

DETECTION OF SHIGELLA IN LETTUCE BY THE USE OF A RAPID MOLECULAR ASSAY WITH INCREASED SENSITIVITY

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ABSTRACT

A Multiplex Polymerase Chain Reaction (PCR) assay to be used as an alternative to the conventional culture method in detecting *Shigella* and enteroinvasive *Escherichia coli* (EIEC) virulence genes *ipaH* and *ial* in lettuce was developed. Efficacy and rapidity of the molecular method were determined as compared to the conventional culture. Lettuce samples were inoculated with different *Shigella flexneri* concentrations (from 10 CFU/ml to 10⁷ CFU/ml). DNA was extracted directly from lettuce after inoculation (direct-PCR) and after an enrichment step (enrichment PCR). Multiplex PCR detection limit was 10⁴ CFU/ml, diagnostic sensitivity and specificity were 100% accurate. An internal amplification control (IAC) of 100 bp was used in order to avoid false negative results. This method produced results in 1 to 2 days while the conventional culture method required 5 to 6 days. Also, the culture method detection limit was 10⁶ CFU/ml, diagnostic sensitivity was 53% and diagnostic specificity was 100%. In this study a Multiplex PCR method for detection of virulence genes in *Shigella* and EIEC was shown to be effective in terms of diagnostic sensitivity, detection limit and amount of time as compared to *Shigella* conventional culture.

Key words: PCR, *Shigella*, rapid method, lettuce, food.

INTRODUCTION

According to the World Health Organization, foodborne diseases represent a major public health problem which has increased in the last decade. The ingestion of pathogenic and virulent microorganisms generally results in foodborne illnesses affecting populations in both developed and developing countries (28).

Shigella spp. is a virulent bacterium that belongs to the *Enterobacteriaceae* family. The infectious dose of *Shigella* is as low as 10 bacterial cells (1,2,9). The transmission occurs

person to person through the faecal-oral pathway, and also by contaminated food and water (4). The symptoms of *Shigella* infection (shigellosis) range from mild watery diarrhea to severe bacillary dysentery with fever, abdominal pain, blood and mucus in stool samples (21).

Only three out of the four recognized *Shigella* species (*S. flexneri*, *S. boydii* and *S. sonnei*) are known to be etiological agents of foodborne gastroenteritis (3, 13). *S. dysenteriae* was described by Shiga in 1898, and is the primary cause of bacillary dysentery (9).

Foodborne diseases, including shigellosis, are substantially

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underestimated, particularly in developing countries (9). Although 99% of the cases of shigellosis occur in the developing world, industrialized countries such as Norway, Denmark, Australia, Portugal and the United States, among others, have reported outbreaks of foodborne shigellosis in the last decade (9, 13, 28). In the United States, *Shigella* is reported as the third highest cause of foodborne disease since 1997, after *Salmonella* and *Campylobacter* (26).

In Costa Rica, *Shigella* has been isolated from faecal, water and food samples (7,8,27). Other studies confirm that *Shigella* spp. is an endemic pathogen in Central American countries (R.Achí *et al.*, manuscript in preparation), causing severe diarrheal disease that requires hospitalization in young children (2).

Traditional microbiological assays for detection of *Shigella* in food include enrichment, culture, and biotype- and serotype identification steps; all of which are time consuming, requiring several days to obtain results. On the other hand, *Shigella* is considered a fastidious pathogen for bacteriological isolation, which in the context of indigenous micro flora and other substances makes detection less feasible (9,16,19). However, the advent of molecular biology assays, such as the Polymerase Chain Reaction (PCR), has made bacterial detection possible without the need for bacteria isolation. PCR has become a powerful diagnostic tool for detection of microorganisms in food and clinical samples (5, 16, 19). It is considered a fast, highly sensitive and specific assay that quickly amplifies specific sequences of the target DNA from bacterial pathogens such as *Shigella* spp. The target DNA is a specific sequence from a gene related to virulence mechanisms. However, despite the advantages compared to conventional culture, PCR-based methods must be reliable according to international criteria such as selectivity (inclusivity and exclusivity), good detection level and the mandatory use of internal amplification controls (IAC) (6, 11, 7).

For the present study, a PCR assay for detection of the invasion antigen loci (*ial*) region and invasion plasmid antigen H (*ipa H*) genes of *Shigella* and enteroinvasive *Escherichia coli* (EIEC) in lettuce samples was developed. These target

sequences are related to the cell invasion process in both pathogens (6,22-25). The *ial* region is encoded in the invasion plasmid while the *ipaH* genes are present in both the bacterial chromosome and the invasion plasmid (9,15,25). This study compared a Multiplex PCR technique to conventional culture by using lettuce samples spiked with serial dilutions of the control strain of *Shigella flexneri* and the diagnostic accuracy was calculated.

MATERIALS AND METHODS

Bacteria strains

Bacterial strains selected to test the diagnostic specificity of PCR included: *Aeromonas hydrophila*, *Citrobacter freundii*, *Citrobacter youngae*, *Edwardisella tarda*, *Enterobacter cloacae*, *Escherichia coli* ATCC 64111, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 13706, *Escherichia coli* O155, *Enterococcus faecalis* ATCC 10741, *Klebsiella onithynolitica*, *Plesiomonas shigelloides*, *Pseudomonas aeruginosae* ATCC 14502, *Pseudomonas fluorescens*, *Providencia* spp., *Proteus* spp, *Salmonella* serovar Enteritidis ATCC 13076, *Salmonella* serovar Typhimurium ATCC14028, *Salmonella* serovar Indiana, *Salmonella* serovar Typhi, *Staphylococcus aureus* ATCC 6538, *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, *Shigella dysenteriae* and *Yersinia enterocolytica*.

DNA Extraction

DNA from the four species of *Shigella* and non *Shigella* strains was obtained by the phenol-chloroform extraction method: 1.5 ml of an overnight broth culture (Trypticase Soy Broth, and GN broth) at 35°C were centrifuged at 2700 g for 5 min (microcentrifuge 5417C Eppendorf Hamburg, Germany). The pellet was resuspended in 567 µl of TE Buffer, 30 µl of 10% SDS and 3 µl of Proteinase K 20 mg/ml (Promega). The mixture was incubated at 37°C for 1 h. After incubation 100 µl of 5M NaCl was added and mixed thoroughly. After incubation, 80 µl of CTAB/NaCl was added, mixed and incubated for 10 min at 65°C. Finally, DNA was collected by centrifugation after phenol-chloroform precipitation, washed

with 70% ethanol and dried with air.

Primers and Internal amplification control (IAC)

The sequences of *ipaH* 600 bp product primer pair (fw 5'-GCCGGTCAGCCACCCT CTGAGACTAC-3' and rv 5'-GTTCCCTTGACCGCCTTTCCGTACCGT C-3') (15), and *ial* 320 bp product primer pair (fw 5'-CTGGATGGTATGGTG AGG-3' and rv 5'-GG AGGCCAACAATTATTTCC-3') (1), were prepared by Innovagen (Lund, Sweden). An internal amplification control (IAC) of 100 bp using *ipaH* primers sequence was designed and purchased from Invitrogen (USA): 5' – GTTCCTTGACCGCCTTTCCGTACCGTCTCT GCACGCAATACCTCCGGACAGAAGTATGAGATGCTG GAGAATGAGTACTCTCAGAGGGTGGCTGACCGGC-3'. The amplification of IAC was performed with ten-fold dilutions of IAC (from 1 µg/ml to 0.01 ng/µl) with two defined amounts of target (*Shigella*) DNA at 50 ng/µl and 100 ng/µl.

PCR protocol

Comparable PCR protocols were standardized using extracted DNA from both *Shigella* (*S.dysenteriae*, *S.flexneri*, *S.boydii* and *S.sonnei*) culture and lettuce samples spiked with bacterial dilutions. A 25 µl reaction volume was used, containing 1X Buffer, 2.0 mM MgCl, 0.5 U Taq polymerase (Promega), 5% DMSO (Sigma Aldrich) 0.2 mM each dNTPs (Applied Biosystems), 0.004 mM *ipaH* primers, 0.016 mM *ial* primers and 10 µl of DNA template. The reaction mixture was placed in a thermocycler (Verity, Applied Biosystems) for 30 cycles, 10 cycles in PCR touch down (each cycle consisting of 1 min at 94°C, 30 s at 65°C decreasing to 55°C, and 1 min at 72°C) and last 20 cycles in normal PCR (1 min at 94°C, 30 s at 55°C and 1 min at 72°C). An additional step at 5 min at 72°C for primer extension was added at the end of the reaction.

The PCR products were detected by 2% agarose gel electrophoresis (140V, at 45 min) in 1X TBE Buffer at pH 8.3 and stained with 0.5 µg/ml ethidium bromide. The products were visualized as 600 bp (*ipaH*), 380 bp (*ial*) and 100bp (IAC) bands using an UVITEC transilluminator (Cambridge, UK).

DNA sequences of the *ipaH* and *ial* products were determined (310 ABI Genetic Analyzer, Applied Biosystems) to confirm amplification of the correct gene.

Inoculation of lettuce samples

Portions of lettuce (*Lactuca sativa* Var. Capitata) were aseptically added to sterile bags and spiked with serial dilutions of *S. flexneri*. Only spiked lettuce samples with *Shigella* were used.

One ml of each serial dilution of *S. flexneri* in sterile peptone buffer (10⁷ CFU/ml to 10 CFU/ml) was inoculated into three portions of lettuce, two of 10 g and one of 25 g. The inoculation experiment was performed to complete 10 repetitions at the detection levels for Enriched-PCR and culture (5). Bacterial dilutions were prepared as follows: A *S.flexneri* strain was inoculated onto Trypticase soy agar (TSA) and incubated overnight at 35°C. After incubation, single colonies from TSA were inoculated into 10 ml of sterile peptone buffer to a concentration of 10⁸ CFU/ml, as determined by the Pour Plate technique (10).

Conventional Culture Samples: Lettuces (25 g) were homogenized for 2 min and 225 ml of GN broth were added. After 1 hour pH was measured and samples were incubated at 35°C for 24 hrs. After incubation they were streaked onto T7, XLD and DC agars (14).

Direct-PCR Samples: Lettuces (10 g) were rinsed with 4 ml of peptone buffer. After 1 min homogenization, 3 ml buffer were recovered with a sterile Pasteur pipette into a polyethylene sterile tube for further centrifugation and DNA extraction for detection of *Shigella* as previously described.

Enriched-PCR Samples: Lettuces (10 g) were rinsed with 4 ml of peptone buffer. After 1 min homogenization, 3 ml of buffer were recovered with a sterile Pasteur pipette into a stomacher sterile bag (Labplas, Canada) with 45 ml of GN broth. These samples were incubated at 35°C for 24 h. After incubation, 1.5 ml portion of enrichment broth was used to DNA extraction for detection of *Shigella* as previously described.

Lettuce inoculation experiment (culture, direct-PCR and enriched-PCR) was performed by triplicate in each dilution (10 to 10⁷ CFU/ml) and with 10 repetitions for dilutions 10³, 10⁴ and 10⁵ CFU/ml (Enriched-PCR) and 10⁵, 10⁶ and 10⁷ CFU/ml (culture). DNA extracted was used to detect *ipaH* (600 bp) and *ial* (320 bp) products by PCR.

PCR and culture accuracy

Diagnostic accuracy of both methods was established according to definitions and formulas as follows:

- Diagnostic specificity is defined as a measure of the degree to which the method is affected by non-target microorganisms present in a food matrix, which may result in false positive responses (17, 18).
- Diagnostic sensitivity is defined as a measure of the

degree to detect the target pathogen in the food matrix, which may result in false negative responses (17, 18).

RESULTS

Internal amplification control (IAC)

The IAC was co-amplified with the target DNA, resulting in a 100 bp amplicon (Figure 1). The lowest IAC concentration which showed a PCR band, visible by gel electrophoresis and ethidium bromide stain, was 0.1 ng/μl per 25 μl reaction. IAC was used in all PCR analysis to detect false negative results because of failure of the amplification reaction. This internal control is highly recommended when PCR-based methods are used to detect bacterial pathogens (5,11,17,18).

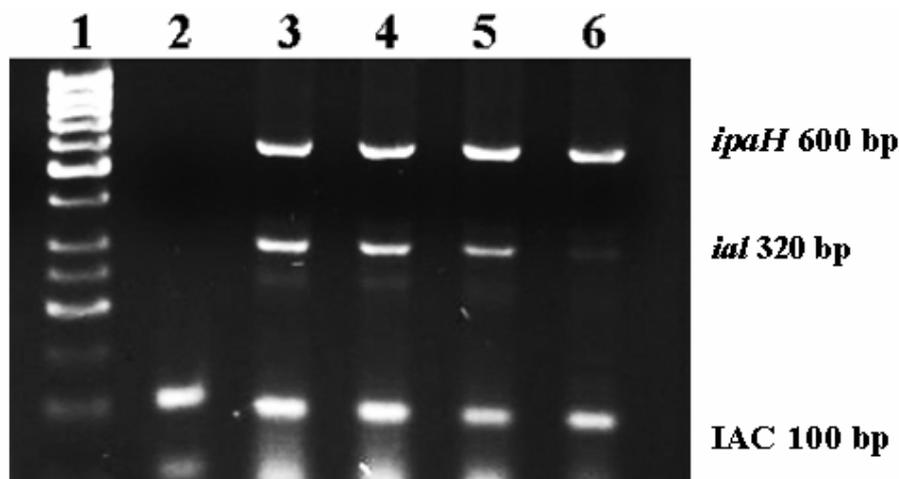


Figure 1. Multiplex PCR standardization results: 2% agarose gel electrophoresis stained with ethidium bromide. Lane 1: molecular size marker; lane 2: negative control, lane 3 and 4: *Shigella flexneri* strain 50(*ipaH*+/*ial*+); lanes 5 and 6: *Shigella flexneri* strain 01 (*ipaH*+/*ial*+).

PCR standardization

The products from DNA amplification by Multiplex PCR are shown in Figure 1. In the amplification reaction, bands of 100 bp (IAC), 320 bp (*ial*) and 600 bp (*ipaH*) were observed with *S.flexneri* DNA. The *ipaH* and *ial* products were not observed with the other bacterial strains (data not shown).

Detection level

Table 1 shows the results of the number of positive repetitions/total of repetitions for each bacterial dilution (10 to 10⁷ CFU/ml), from enrichment broth (Enriched-PCR), direct rinse (Direct-PCR) and conventional culture. The detection level of *S. flexneri* in lettuce by PCR was better than culture

(10^4 to 10^7 CFU/sample by PCR and 10^6 to 10^7 CFU/sample by culture). It was detected an inhibitory effect in the PCR-Direct samples since IAC was negative for all dilutions with 3 repetitions (Table 1, Figure 3), probably because of the matrix used. For this reason, only 3 repetitions were tested in the PCR-Direct experiment and these results were excluded to calculate PCR diagnostic accuracy.

PCR diagnostic accuracy

According to Table 1, diagnostic sensitivity and specificity

of Multiplex PCR and conventional culture were calculated using the number of repetitions from all dilutions that showed at least one positive result: for Multiplex PCR 10^4 CFU/ml to 10^7 CFU/ml and for conventional 10^5 CFU/ml to 10^7 CFU/ml. Multiplex PCR showed diagnostic specificity and sensitivity of 100% to detect *Shigella flexneri*. Conventional culture showed a diagnostic specificity of 100%, but sensitivity of 53% from a detection limit of *S. flexneri* 10^5 CFU/ml.

Table 1. Detection level of the PCR assay (Direct and Enriched) as compared to the conventional culture, using serial dilutions of *Shigella flexneri* spiked to lettuce samples.

Inoculum <i>Shigella</i> (CFU/sample)	Multiplex PCR positive results		Culture positive results
	Enriched-PCR samples	Direct-PCR samples	
10^7	3/3	0/3	10/10
10^6	3/3	0/3	5/10
10^5	10/10	0/3	1/10
10^4	10/10	0/3	0/3
10^3	0/10	0/3	0/3
10^2	0/3	0/3	0/3
10	0/3	0/3	0/3
Noninoculated	0/10	0/3	0/10

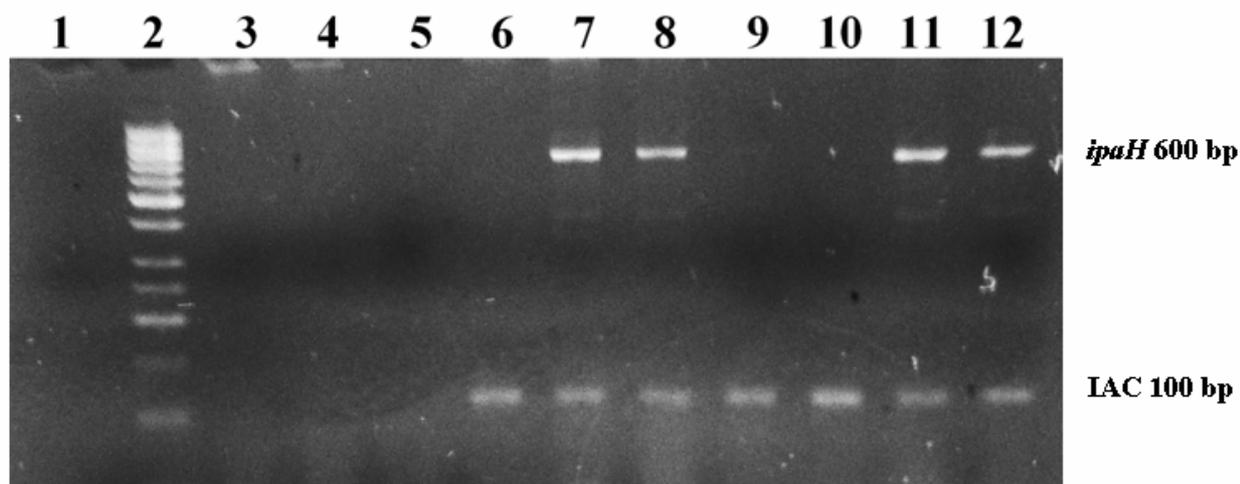


Figure 3. Multiplex PCR results from direct-PCR samples at different concentrations of *Shigella flexneri*. 2% agarose gel electrophoresis stained with ethidium bromide. Lane 1: empty; lane 2: molecular size marker; lane 3: 10^7 *Shigella* direct-PCR sample, lane 4: 10^6 *Shigella* direct-PCR sample; lane 5: 10^5 *Shigella* direct-PCR sample; lanes 6 and 9: negative control; lanes 7 and 11, 10^5 *Shigella* enriched sample; lane 8 and 12: 10^6 *Shigella* enriched sample; lane 10: *Escherichia coli*.

DISCUSSION

In this study, a Multiplex PCR assay for detection of virulence genes of *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) was standardized and compared to the reference culture method to detect *Shigella* spp. PCR detection of virulence genes in *Shigella*, such as *ipaH*, are considered a good and specific diagnostic tool of *Shigella* from clinical and food samples (9,15,23-25). Although *ipaH* and *ial* genes are also carried by EIEC, *Shigella* has been considered the most incident pathogen related to foodborne outbreaks in Costa Rica during 2005 and 2006 (12).

Theoretically, the detection limit of one bacterial cell per PCR reaction is equivalent of 10^3 - 10^4 cells per ml of preenriched sample, and our results showed a detection limit for the PCR method of 10^4 CFU/sample, comparable to this criterion (18). We also obtained superior results using an enrichment step prior to PCR analysis rather than the direct PCR analysis of samples. The use of enrichment provides advantages such as multiplication of the bacterial target levels

to obtain detectable concentrations, dilution of inhibitory substances present in food and dilution of dead target cells, which provides some assurance that the detected DNA belongs from viable target cells (16).

According to international standards, the use of an internal amplification control (IAC) is mandatory in any PCR-based method to detect foodborne pathogens (5, 11 17). The IAC band should always be present in both negative and positive samples since IAC specific primers are added to the sample tube and to be coamplified at same time with the target sequence (11). In this study, addition of the IAC allowed the differentiation of a true negative result from an inhibitory effect in Direct PCR samples, since no IAC band was obtained (Figure 3), conversely to Enriched samples, which showed the IAC band even in negative samples (Figure 2). These results confirm the importance of these controls as indicators of failure of the amplification reaction; therefore this aspect should be considered as part of a good diagnostic performance of any PCR detection method (5, 11, 17).

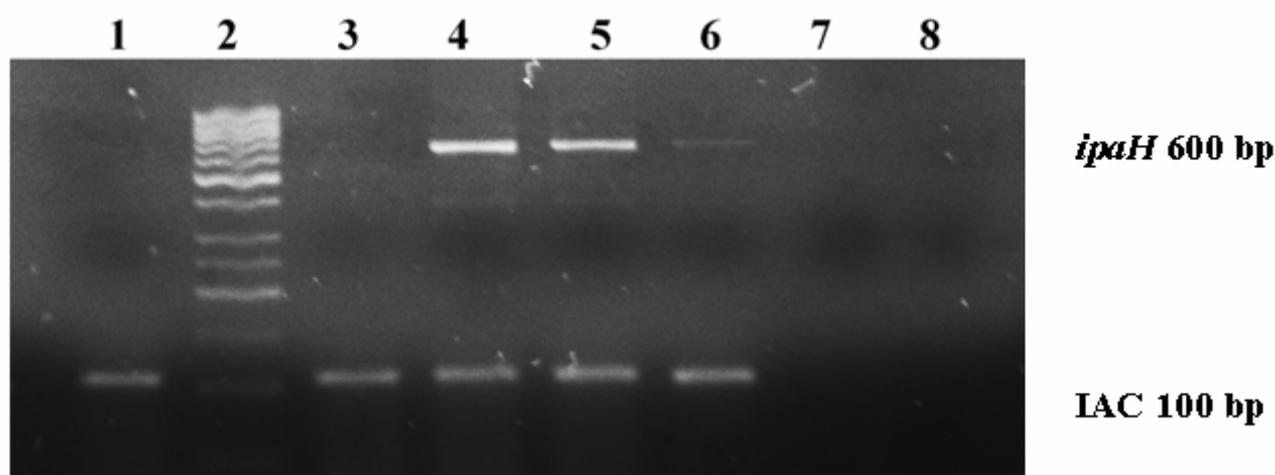


Figure 2. Multiplex PCR results from Enriched-PCR samples at different concentrations of *Shigella flexneri*. 2% agarose gel electrophoresis stained with ethidium bromide. Lane 1: negative control, lane 2: molecular size marker; lane 3: *Escherichia coli*; lane 4: *Shigella* 10^6 enriched-PCR sample; lane 5: *Shigella* 10^5 enriched-PCR sample; lane 6: *Shigella* 10^4 enriched-PCR sample; lanes 7 and 8: empty.

Some protocols have described a simplex PCR method specific to the *ial* region to detect *Shigella* from food. In this study the *ial* product was detected only from pure culture but not from enriched samples. The *ial* region is encoded into the *Shigella* invasion plasmid, and it has been described that avirulent variants (pINV-) often arise owing to the loss or deletion of large fragments of the pINV spontaneously during growth at *in-vitro* conditions (9). Schuch *et al.* have described the stability of invasive phenotype at 30°C but not at 37°C, presumably attributed to absence of high level activity and/or repression of virulence genes expression into pINV (22). The instability of the *Shigella* invasion plasmid may contribute also to false-negative result because this extrachromosomal element is prone to loss or deletions (9,20,25). Invasive phenotype *Shigella* colonies could also be directly detected from culture by addition of Congo red dye to the agar medium (9). Virulent strains absorb the dye from agar and thus appear pigmented or Congo red positive (Crb+), which allows identification by culture of potentially invasive colonies (9,22,25). In this study the growth temperature at 35°C could possibly affect the detection from enrichment samples by PCR of the *ial* target. The use of the Congo red dye will be included in future studies for identification of *Shigella* invasive phenotype *in vitro* when these virulence genes are the targets to be detected.

Both *Shigella* Multiplex PCR and conventional culture showed good analytical and diagnostic accuracy, which means that non target microorganisms tested were negative both by PCR and culture while all *Shigella* strains were positive by both methods. The molecular method showed a higher diagnostic sensitivity and a faster result as compared to the conventional culture. Even though conventional culture is considered the gold standard for *Shigella* detection, the PCR method is a useful tool which complements detection of foodborne pathogens such as *Shigella*.

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