DETECTION OF THE VIRULENCE PLASMID pINV, USING *inv*E GENE IN *Shigella* sp. IN SEWAGE SAMPLES BY PCR ASSAY.

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ABSTRACT

Virulent phenotype of *Shigella* is directly related to the presence of pINV plasmid of 210 to 230 kb. IpaBCD proteins encoded by the plasmid direct the bacterial invasion into the host epithelial cells. InvE protein positively regulates the expression of virulence genes *ipa*BCD. The polymerase chain reaction was used to amplify a 362 base-pair fragment specific for the *inv*E gene in sewage samples. Sewage samples were used in the assays because invasive bacteria like *Shigella* can used this way to infect human. The amplification products were digested with the restriction enzyme *Bgl*I and the resulting fragments confirmed the specificity of the amplification. A detection limit of 10^5 CFU/mL of *Shigella* was obtained for cultured bacteria and also for sewage samples seeded with the pathogen. Sewage samples were seeded with *S. dysenteriae* in enrichment culture and incubated for 8h at 37ºC. After incubation DNA was extracted and the PCR assay ensued. A minimal concentration of 10^2 CFU/mL was enough for the detection of the microorganisms after the enrichment of the sample.
Key words: PCR, sewage, *Shigella* sp., *inv*E gene, enrichment culture.

DETECÇÃO DO PLASMIDEO DE VIRULÊNCIA pINV EM *Shigella* sp. PRESENTE NO ESGOTO ATRAVÉS DA TÉCNICA PCR USANDO O GENE *inv*E

RESUMO

O fenótipo virulento em linhagens de *Shigella* é dependente da presença do plasmídeo pINV de 210 a 230 Kb. As proteínas IpaBCD codificadas no plamideo de virulência pINV, direcionam a entrada destas bactérias nas células epiteliais do hospedeiro. A regulação positiva dos genes que codificam tais proteínas (*ipa*BCD) é realizada pela proteína InvE. A reação em cadeia da polimerase foi usada para amplificar um fragmento de 362 pb específico para o gene *inv*E. Amostras de esgoto foram utilizadas nos ensaios, visto que bactérias invasivas tais como *Shigella* sp. podem ser transmitidas por essa rota. A detecção limite de 10^5 UFC/mL foi obtida em cultura de *Shigella* e o resultado repetiu-se quando amostras de água de esgoto foram contaminadas com a mesma bactéria. Quando amostras de esgoto contaminadas com *S. dysenteriae* foram submetidas ao enriquecimento com meio de cultura apropriado, um mínimo de 10^2 CFU/mL na amostra foi suficiente para a detecção do microrganismo. O produto de amplificação foi digerido com a endonuclease de restrição *Bgl*I e os fragmento resultantes confirmaram a especificidade do produto de amplificação.
Palavras-chave: PCR, esgoto, *Shigella* sp., gene *inv*E, cultura de enriquecimento.

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INTRODUCTION

Virulence in all *Shigella* strains is dependent on the presence of a large 210 – 230 kb plasmid. The plasmid is determinant for the invasiveness and the ability to cause disease (HALE, 1991). A common 31 kb core region within pINV, essential for the virulence phenotype, includes genes for invasions, molecular chaperones, motility regulation, and a specialized type III secretion apparatus. The plasmid pINV seems to be required for virulence, but virulence can be enhanced by genes on the chromosome (LAN and REEVES, 2002). The invasive mode has been acquired by plasmid transfer in several lineages giving multiple origins of *Shigella*. The *ipa*BCD genes encoded on virulence plasmid pINV are required for host cell invasion (MENARD, et al., 1993). VirF and *invE* (*vir*B and *ipa*R) are two positive regulators involved in the regulation of *ipa*BCD expression dependent on environmental conditions (ADLER, et al., 1998; WATANABE, et al., 1990). VirF induces the tanscription of *inv*E. InvE induces the transcription of the *ipa*BCD operon and the results obtained indicated that InvE bound directly to the promoter region of this operon (TANIYA, et al., 2003). The InvE has significant homology with ParB of plasmid P1, which is in turn a protein with DNA binding ability involved in plasmid partitioning factor.

Shigella infectivity is determined by the remarkably low amount of microorganisms required to fully establish the infectious process: as little as 10 to 100 microorganisms is enough to start an infection in healthy individuals. In a study carried out in which Brazilian children up to one year of age were tested for diarrhea, *Shigella* sp. was the pathogen most frequently detected among those over six months of age (Gomes, et al., 1991). In this way many research have been aiming to reach the most efficient method to detect those microorganism in aquatic environment (FRICKER and FRICKER, 1994; TSAI, et al., 1993).

The aim of this study was to develop a rapid and sensitive laboratory routine to detect the plasmid pINV, present in *Shigella* sp. with the amplification of the *inv*E gene.

MATERIAL AND METHODS

SENSITIVITY OF THE PCR TEST

Shigella dysenteriae IAL 1496 was used to determine the sensitivity of the PCR assay. The bacteria were grown until late exponential phase on BHI (Brain heart infusion) broth ($3x10^9$ CFU/mL). Appropriate concentrations of cells were obtained by 10-fold dilutions in peptonated water. To enumerate the bacteria 0.1 mL aliquots were spread onto BHI agar plates and incubated at 37ºC overnight and the bacterial concentration was estimated by calculating the average colony counts on plates. All the dilutions underwent the extraction of plasmid DNA using the alkaline lysis method (SAMBROOK, et al., 1989). Raw sewage samples were seeded with *S. dysenteriae* and underwent the same protocol.

PREPARATION OF SEEDED SEWAGE SAMPLE WITH *S. DYSENTERIAE*

A 14-h culture of *Shigella dysenteriae* was serially diluted on peptonated water and enumerated by plate counting. The same culture was serially diluted in sewage samples. Each dilution was added to a flask containing 30mL BHI broth medium and incubated at 37^o C at 150 rpm for 8 hours in rotatory shaker. After the incubation 1mL was used for plasmid DNA extraction (SAMBROOK, et al., 1989).

MOLECULAR FINGERPRINTING – PCR

Standard *Shigella* sp. was used in the PCR tests (Table 1). The plasmid DNA of the standard strains, of the isolated samples, and from the sewage samples was extracted using alkaline lysis method. The primers SHI (5' GAG CAT AGC ATC CGA GAA CT 3'), in the position 651 sense, and SHII (5'- GGG TTT TGC TTT CTG TTT TT -3') in the position 1012 anti-sense were designed from the complete sequence of the gene *inv*E (WATANABE, et al., 1990). The PCR reactions were performed in a final volume of 25 μL, containing 1X buffer (100 mM tris-HCl, 500 mM KCl, 2.5 mM $MgCl_2$), 2.5mM of each deoxynucleotides, 100 ng bovine albumin serum (BSA), 30 ng of each primer SHI and SHII, 0.5 U Taq DNA polymerase, and 30 ng template DNA. Reaction tubes were placed in a thermal cycler. PCR reactions were performed as follows: one initial cycle (1 min at 95^o C, 5 min at 60^o C, and 2 min at 72^o C), and 35 cycles (1 min at 95^o C, 1 min at 60^o C, and 2 min at 72^o C). The PCR products were resolved on a 1.2 % agarose gel. A 100 pb ladder was used as a molecular size marker. The restriction endonuclease *Bgl*I was used to confirm the specificity of the PCR amplification products using primers SHI and SHII.

RESULTS

SPECIFICITY OF THE PCR REACTION

To investigate the specificity of the primers SHI and SHII four strains of *Shigella* and fourteen strains belonging to other genera were used (Table 1). The primers designed for the gene *inv*E amplified specific products of the expected size (362 bp) for all *Shigella* samples. No cross-

reaction with the negative controls was observed. To validate the amplification product, restriction enzyme digestions were performed with *Bgl*I on the PCR product. The results confirmed the pattern expected of 118 bp and 244 bp fragments for all *Shigella* samples (results not shown).

SENSITIVITY OF THE PCR ASSAY

PCR detection of indicator and pathogenic microorganisms requires specificity and sensitivity of the reaction in order to ensure safety and quality of water. In order to evaluate the minimal detectable number of *Shigella* organisms with the primers SHI and SHII a culture of *S. dysenteriae* IAL 1496 was serially diluted in sterile water and template DNA was prepared. The sensitivity of detection for the PCR assay was 10^5 CFU/mL of *S. dysenteriae* were necessary to obtain a visible amplification, with the conditions used in this work (Figure 1).

PCR ASSAY OF SEWAGE SAMPLES FOR DETECT *Shigella* sp.

Raw sewage samples were spiked with *Shigella* for the PCR amplification. It was found that *S. dysenteriae* detection level was similar to that obtained for the bacterial culture diluted in water, 10^5 CFU/mL of *S. dysenteriae*. However, when the PCR assay was applied to sample seeded with *S. dysenteriae*, and left to grow for 8h on enrichment BHI medium the concentration of 10^2 CFU/mL was necessary to detect the isolate in the PCR assay (Figure 2).

DISCUSSION

The primers SHI and SHII, designed for this study were based on the sequence of the *Shigella inv*E gene. All the *Shigella* strains tested, when screened by the PCR assay amplified the predicted 362 bp fragment. Homollogous sequences are present in *Shigella* sp. and enteroinvasive *E. coli* plasmids. This was demonstrated by many authors working with hybridization, ribotyping, and studies using multilocus enzyme eletrophoresis (MLEE) (SANSONETTI, et al., 1983; OCHMAN, et al., 1983; ROLLAND, et al., 1988). The sequencing of the genes *ipg*D, *mxi*C and *mxi*A within the invasion region for the pINV, from *Shigella* strains and EIEC *E. coli* revealed a phylogenetic relationship of the invasion plasmid in these strains (LAN, et al., 2001). The author's proposition are that *E. coli* must have acquired these plasmid by lateral transfer and since the are virulence differences between EIEC and *Shigella* the virulence may no lie in plasmid, unless the difference is in regulation of virulence genes or interaction of chromosomal genes with plasmid encoded genes (LAN, et al., 2001).

The sensitivity of the PCR protocol to detect microorganisms depends on the conditions in which amplification takes place. By setting the temperature at 60^o C the amplification of *inv*E was possible when 10^5 CFU/mL were present in the sample. The higher the annealing temperature, the more selective will be the amplification product under study. Yet, under this circumstance, sensitivity in detecting those cells may be impaired (BEJ, et al., 1990). Thought high temperatures of annealing are better for the amplification of specificity the sensitivity of the method is still the major problem associated with this methodology (COHEN, et al., 1994). A low detection level is, however, indispensable for analysis of environmental water samples, since the infections dose of *Shigella* being very low, around 10^1-10^2 microorganisms (LEVINE, et al., 1973). When samples from the environment are used contaminants, present in the environment, are the main problem for the sensitivity of the assay. Usually, a low sensitivity is obtained in these cases (BEJ, 1995, TREVORS and VAN ELSAS, 1995). For this reason an enrichment step was done with the samples in this work. And after the enrichment procedure, a concentration of 10^2 CFU/mL of the sample was enough for the detection of the microorganisms with the conditions that were used in this work. ZHU, et al. (1996) point to selective pre-enrichment and enrichment routines as a key measure to detect low microorganism concentrations, stressed cells, and also to rule out any interference in the amplification process caused by inhibiting agents.

The PCR technique can detect a small number of culturable as well non culturable organisms. It is fast and an efficient way to get results in a few hours (JOSEPHSON, et al., 1993; ISLAM, et al., 1993; RAHMAN, et al, 1996). Since the PCR has been frequently used for the identification of clinic pathogens the same technique can be used to monitor and control of these microorganisms in the environment (ARNHEIM and ERLICH, 1992; ATLAS and BEJ, 1990; FRICKER and FRICKER, 1994). The classical bioassays is able to identify microorganisms that are phenotipicaly pathogens, however they are not very useful for the detection of toxigenic microorganisms. Accordingly to MARTINS, et al. (1992) when water has been analyzed, not only phenotype but also the genotype must be evaluated. And only DNA reveals the genetic potential of the microorganisms for the virulence production. The use of PCR in water samples has shown to be very efficient to diagnose the pathogenic microorganisms (LANG, et al., 1994).

Plasmid pINV present in S*higella* sp. strains that can cause diarrhea outbreaks due to water contamination can be detected via PCR using primers SHI and SHII designed for the *inv*E gene, within the amplification conditions

employed in this study. The PCR protocol is an alternative to detect plasmid pINV carrying virulence-codifying genes.

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Table 1: Bacterial strains used in the specificity assay of a PCR protocol to identify pINV in raw sewage samples.

Strains	Origin
Enterotoxigenic *Escherichia coli* (ST^+ LT^+)	[1]ATCC 11105
Enterotoxigenic *Escherichia coli* (ST^-LT^+)	[3]IAL 1895
Enterotoxigenic *Escherichia coli* (ST^+LT^-)	[3]IAL 1878
Enterotoxigenic *Escherichia coli O124:NM*	*ATCC*43893
Enterobacter aerogenes	[1]ATCC 13048
Enterobacter cloacae	[2]INCQS 00146
Klebsiella pneumoniae	[1]ATCC 10031
Listeria monocytogenes	[2]INCQS 7644
Proteus mirabilis	[2]INCQS 0098
Proteus vulgaris	[2]INCQS 00106
Pseudomonas aeruginosa	[1]ATCC 25619
Salmonella enteritidis	[1]ATCC 13076
Salmonella typhimurium	[1]ATCC 14028
Shigella dysenteriae	[3]IAL 1496
Shigella flexneri	[3]IAL 1541
Shigella boydii	[3]IAL 1548
Shigella sonnei	[1]ATCC 25931
Vibrio cholerae	[3]IAL 1941
Yersinia enterocolitica	[1]ATCC 9610

[1]American Type Culture Collection; [2]Instituto Nacional de Controle de Qualidade em Saúde (Fundação Osvaldo Cruz, Rio de Janeiro, Brasil), [3]Instituto Adolfo Lutz (São Paulo, Brasil).

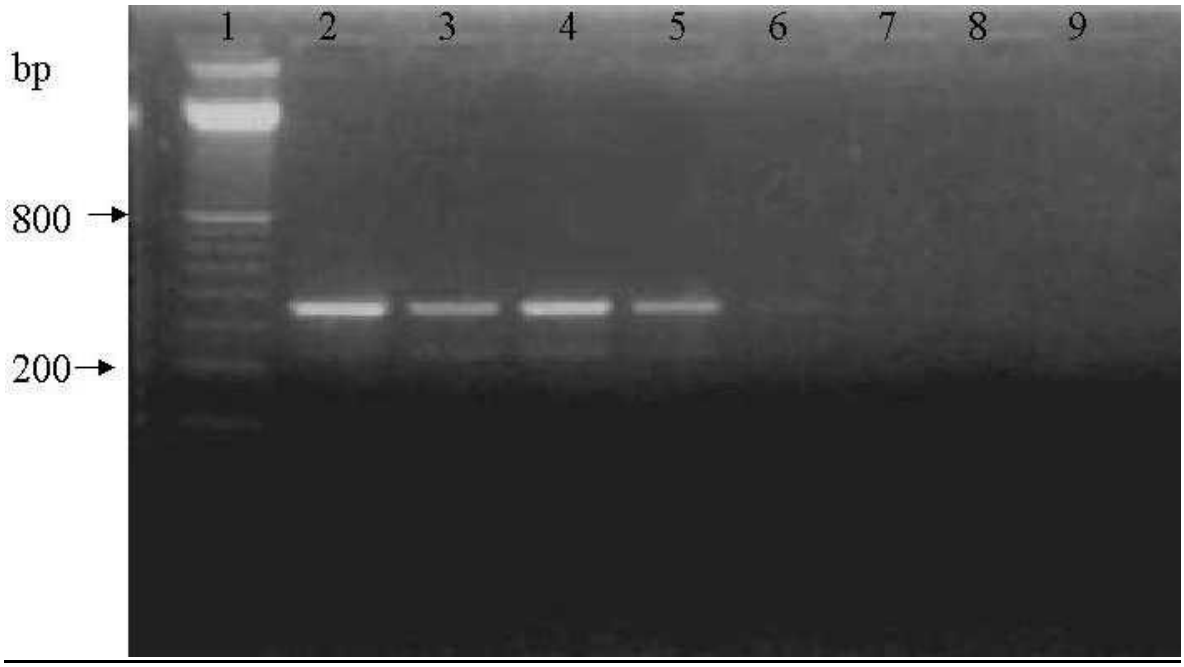

Figure 1. Electrophoresis in 1.2% agarose gel stained with ethydium bromide, showing the results of the sensitivity assay in sewage samples contaminated with *Shigella dysenteriae*. (1) 100-bp ladder; (2) 10^9 CFU/mL; (3) 10^8 CFU/mL; (4) 10^7 CFU/mL; (5) 10^6 CFU/mL; (6) 10^5 CFU/mL; (7) 10^4 CFU/mL; (8) 10^3 CFU/mL; (9) negative control.

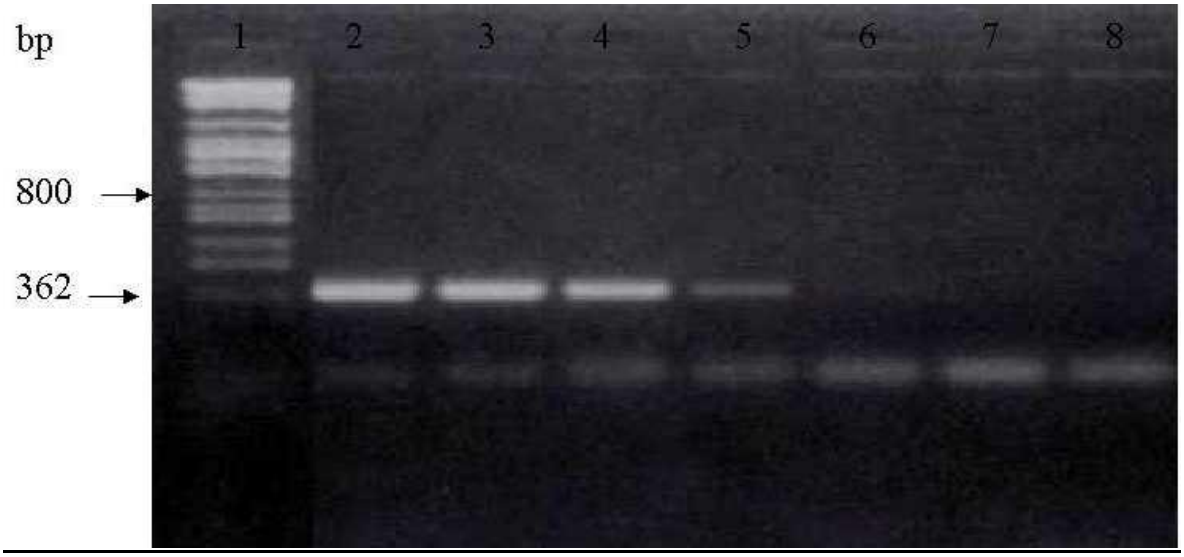

Figure 2. Electrophoresis in 1.2% agarose gel stained with ethydium bromide, showing the results of the sensitivity assay for CFU/mL in sewage samples contaminated with *Shigella dysenteriae* after enrichment culture. (1) λ (*Eco* RI/ *Hind* III/*Cla* I); (2) 10^5 CFU/mL; (3) 10^4 CFU/mL; (4) 10^3 CFU/mL; (5) 10^2 CFU/mL; (6) 10^1 CFU/mL; (7) 10^0 CFU/mL; (8) negative control.