Molecular and Cellular Probes 27 (2013) 53–59

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Real-time PCR using SYBR Green for the detection of *Shigella* spp. in food and stool samples

W. Mokhtari [a,b], S. Nsaibia [a,*], A. Gharbi [a], M. Aouni [b]

[a] *Laboratoire Régional de Santé Publique de Nabeul, 8, Rue Bab Ezzaouia, Nabeul 8000, Tunisia*
[b] *Laboratoire des Maladies Transmissibles et Substances Biologiquement Actives (LR99-ES27), Faculté de Pharmacie de Monastir, Tunisia*

ARTICLE INFO

Article history:
Received 2 August 2012
Received in revised form
22 September 2012
Accepted 25 September 2012
Available online 5 October 2012

Keywords:
Shigella detection
Stool
Food
ipaH
SYBR Green
Real-time PCR

ABSTRACT

Shigella spp are exquisitely fastidious Gram negative organisms that frequently get missed in the detection by traditional culture methods. For this reason, this work has adapted a classical PCR for detection of *Shigella* in food and stool specimens to real-time PCR using the SYBR Green format. This method follows a melting curve analysis to be more rapid and provide both qualitative and quantitative data about the targeted pathogen.

A total of 117 stool samples with diarrhea and 102 food samples were analyzed in Public Health Regional Laboratory of Nabeul by traditional culture methods and real-time PCR. To validate the real-time PCR assay, an experiment was conducted with both spiked and naturally contaminated stool samples. All *Shigella* strains tested were ipaH positive and all non-*Shigella* strains yielded no amplification products. The melting temperature ($T_m = 81.5 \pm 0.5$ °C) was consistently specific for the amplicon. Correlation coefficients of standard curves constructed using the quantification cycle (C_q) versus copy numbers of *Shigella* showed good linearity ($R^2 = 0.995$; slope = 2.952) and the minimum level of detection was 1.5×10^3 CFU/g feces. All food samples analyzed were negative for *Shigella* by standard culture methods, whereas ipaH was detected in 8.8% culture negative food products. Moreover, the ipaH specific PCR system increased the detection rate over that by culture alone from 1.7% to 11.1% among patients with diarrhea.

The data presented here shows that the SYBR Green I was suitable for use in the real-time PCR assay, which provided a specific, sensitive and efficient method for the detection and quantification of *Shigella* spp in food and stool samples.

1. Introduction

Illness caused by food contaminated with infectious or toxigenic microorganisms is a major and significant cause of suffering and death throughout the world [1,2]. *Shigella* spp continues to be a significant agent of foodborne illness especially with foods that require hand processing and/or are prepared from raw or previously cooked products without re-heating [3], which is usually confirmed by clinical isolates before the contaminated food is found. The low infectious dose (10 cells) [4], allows the disease to be spread effectively by infected food or water, and also by person-to person contact [5].

According to several reports in the literature, *Shigella* spp are exquisitely fastidious Gram negative organisms which frequently get missed in the detection by traditional culture methods [6,7]. Consequently, there is not much information about the prevalence of *Shigella* on food or among food handlers while *Shigella* can survive in many types of food [8] and has been classified as a potential agent for biological terrorism [9]. Several molecular microbiological methods have been developed to overcome some of the shortcomings of traditional culture methods. Most of the time, the assay is based on the detection of an invasion plasmid antigen, *ipaH*, a gene found in all *Shigella* species as well as in entero- invasive *Escherichia coli* (EIEC) [10,11]. Recently, real-time PCR has emerged as a rapid and reliable tool for the detection of microorganisms in different samples [12–14]. In contrast to conventional single and multiplex PCR which is based on DNA amplification with subsequent electrophoresis and visualization steps, the real-time PCR technology allows sensitive, high-throughput results with easy automation and does not require post-PCR detection procedures [15].

In 2007, our laboratory carried out research, for the first time in Tunisia, to detect *Shigella* prevalence in food elements as well as

* Corresponding author.
E-mail address: nsaibia.siwar@hotmail.com (S. Nsaibia).

0890-8508/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mcp.2012.09.002

using molecular typing techniques to investigate possible clonal relationships among strains isolated from naturally contaminated food samples and compare it with those isolated from human stools in order to evaluate the potential association between food product contaminated with *Shigella* and human infection [28]. In order to be more sensitive and to eliminate the need for gel electrophoresis we applied real-time PCR assay to detect *Shigella* spp from food products and fecal samples without the use of fluorescent probes. Protocols that have already used for conventional PCR can thus be used with only slight modifications [17]. Based on the combination of enrichment stage (ISO 21567:2004) and a real-time PCR technique this is the first report of our knowledge to detect *Shigella* spp from direct food product samples collected (various food matrices) not from artificially contamination like in some studies [11,12]. A number of fluorescent-probe-based real-time PCR studies have been carried out to detect *Shigella* in stool samples and water using TaqMan probes [7,14,16]. However in the absence or instability of probes, the intercalating dye, SYBR® Green I, offers direct detection of double-stranded (ds) DNA during amplification. With a little optimization the much cheaper option of SYBR Green can be used as an effective alternative tool of laboratory diagnosis to obtain both qualitative (for food samples) and quantitative (for stool specimens) measurement of the targeted pathogen. The specificity of the reaction is determined by melting temperature (*Tm*) of the amplicon obtained, defined as the temperature at which 50% of the DNA amplicon is in a double-stranded configuration [18]. A number of SYBR Green real-time PCR assays for detection of microbial pathogens such as *E. coli* O157:H7 [19], *Campylobacter* [20], *Vibrio* [21], *Salmonella* [22] and *Clostridium botulinum* [23] have been reported.

The objectives of the present study are to establish a simple and robust real-time PCR method using SYBR Green for simultaneous detection of *Shigella* spp. in naturally contaminated food and quantification in stool samples, and compare it with traditional microbiological methods.

2. Materials and methods

2.1. Bacterial strains

The specificity of the primer used in this study has also been well demonstrated by other researchers using fluorogenic probes real-time PCR assays [6,7,16]. For this reason, bacterial strains numbers used in this work was not highly compared to others studies. *Shigella flexneri (Sh.)* ATCC 12022 and *Shigella sonnei* ATCC 25931 were used as the reference strains. Various other bacterial strains were used to test the specificity of the PCR are listed below: one strain of *E. coli* ATCC 10536, *Salmonella enterica* ATCC 13076, *Salmonella thyphi* ATCC 10749, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 19434, *Listeria monocytogenes* ATCC 15313, *Listeria ivanovii* ATCC 19119, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 27799, *Enterobacter cloaceae* ATCC 13047, *Vibrio parahaemolyticus* ATCC 17802.

2.2. Sample collection and culture conditions

From May to October 2010, a total of 102 food samples were collected and transported in an icebox by the department of hygiene of Nabeul (northeast of Tunisia). These samples represented the following products: Raw salads (67 samples) and poultry (35 samples). All samples were tested within 24 h of collection according to international norms for *Shigella* ISO 21567:2004 [24] and food samples that were refrigerated have been rejected. After incubation, enrichment cultures from *Shigella* broth (HiMedia Laboratories, USA) were subdivided into two aliquots: one aliquot was analyzed by the standard culture method ISO 21567, and the other aliquot was subjected to DNA extraction for SYBR Green I real-time PCR and melting curve analysis.

In addition, all 102 food samples were subcultured onto Tryptone Bile X-glucuronide agar plates (Pronadisa, Laboratoires Conda, Madrid, Spain) for the enumeration of *E. coli* according to the procedure described in ISO16649-2: 2001 [25].

Furthermore, a total of 117 stool specimens were collected from Adult Gastroenterology Unit-Nabeul Regional Hospital and examined for the detection of *Shigella* spp. Patients (>18 years of age) would enrolled in the study if they had acute diarrhea characterized by watery stools with or without blood. They were divided into two groups: 75 males and 42 females. Most patients had watery diarrhea (72.6%); only 27.4% (32 of 117) had bloody diarrhea. Two samples were obtained from each patient. One sample was suspended into phosphate-buffered saline (PBS) [16] for real-time PCR and the other was performed according to the local standard operating procedures using *Salmonella-Shigella* agar (Pronadisa, Laboratoires Conda, Madrid, Spain) and incubated at 37 °C for 18–24 h in an aerobic environment. Transparent colonies (3–5 colonies from each culture plate) were screened by using triple sugar iron agar and motility indole–urea medium. The presumptive *Shigella* spp isolates were identified by using API 20E system (bioMérieux, Marcy-l'Etoile, France).

2.3. Sensitivity study

This study was based on a modification described by [26]. Feces were suspended in 10 times volumes (v/w) of PBS. An overnight culture of *S. flexneri* ATCC 12022 were adjusted at 0.5 Mac Farland (1.5×10^8 CFU/ml) and diluted 10-fold to determine the lower detection limit of the real-time PCR assay. The precise number of CFU in the dilutions was obtained by the plate count method. 100 μl of each dilution was plated onto SS agar plates (Pronadisa, Laboratoires Conda, Madrid, Spain) and incubated overnight at 37 °C. Cultures containing less than 300 CFU/ml were counted (10^{-5} dilution yielded 1.68×10^3 CFU/ml).

Then, Standard curves were prepared by mixing the fecal suspensions to the appropriately diluted suspension of *S. flexneri*. The DNA was extracted using the method described below.

2.4. DNA isolation

For PCR, 0.5 ml of the bacterial suspensions or from broth cultures was centrifuged for 5 min at 10,000 × g, resuspended in 250 μl of Aquadien™ lysis solution (Bio-Rad) and heated at 95 °C for 15 min. After centrifugation, the supernatant was directly used for amplification or stored at −20 °C until analyzed.

2.5. Conventional PCR

Primers used were previously published by [6,16]. The forward primer *ipaH*-U1 [5′-CCT TTT CCG CGT TCC TTG A-3′] and reverse primer *ipaH*-L1 [5′-CGG AAT CCG GAG GTA TTG C-3′] were designed on the basis of *ipaH* gene sequence (Genbank accession No. M32063). The 25 μl reaction mixture contained: 1 × PCR Green buffer (Promega, USA), 2 mM $MgCl_2$ (Promega, USA), 100 μM of dNTP mix (Promega, USA), 1 μM of *ipaH*-U1 and L1 primers, 1U of Ampli Taq Gold™ (Applied Biosystems) and 2 μl of DNA template. Cycling conditions were as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C, 5 s; 60 °C, 60 s and 72 °C, 60 s. A final extension at 72 °C for 7 min concluded all amplification reactions.