;7:1513-1523
9. Caruthers MH, Beaucage SL, Becker C, Efcavitch W, Fisher EF, Galluppi G, Goldman R, de Haseth F, Martin F, Matteucci M, Stabinsky Y. New method of synthesizing deoxyoligonucleotides. In: Selowd A, Hollaender SK, eds. Generic engineering principles with methods. Vol 4. New York: Plenum Publishing, 1982:1-17
10. Jablonski E, Moomaw EW, Tullis RH, Ruth JL. Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res* 1986;14:6115-6128
11. Saiki RK, Bugawan TL, Jorn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified β -globin and HLA-DQ- β DNA with allele specific oligonucleotide probes. *Nature* 1986;324:163-166
12. Saiki RK, Gelfand DG, Stoffel S, Schare SJ, Higuchi R, Torn GT, Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 1988;239:487-491
13. Devereux J, Haeberli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 1984;12:387-395
14. Venkatesan M, Buysse JM, Vandendries E, Kopecko DJ. Development and testing of invasion-associated DNA probes for detection of *Shigella* spp. and enteroinvasive *Escherichia coli*. *J Clin Microbiol* 1988; 26:261-266
15. Gomes TAT, Toledo MRF, Trabulsi LR, Wood PK, Morris JG Jr. DNA probes for identification of enteroinvasive *Escherichia coli*. *J Clin Microbiol* 1987;25:2025-2027