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RESEARCH PAPER

Detection of *Shigella dysenteriae* Type 1 in Milk by PCR

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ABSTRACT

Shigellosis is the severest form of bacillary dysentery, a disease limited to humans and certain other primates. Conventional microbiological techniques used for the isolation and biochemical identification of microbes are time-consuming as well as labour intensive. Due to the fastidious nature of *Shigella* and the lengthy culture time, a rapid and sensitive system for the detection of bacteria is highly desirable. Nucleic acid-based techniques have enormous potential in the detection of biological weapons because of their specificity, sensitivity, and the speed with which results can be obtained. The application of polymerase chain reaction in sensitive detection of bacterial pathogens in direct food samples is largely affected by the quality of the template DNA. To overcome the shortcomings of conventional methods, simple method of sample preparation that facilitates PCR based detection of *Shigella dysenteriae* in milk is defined. In present study, gene specific primers were designed to detect *stxA* gene. The genomic DNA extraction from the spiked milk was carried out using proteinase K, lysozyme, chloroform-isoamyl alcohol and heat treatments. For detection extraction of DNA by addition of chloroform-isoamyl alcohol proved to be the most satisfactory amongst all, with limit of detection of 2.0 x 10^3 cfu/reaction and was completed in 4 h.

Keywords: *Shigella dysenteriae*; Shiga toxin; Detection; Spiked milk; PCR

1. INTRODUCTION

A Japanese bacteriologist, Kiyoshi Shiga discovered *Shigella dysenteriae,* in 1896. An epidemic of acute dysentery occurred in Japan and Kiyoshi Shiga isolated bacterial culture to investigate it. *Shigella* is a significant cause of food-borne disease, though classically it is known as a water-borne pathogen[6]. The main factors which have led to outbreaks of food-borne shigellosis are poor personal hygiene of food handlers as well as improper holding temperature of contaminated foods[17]. Hence, the foods that require hand processing or are prepared from raw or previously cooked products without re-heating are those involved in the outbreaks. Enteric pathogens are also classic potential agents of bioterrorism. In an incident, a medical technologist obtained *S. dysenteriae* from a laboratory and used it as an agent to sicken coworkers[8].

Genome of *S. dysenteriae* comprises of single circular chromosome with 4,369,232 base pairs[19]. The genes that code for the production of Shiga toxin are coded by the invasion-associated plasmid. Shiga toxin is fundamental to the pathogenicity of *S. dysenteriae* type (i). It is a AB_5 type of toxin with 70 kDa protein, comprising an enzymatic active A chain (32 kDa) which is responsible for the inhibition of protein synthesis after translocation into the cytosol and a pentamer of receptor binding B chain (7.7 kDa) that binds to globotriosylceramide (Gb3) receptor at the cell surface and plays role in endocytosis of the toxin[9]. The toxin inhibits protein synthesis by cleaving a specific adenine residue from the 28S ribosomal RNA in the 60S ribosome and causes cell death[12].

In conventional methods bacteria can be detected by culturing on selective media which is labour intensive and time consuming[3-4,18]. Reliable results may be obtained by some of the methods in single step, but mostly additional steps are essential for confirmation of the results[7,14]. PCR is one of the approaches to overcome the difficulties of the standard methods used in actual food borne pathogen detection[11]. Direct identification of the pathogens is possible by *in vitro* amplification of specific DNA sequences by PCR. It was observed that PCR was inhibited by the inhibitors present in food matrices, such as fats, proteins, metal ions, and also the chemicals required in the media for selective enrichment[15]. Milk is well known as complex combination of fats, proteins and other inhibitory components therefore, efficient extraction of the DNA is a crucial step and needs to be attentively dealt with for a successful amplification. About four-fifths of milk protein consists of casein which can bind cations, primarily calcium and magnesium[13]. As a food matrix, milk contains high concentrations of several inhibitors of PCR. DNA extraction from milk is interfered not only by the presence of fat and suspension particles, but also by proteinase and calcium ions[2]. When DNA extraction procedures for pure cultures were applied to spiked milk PCR detection limits dropped dramatically. In the present study the objective of the work was to optimise PCR based assay to detect *S. dysenteriae* type 1 in milk samples by using simple method for sample preparation.

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2. MATERIALS AND METHODS

2.1 Isolation of Genomic DNA and Optimisation of PCR Conditions

Genomic DNA was isolated from *S. dysenteriae* type 1 obtained from NICED (National Institute of Cholera and Enteric Disease, Kolkata) by using kit as per manufacturer's protocol (Qiagen, USA). Gradient PCR was used to optimise the annealing temperature. The sequence of the primers used is shown as follows:

Name of primer	Sequence (5' -3')	Melting temp (°C)
Forward *Stx af*	GTGGTTGCG AAG GAA TTT ACC	57.9
Reverse *Stx ar*	AGTTCTGCG CAT CAG AAT TGC	57.9

Amplification reaction (50 µl) contained DNA template (50 ng), PCR Buffer, 2.5 mM of $MgCl_2$, 200 µM of each dNTPs, 2U Taq polymerase, 0.5 µM of each primers and nuclease free water to adjust the total volume. The amplification profile comprised of initial denaturation at 95 °C for 10 min and 30 cycles of denaturation at 94 °C for 1 min. The annealing of primers from 54.4 °C to 59.8 °C for 1 min and primer extension step at 72 °C for 1 min. The final extension was carried out at 72 °C for 10 min. Agarose gel electrophoresis was performed to verify the size of PCR product (5µl)[16]. After completion of the electrophoresis, the DNA bands were visualised on gel documentation system.

2.2 Detection of *Shigella dysenteriae* in Spiked PBS

Gram staining was performed initially to study the colony morphology. In order to optimise the conditions for the detection of *S. dysenteriae* in spiked milk, initially PBS was used. For this, PBS was spiked with the standard culture of *S. dysenteriae* and the conditions were optimised for PCR. Standard culture of *S. dysenteriae* was inoculated in 5 ml of nutrient broth and incubated overnight at 37 °C at 200 rpm. Ten fold serial dilutions (1 ml each) of PBS spiked with overnight grown culture were prepared. Hundred µl of dilutions (10^{-6} to 10^{-8}) was spread on nutrient agar plate and kept in incubator at 37 °C for 24 h to obtain the bacterial cell counts.

The following three methods were used to obtain the template for PCR:

(i) 100 µl was taken from each dilution tube (10^{-3} to 10^{-8}) and centrifugation was done at 15,000 g for 5 min. Supernatant was decanted and 20 µl of PCR master mix was dispensed in each tube containing the pellet.

(ii) One ml sample from dilution tubes (10^{-3} to 10^{-8}) was centrifuged, supernatant was decanted and the pellets were resuspended in 10 µl of deionised water. Subsequently, this suspension (1 µl) was used in PCR as a template and the remaining 9 µl was heated at 100 °C for 10 min, of which 1 µl was used as template.

(iii) From the dilutions of the overnight grown cultures, pellets were resuspended in 200 µl TE buffer and kept for heating (10 min at 100 °C), followed by chilling on ice for 5 min. The cell pellet was removed by centrifuging at 15,000 g for 5 min. Finally, 1 µl of supernatant was used as template for PCR.

2.3 Detection of *Shigella dysenteriae* in Spiked Milk

Optimisation of detection of *S. dysenteriae* type 1 in spiked milk was carried out using different DNA extraction methods. Ten fold serial dilutions from 10^{-1} to 10^{-8} (2 ml each) of milk spiked with overnight grown culture were prepared. To obtain the bacterial counts 100 µl of dilutions (10^{-6} to 10^{-8}) spread on NA plate and was kept in incubator at 37 °C for 24 h. All the dilutions (10^{-1} to 10^{-8}) were incubated at 37 °C for 1 after spiking and cells were harvested at 15,000 x g for 10 min from 1 ml of each dilution. The resulting cell pellets were resuspended in 1 ml sterile water. The bacterial cells were lysed by heating at 100 °C for 10 min and cell pellet was removed after centrifugation of the suspension. Finally, 1µl volume of the supernatant was used as template while performing PCR.

In another method, chloroform-isoamyl alcohol was used during DNA extraction from spiked milk. Serial dilutions were prepared and colonies were counted as mentioned earlier. The dilutions were then incubated at 37 °C for 1h. From the dilutions (10^{-1} -10^{4}), 1 ml was centrifuged and the pellets were resuspended in 1 ml sterile water. The remaining 1 ml of the same dilutions was used without centrifugation. In both the cases, cells were lysed and the cell debris was removed. Chloroform-isoamyl alcohol (24:1) was added in the ratio of 1:1 and mixed by inverting the tubes several times. Centrifugation was carried out for 10 min at 3,700 x g, 4 °C and 1 µl of aqueous phase was used as a template for PCR. Genomic DNA isolated from *S. dysenteriae* and non-spiked milk was used as positive and negative controls respectively.

In another method, milk was autoclaved and the sample preparation was carried out as before, with an additional step of chloroform-isoamyl alcohol extraction. One microliter of the aqueous phase was used as a template for PCR.

3. RESULTS

Two specific primers flanking a 879 bp region of the *S. dysenteriae* gene of A chain (Acc. No. AM230663) were designed. These primers amplified the DNA isolated from different clinical samples of *S. dysenteriae* as well as of Shiga like toxin producing *E. coli*. The best amplification was obtained at 59.1 °C (data not shown). Besides this, The morphology of bacterial cells was confirmed by Gram staining with the presence of Gram negative rods. Initially, the optimisation procedure was carried out in PBS. In all the experiments, overnight grown broth culture was used to spike PBS and milk. Ten-fold serial dilutions of this culture were prepared by spiking in PBS and milk. Spread plating of higher dilutions was done on nutrient agar plate. Detection sensitivity of PCR was determined based on the average count of dilution, of which amplicon is visible on agarose gel and expressed as colony forming unit (cfu) / reaction.

When the cell pellet without any chemical or heat treatment was used as template, the detection sensitivity of PCR was found to be $1.2x10^2$ cfu / reaction data not shown. In another treatment when pellets were resuspended in deionised water followed by heating, the detection sensitivity was found to be $1.4x10^2$ cfu/reaction. Similar results were

obtained when samples were devoid of heating is as shown in Fig. 1. The detection was $1.3x10^3$ cfu / reaction by using TE buffer in the sample preparation which is as shown in Fig. 2. Amongst the different methods used in sample preparations, pellets used directly as the template proved to be the most sensitive for detection.

Optimisation of detection of *S. dysenteriae* type 1 in spiked milk was carried out using different treatments during sample preparation. Initially lysis buffer containing 1 mg/ml lysozyme and 650 µg Proteinase K in TE buffer (pH 8) were used to extract DNA from spiked milk but amplification could not be achieved (data not shown). Chloroform-isoamyl alcohol was used, in addition to this boiling was done to lyse the bacterial cells and amplification could be achieved. Figure 3 shows that the detection limit was extremely low (1.4×10^9 cfu/reaction) when samples were prepared by heating to lyse the cells. During DNA extraction when chloroform-isoamyl alcohol was used along with centrifugation the detection sensitivity was $3.4x10^5$ cfu / reaction, while it was 3.4×10^4 cfu / reaction without centrifugation, as shown in Fig. 4. The detection sensitivity of PCR was enhanced to 2.0×10^3cfu / reaction when chloroform-isoamyl alcohol was used twice which is as shown in Fig. 5.

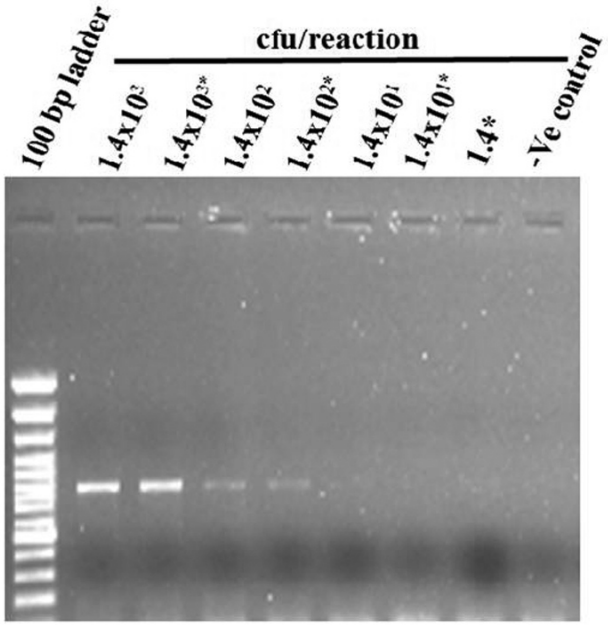

Figure 1. PCR of spiked PBS-DNA extraction by heating *and without heating.

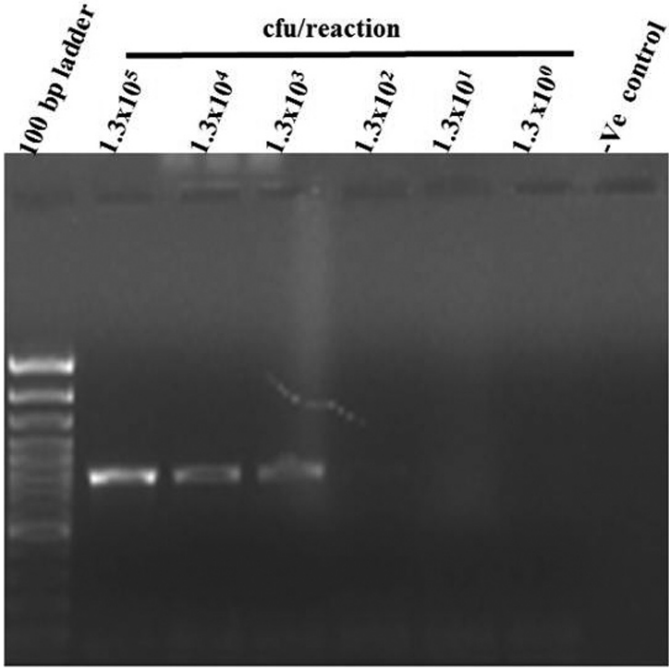

Figure 2. PCR of spiked PBS-DNA extraction-by using TE buffer.

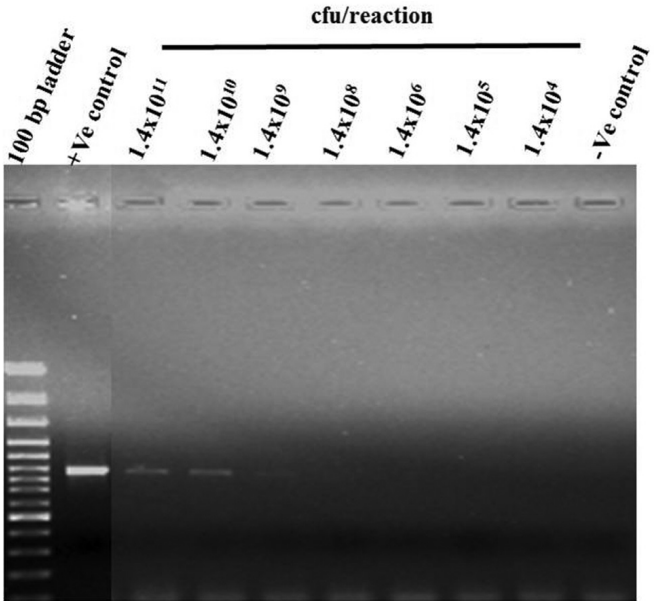

Figure 3. Optimisation of PCR in spiked milk-DNA extraction by boiling.

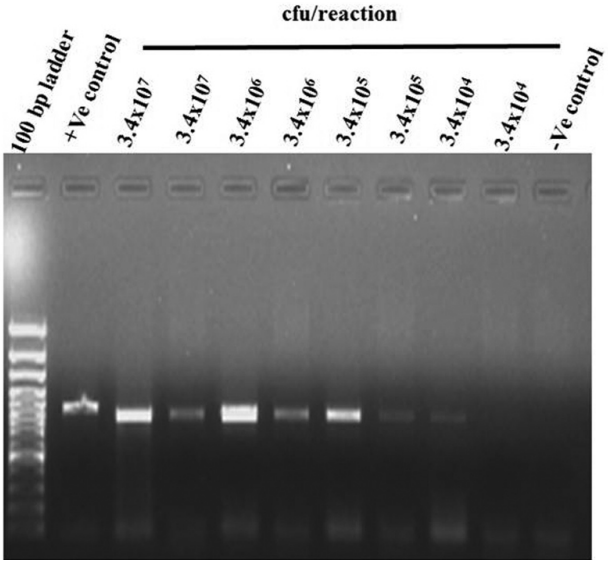

Figure 4. Optimisation of PCR in spiked milk - DNA extraction by chloroform-isoamylalchohal

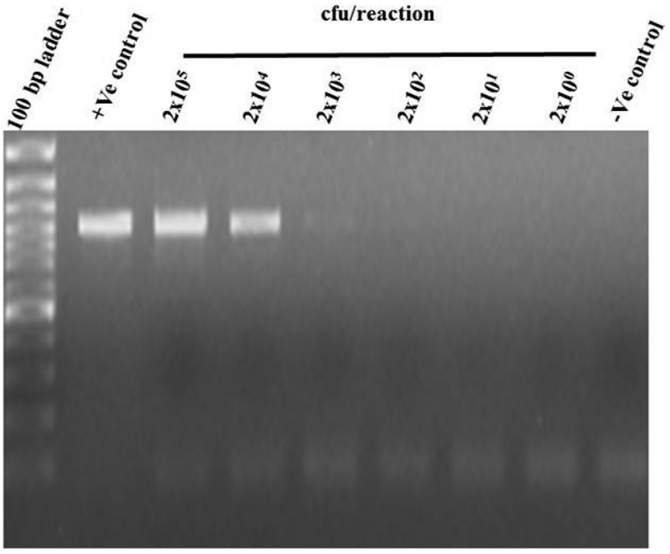

Figure 5. DNA extraction by repeating additions of chloroform-isoamylalcohal in spiked milk.

4. DISCUSSION

Detection of bacterial pathogens in food using microbiological methods as well as the biochemical identification techniques are labour intensive and consume lot of time. *Shigella* are fastidious in nature and the lengthy culture time makes it more difficult. To overcome the shortcomings of the conventional methods for detection of pathogens, we have chosen PCR based detection of *S. dysenteriae* in milk. A research group has described combination of enrichment, buoyant density centrifugation and nested PCR that could be accomplished in a time period of less than two working days[10]. *S flexneri* was recovered from milk, inoculated with 10 cfu g^{-1} by this treatment. It has been reported that *S. dysenteriae was* detected in feces by immune-magnetic isolation and PCR in about 7 h[5]. As stated by a group, the presence of inhibitors is one of the potential problems of PCR and may cause false results also[1]. Sensitivity of PCR for detection of pathogens in food products depends strongly on the procedure of DNA extraction. The method followed in this piece of research work is not only rapid and sensitive, but also avoids interference from potential PCR inhibitors. It provides the potential of detecting *S. dysenteriae* in milk without any enrichment techniques. This simple method of sample preparation can also be exploited to detect other food borne pathogens from complex matrices.

5. CONCLUSIONS

Shiga toxin is an important bio-warfare agent and our study gives emphasis on detection of the toxin gene. Detection of the agent is always a challenging task in sample matrices. Our work gives significant output regarding detection of Shigella dysenteriae from complex milk matrix. Different protocols were used in the sample preparation so as to optimise the PCR conditions in spiked milk. The molecular detection of Shiga toxin which we attempted is based on PCR and uses simple methods of sample preparation which can also be utilised for such other toxins or biological agent detection.

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Contribution in the current study, he designed the experiments and checked the manuscript.