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Improvement of the Detection of *E. coli* O157:H7 in Ground Beef as A Result of the Optimization of PCR Assay and the Use of Pellet Paint

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Abstract: *Escherichia coli* 0157:H7 is now recognized as an important human enteropathogen. PCR technology has proven to be very efficient for the detection of *E. coli* 0157:H7 in incriminated samples. Development of an extraction procedure resulting in 57% ± 6 recovery of *E. coli* 0157:H7 from seeded ground beef using 0.01 M phosphate buffered saline pH 6.0 with the aid of differential centrifugation. The optimized PCR reaction mixture consisted of 5 µl of 10X PCR mix (final concentrations: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 1 mM $MgCl_2$, , 1.6 µM each of the two primers, 0.2 mM of dNTPs and *Taq* polymerase (2.5 units for SLT-1 and 5 units for SLT-2) in a final volume of 50 µl. Prior to optimization, the minimum limit of detection for SLT-1 DNA sequence was 200 DNA targets compared to 100 after complete optimization. With SLT-2 DNA sequence, the minimum level of detection prior to optimization was 150 DNA targets compared to 20 after complete optimization. Optimization of the PCR allowed the detection of 10 SLT-1 and 5 SLT-2 targets per one gram of ground beef with the aid of pellet paint and pre-enrichment at 37°C for 5.5 hours.

Key words:PCR · Polymerase chain reaction · $MgCl_2$ · dNTPs · *Taq* polymerase · SLT-1 · SLT-2 · TZ lysis solution · *Escherichia coli* O157:H7 · Ground beef

INTRODUCTION

For the past two decades verotoxin-producing *Escherichia coli* (VTE) have been recognized as important pathogens of the gastrointestinal tract [1]. The most frequently identified VTEC serotype, *E. coli* 0157:H7, is strongly associated with hemolytic-uremic syndrome (HUS), a leading cause of acute renal failure in childhood [2]. Molecular methods, particularly PCR, have proven useful as screening tests for *E. coli* O157:H7 and for confirmatory identification of putative isolates. Meat samples are among the most complex specimens for PCR testing due to the presence of natural inherent PCR inhibitors that are often coextracted along with bacterial DNA [3]. Samples often contain low numbers of VTEC, with high levels of background flora which can interfere with isolation and subsequent detection of the pathogen [4, 5, 6]. In the case of raw ground beef, the target organism (*E. coli* O157:H7) is often present at very low levels (<100 CFU/g), while the level of other organisms may be as high as 104 to 106 CFU/g [7].

PCR is a very sensitive procedure and requires more precautions and attention to cycling parameters, PCR reaction components to avoid non-specific amplification [8, 9]. Consequently, each new PCR application is likely to require optimization [10]. Alteration of PCR components such as PCR buffer constituents, dNTPs and enzyme concentrations in multiplex PCR was found to improve the sensitivity and the specificity of the assay. Elfath *et al*. [11] and Kim *et al*. [12], found that collagen, a major component of several foods, inhibited the PCR. The inhibitory action of collagen on PCR could be partially reversed by adjusting the concentration of magnesium ion in the reaction mixture and by the use of various DNA extraction methods to remove the collagen from the DNA. It was also suggested that rapid detection of enterotoxigenic Clostridium perfringens by PCR requires an optimization of the PCR reaction mixture composition [12]. Unquestionably, no single protocol can be appropriate to all situations.

Two variables, which are reported to greatly influence the specificity of the PCR reaction, are Mg++ and dNTP concentration [13]. The magnesium concentration is a

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crucial factor affecting the performance of *Taq* DNA polymerase. Mg^{++} is used to stabilize *Taq* Polymerase and in the absence of adequate free magnesium, *Taq* DNA Polymerase is inactive [14]. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total of dNTP concentration [10]. Reaction components, including template DNA, chelating agents (e.g., EDTA or citrate), dNTPs and proteins all affect the amount of free magnesium in the PCR reaction mixture [14]. Mg^{++} ions form a soluble complex with dNTP's which is essential for dNTP incorporation as a necessary co-factor for *Taq* polymerase activity and increases the Tm (melting temperature) of primer/template interaction [8, 15, 16, 13]. The sequestration of Mg^{++} ions by various compounds and interference by Ca^{++} ions may inhibit amplification [17, 18]. Conversely, excess free magnesium reduces enzyme fidelity [19] and may increase the level of nonspecific amplification [20, 21].

VTEC are often present in an injured or stressed condition that are not recoverable and can result in false negatives [22, 23, 24]. A recovery step such as the use of pre-enrichment and/or enrichment media must therefore be built into the protocol to allow the resuscitation of injured or stressed bacteria [25-27]. The efficiency of PCR is limited by the purity of DNA lysates and the amplification conditions which includes the composition of PCR reaction mixtures and thermo cycling profile for PCR protocols. The objective of this study is to increase the sensitivity of PCR assay by optimizing the PCR reaction mixture ($MgCl_2$, dNTPs, *Taq* polymerase and primers concentration) with the aid of differential centrifugation for the extraction of target cells and the use of pellet paint for the isolation of DNA lysates.

MATERIALS AND METHODS

Microorganisms and Routine Cultivation: *E. coli* O157:H7 strain C9490 was obtained from The Centers for Disease Control, Atlanta GA 30333 and was routinely grown in 50 ml of Tryptic Soy Broth plus 0.5% glucose (Difco) at 37°C in 250 ml baffled flasks with rotary agitation (200 rpm). Exponentially growing cells (~1 x 10^9 cells/ml) were harvested by centrifuging broth cultures at 10,000g for 10 min. at 4°C. Pellets were resuspended in 30 ml of saline, pelleted again and the concentration of cells adjusted as required for lysing by resuspending in d-H_2O. Cell densities were determined with the use of a Petroff-Hausser bacterial counting chamber and by plate counts with Tryptic Soy Agar containing 0.5% glucose (Difco).

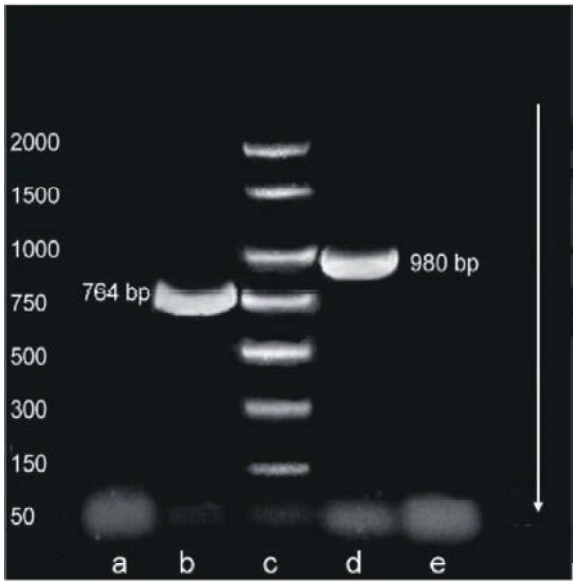

Fig. 1: Gel electrophoresis of amplified *E. coli* 0157:H7 verotoxins genes VT1 and VT2.
DNA sample (1 µl) containing 1 x 10^7 templates (derived from number of CFU) was added to the 50 µl of PCR mixture as described by Ramotar *et al*.[29]. DNA lysates were obtained from cell suspension of pure culture using TZ lysis buffer. Lane a: Negative control with SLT-1, Lane b: Positive control with SLT-1, Lane c: DNA ladder (3.4 µl) obtained from Sigma, Lane d: Positive control with SLT-2 and Lane e: Negative control with SLT-2.

Lysing of Cell Suspensions with the Aid of TZ Buffer: A cell suspension (0.1 ml) in d-H_2O containing a total of 2.0 x 10^7 cells, unless otherwise stated, was added to 0.1 ml of 2X TZ lysing solution (2.0% Triton X-100 in 0.1 M Tris-HCl buffer plus 2.5 mg/ml sodium azide, pH 8.0) was prepared and used as described by Abolmaaty *et al*. [28]. The suspension was heated in a water bath at 100°Cfor 10 min and then cooled to 5°C in ice. The lysates were centrifuged at 16,000 x g for 10 min at room temperature. Pellets were discarded after the very clear portion at the top of the supernatant was taken for the PCR procedure.

PCR Protocol and Optimization Studies: The primers selected to amplify the VT-1 gene segment were: 5'-TAAAACGCCGTCCTTC-3' and 5'TACTCAACCTTCCCCAGTT-3' and amplify a 764-bp fragment (nucleotides 256 to 1019) of the VT1 gene (Figure 1). The primers selected to amplify the VT-2 gene segment were: 5' -TCTTCGGTATCCTATTCCC-3' and

5'-GCCATTGCATTAACGAAAC-3' and amplify a 980-bp fragment (nucleotides 256 to 1235) of the VT-2 gene (Figure 1). The primers were synthesized by National Biosciences Inc. (3650 Annapolis Lane North, 140 Plymouth, MN) and were designed with the use of the Wisconsin Software Package version 8.1. The PCR protocol described by Ramotar *et al*., [29] was used as a control. Unless otherwise stated, the final standard PCR mixture consisted of 5 µl of 10x PCR mix: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 0.001 mM each of the two primers and 1 µl of DNA sample containing up to 1 x 10^4 templates and 2.5 units of *Taq* polymerase (Promega) in a final volume of 50µl. Optimization of the PCR reaction mixture was performed in a final volume of 50 µl of PCR reaction mixture but with varying concentrations of one of the following components while the others remained constant: 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mM of $MgCl_2$; 0.2, 0.4, 0.6, 0.8, 1.0 mM of dNTPs; 1.25, 2.5, 3.75, 5.0, , 6.25, 7.5 U of Taq DNA polymerase; and 0.5, 1.0, 1.2, 1.4, 1.6, 1.8 µM of each primer. After achieving the maximum amplification, the PCR was conducted with different concentrations of target DNA to generate standard curves for both SLT-1 and SLT-2. The cycling conditions were conducted according to Ramotar *et al*., [29] and consisted of initial denaturation at 95°C for 5 min and then 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. Final extension was performed at 72°C for 7 min. Two blanks were routinely used containing all components of the reaction mixture except the DNA sample or DNA *Taq* polymerase. PCR vials were placed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA). Amplified products were subjected to electrophoreses in 1.0% agarose gels and were stained with 1 mg/ml of ethidium bromide in the electrophoresis buffer (0.2 M Tris base, 0.1 M sodium acetate, 0.01 M Na_2.EDTA and 0.02 M sodium azide at pH 7.8). Digital images were obtained using a Spectroline Model EAS-1000 Electronic Archival System (Spectronics Corporation) and NIH Image 1.59 software was then used for relative quantitation of DNA bands. All assays were performed at least three and the mean values reported.

Inactivation of *E. coli* in Ground Beef Samples: Ground beef containing 5% fat was purchased from a local retail store and then distributed into plastic zipper bags in a thin layer (0.5 cm). Packed bags were frozen and thawed at least three times in liquid nitrogen and then stored at -20°C for future use. Aseptically, a sample was thawed and Ten g of ground beef samples were added to 90 ml of Tryptic Soy Broth plus 0.5% dextrose into a stomacher bag with inserted mesh (Whirl-pak, NASCO, Fort Atkinson, Wisconsin, USA). Bags were then stomached with stomacher 400 BA 7021 (Seward, Tekmar, Cincinnati, Ohio, USA) at normal speed (230 rpm) for 90 second. The homogenate was transferred to a 1 litre flask and then incubated with rotary agitation (300 rpm) at 37°C for two days. Culture media was centrifuged at 1000 rpm for 3 min to remove large debris. One loop of the supernatant was streaked onto Violet Red Bile Agar (VRBA) plates which were incubated at 37°C for two days. If the freeze and thaw process was successful, there would be no colonies on the plates after incubation. A 10 ml volume of filtrate was transferred into 100 ml TSB^+ in 250 ml baffled flasks. Flasks were then incubated for two days at 37°C. The growth was tested for *E.coli* 0157:H7 using PCR for VT1 and VT2 genes. All assays were performed at least three and the mean values reported.

Extraction of Target Cells from Ground Beef Samples: Aseptically, 0.2 ml of cell suspension containing ~ 2 x 10^4 cells in PBS prepared as described above was added to 10 g of thawed ground beef. The bacterial cells were allowed to bind for 15 min in a refrigerator at 5-8°C. Homogenate was prepared by stomaching 10 g of ground beef (5% fat) with 90 ml of PBS in a stomaching bag. Filtration was performed in three steps: first, through a coffee filter to remove large beef debris; second, at low speed centrifugation to precipitate the medium particles; and third at high speed centrifugation to precipitate target cells with small particles. The homogenate containing bacteria was filtered through a paper coffee filter in a sterile Buchner funnel using a vacuum pump. The filtrate was then centrifuged at 1000 rpm for 2 min at 4°C. After discarding the pellet, the supernatant was centrifuged at 10,000 rpm for 10 min at 4°C. Pellets were resuspended in 10 ml of sterile saline (0.85% NaCl) and then 0.1 ml surface plated onto Violet Red Bile Agar (VRBA) plates. After overnight incubation at 37°C, characteristic purple colonies were counted to determine the percent recovery of the seeded *E. coli*. All assays were performed at least seven and the mean values reported.

Enrichment of *E. coli* O157:H7 Isolates: Cell suspensions containing varying cell numbers (10, 50, 100, 200, 500 cells prepared in 0.2 ml PBS) prepared as described above were seeded into 10 g samples of ground beef. Bacterial cells were allowed to bind for 15 min in the refrigerator at 5-8°C as described above.

Homogenates and filtrates were prepared using differential centrifugation with the aid of coffee filter method as previously described. Pellets were resuspended in 30 ml of Tryptic Soy Broth plus 0.5% dextrose in 250 ml baffled flasks. Flasks were incubated with rotary agitation (300 rpm) at 37°C for different periods of incubation (3.5, 4.5 and 5.5 h). Cells were then harvested by centrifuging broth cultures at 16,000g for 10 min at 4°C. After reresuspended the pellets in 1.0 ml of d-H_2O in microcentrifuge tubes, a serial dilutions were prepared and then 0.1 ml was plated onto VRBA plates. All assays were performed at least five times and the mean values reported.

Detection of *E. coli* O157:H7 in Ground beef with the aid of Pellet Paint: Cell lysis procedure was performed using the lysis solution (TZ) as described above by Abolmaaty *et al*. [28]. TZ lysing buffer was added to resuspended pellets obtained from differential centrifugation. DNA Lysates were precipitated with ethanol with the aid of pellet paint (2 µl, Novagen) which is a visible flourescent dye-labeled carrier. Pellet paint was added followed by the addition of 40 µl of 3M sodium acetate. The samples were then briefly mixed. Two volumes of absolute ethanol were added, the samples briefly vortexed and then were allowed to stand at room temperature for 2 min. The vials were then centrifuged at 10,000 g for 10 min. A pink pellet with captured DNA was visible at the bottom of the tubes. The supernatants were removed and residual ethanol was evaporated by incubating the vials at 75°C for 8 min. The fluorescent pellets were resuspended in 50 µl of mili-Q water. The PCR protocol was performed by adding 10 µl of fluorescent pellets suspension to the PCR mixture consisted of 5 µl of 10X PCR mix (final: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 1.6 µM each of the two primers, 0.2 mM each of dATP, dCTP, dGTP and dTTP ,1.0 mM $MgCl_2$ in a final volume of 50 µl in a final volume of 50 µl. The concentrations of *Taq* polymerase were 2.5 and 5.0 units for *SLT-1* and *SLT-2* respectively. The PCR vials were placed in the thermocycler for amplification as described above. Image analysis was performed as described earlier. All assays were performed at least three times and the mean values reported.

RESULTS AND DISCUSSION

Effect of $MgCl_2$ Concentration on DNA Amplification: When the concentration of $MgCl_2$ was varied, the maximum yield of amplified DNA occurred when the PCR reaction mixture included 1.0 mM of $MgCl_2$ with both

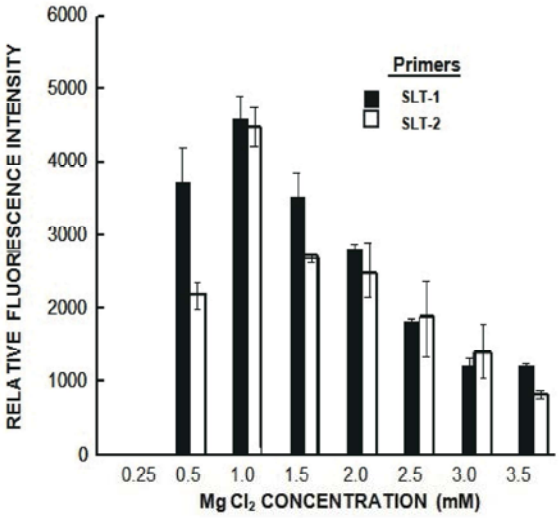

Fig. 2: Effect of $MgCl_2$ concentration on the amplification of *SLT-1* and *SLT-2* target sequences. DNA samples containing 1 x 10^5 templates were subjected to a variety of $MgCl_2$ concentrations in PCR reaction mixtures. Final PCR reaction mixtures consisted of: 5 µl of 10X PCR mix, 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 1 µM each of the two primers and 2.5 units of *Taq* polymerase (Promega) in a final volume of 50 µl. Bar graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments.

SLT-1 and SLT-2 (Figure 2). Concentrations of $MgCl_2$ greater that 1.0 mM resulted in a descending pattern of amplified DNA (Figure 2). Amplification of *SLT-1* and *SLT-2* gene sequences did not occur with 0.25 mM $MgCl_2$ (Figure 2). Baumforth *et al*. [30] found that higher Mg^{++} generally results in higher amplification yields, but if too high will often result in amplification of non-specific products. Both EDTA and dNTPs chelate the Mg^{++} and lower its effective concentration in the reaction [30]. If the dNTP concentrations are too high, then Mg++ will be rapidly depleted and PCR will be inhibited. If Mg^{++} content is too low, the result is little or no PCR product. If the Mg^{++} is too high then mispriming can result. Dwivedi *et al*. [13] studied the effect of Mg^{++} in the amplification of a 38 kDa gene and found that a concentration of 2-mM yielded the best results while higher and lower concentrations of Mg^{++} resulted in reduced product yield.

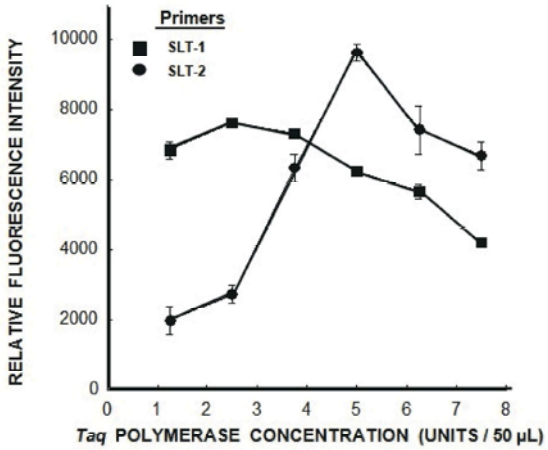

Fig. 3: Effect of Taq polymerase concentrations on the amplification of target DNA. DNA samples containing 1 x 10^5 templates were subjected to a variety of *Taq* polymerase concentrations in PCR reaction mixtures. The composition of PCR reaction mixtures was as described in legend to Figure 2 except for the concentration of $MgCl_2$ which included 1.0 mM for both *SLT-1* and *SLT-2*. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments.

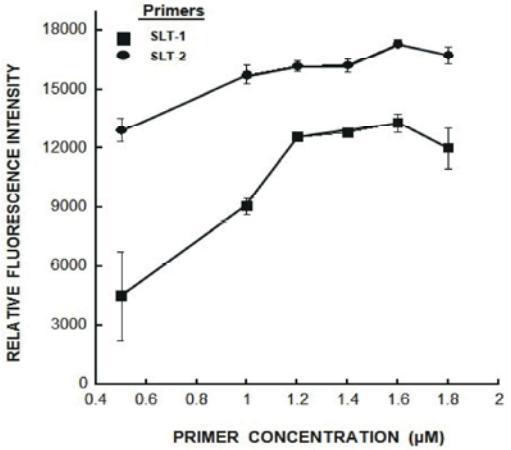

Fig. 4: Effect of varying the concentration of *SLT-1* and *SLT-2* primers on the amplification of DNA. DNA samples containing 1 x 10^5 templates were subjected to a variety of primer concentrations in PCR reaction mixtures which consisted of 5 µl of 10X PCR mix (final: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 1.0 mM of $MgCl_2$ in a final volume of 50 µl. The concentrations of *Taq* polymerase were 2.5 and 5.0 units for *SLT-1* and *SLT-2* respectively. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments.

Effect of Taq Polymerase Concentration on the Amplification of DNA: Varying the level of *Taq* polymerase in PCR reaction mixtures resulted in maximum amplification of *SLT-1* and *SLT-2* target sequences with 2.5 and 5 units of *Taq* polymerase respectively (Figure 3). With respect to SLT-2 target sequences, maximum amplification required 5 units which is higher than the 2.5 units recommended by Ramotar *et al*., 1995 (2.5 units). From these results, it was concluded that the concentration of *Taq* polymerase is critical and should be optimized (Figure 3). The concentration of *Taq* polymerase needed for SLT-2 target sequences is 2.5 units that required for SLT-1. A recommended concentration range for *Taq* Polymerase is between 1 and 2.5 units [31] per 100 µl reaction mixture when other parameters are optimized. Enzyme requirements may vary with respect to individual target templates or primers. When optimizing a PCR, [10] recommended testing enzyme concentrations ranging from 0.5 to 5 units /100 µl reaction. If the enzyme concentration is too high, nonspecific background product may accumulate and if too low, an insufficient amount of desired product is made.

Effect of varying the Concentrations of SLT-1 and SLT-2 Primers on the Amplification of DNA: When the primer concentrations increased from 1 µM to 1.6 µM as recommended by Ramotar *et al*., [29], a significant increase in the amplification occurred with SLT-1 while only a slight increase was noticed with SLT-2 target sequences. In the present study, the highest amplification of *SLT-1 & SLT-2* target sequences was obtained with 1.6 µM primers in the PCR reaction mixture followed by a decreasing pattern (Figure 4). Primer concentrations between 0.1 and 0.5 µM are generally optimal [10] and the higher concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent artifact termed a primer-dimer. Nonspecific products and primer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers, resulting in a lower yield of the desired product [10].

Influence of PCR Nucleotide Mixture Concentration on the Amplification of Target DNA: Visible amplification of *SLT-1 and SLT-2* target sequences was completely inhibited when the concentration of the dNTP's mix were higher than 0.2 mM for *SLT-1* and higher than 0.4 mM for *SLT-2* (data is not presented). Detectable amplification occurred with 0.2 mM of dNTP's with both *SLT-1* and *SLT-2*. According to Innis and Gelfand [10], dNTP concentrations between 0.02 to 0.2 mM each result in the optimal balance among yield, specificity and fidelity. The four dNTPs should be used at equivalent concentrations to minimize incorporation errors. Both the specificity and the fidelity of the PCR are increased by using lower dNTPs concentrations than those originally recommended. Innis *et al*. [9] found that low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extension incorporated nucleotides. Ehlen and Dubeau [32], recommended that one should decide on the lowest dNTP concentration appropriate for the length and composition of target sequences; e.g., 20 µM of each dNTP in a 100 µl reaction is theoretically sufficient to synthesize 2.6 µg of DNA or 10 pmol of a 400-bp sequence. The use of low uniform dNTP concentrations (2 µM) of each enabled highly sensitive allele-specific amplification [34].

Amplification of Different Concentration of SLT-1 and SLT-2 Templates: Optimization of the PCR allowed amplified bands of 100 DNA templates of SLT-1 (Figure 5) and of 20 DNA templates of SLT-2 (Figure 6) to be detected in agarose gels. To generate a standard curve, samples (1 µl) containing different concentrations of SLT-1 and SLT-2 target sequences were subjected to the optimized PCR reaction mixtures which consisted of 5 µl of 10X PCR mix (final: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 1.0 mM $MgCl_2$, 1.6 µM each of the two primers in a final volume of 50 µl. The concentrations of *Taq* polymerase were 2.5 and 5 units for *SLT-1* and *SLT-2* respectively. A complete linear relationship was obtained when different concentrations of SLT-1 target sequences derived from CFU of 4 h old culture of *E. coli* 157:H7 (100 to 5,000) were plotted against the relative integrated areas of the amplified DNA bands using a semi-log plot (Figure 5). The detectable limit was 100 DNA templates for the SLT-1 target sequence. A similar linear relationship was obtained but with a lower detectable limit of 20 DNA templates when the number of SLT-2 templates derived from the CFU of a-4 h old culture was varied at a lower range from 20 to 1000 (Figure 6).

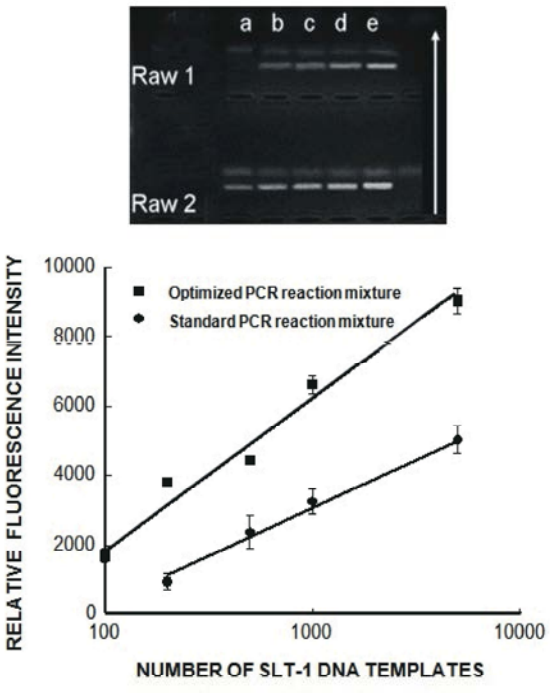

Fig. 5: Amplification of SLT-1 DNA as a result of varying the concentration of DNA template. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments. Inserted is the Gel electrophoresis of PCR amplification products with DNA samples (1-µl) representing a different number of cells (Lanes; a, 100; b, 200; c, 500; d, 1000, e, 5000) subjected to two different reaction mixtures; PCR reaction mixtures (Row 1) described by Ramotar *et al*. (1995) and the optimized reaction mixture (Row 2). The optimized formula consisted of 5 µl of 10X PCR mix (final: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 1.6 µM each of the two primers, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 2.5 units of Taq polymerase,1.0 mM $MgCl_2$ in a final volume of 50 µl. DNA lysates were obtained by lysing cell suspensions derived from CFU of 4 h pure culture.

It is of interest to notice that the optimozed PCR reaction mixture increased the sensitivity of the PCR assay resulting in the detection of as low as 100 SLT-1 and 20 SLT-2 templates while the reaction mixture described by [29] failed to detect them and was able to detect at least 500 SLT-1 and 150 SLT-2 DNA templates (Figures 5 and 6).

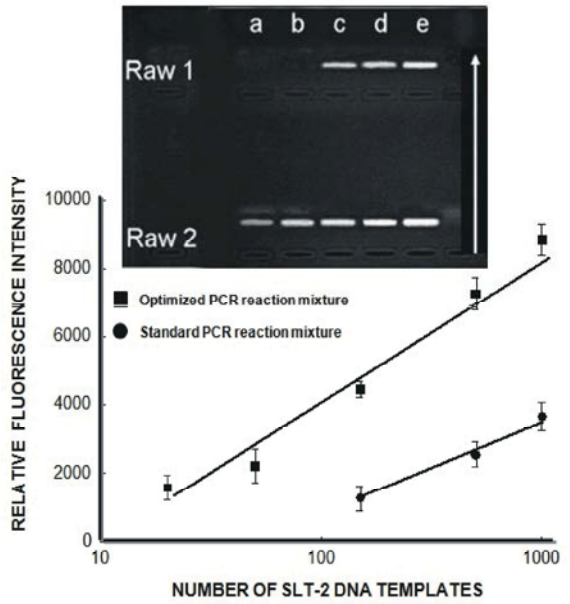

Fig. 6: Amplification of SLT-2 DNA as a result of varying the concentration of DNA template. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments. Inserted is the Gel electrophoresis of PCR amplification products with DNA samples (1-µl) representing a different number of cells (Lanes; a, 20; b, 50; c, 150; d, 500; e, 1000) subjected to two different reaction mixtures; PCR reaction mixtures (Row 1) described by Ramotar *et al*. (1995) and the optimized reaction mixture (Row 2). which consisted of 5 µl of 10X PCR mix (final: 50 mM KCl ,0.01% gelatin, 10 mM Tris-HCl at pH 9.0), 1.6 µM each of the two primers, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 5 units of Taq polymerase,1.0 mM $MgCl_2$ in a final volume of 50 µl. DNA lysates were obtained by lysing cell suspensions derived from CFU of 4 h pure culture.

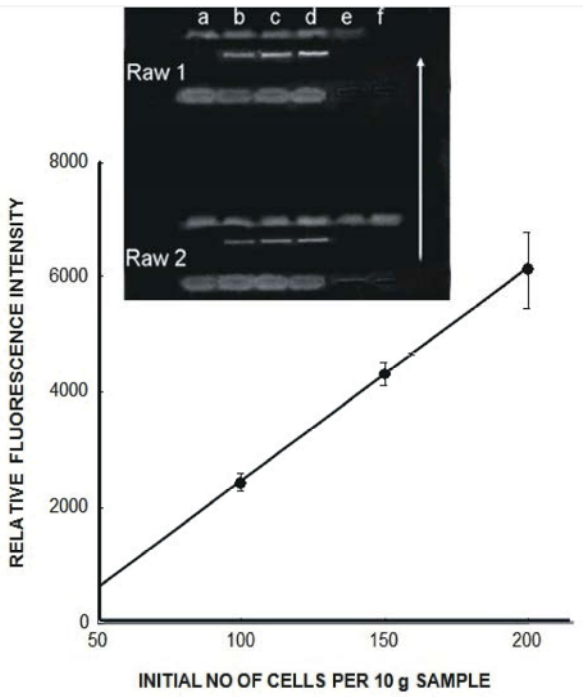

Fig. 7: Detection of *E. coli* 0157:H7 (SLT-1 templates) in ground beef after an incubation time of 5.5 hours in TSB+ at 37°C. Ground beef was seeded with different initial number of cells. After differential centrifugation, pellets were resuspended in TSB+ pre-enriccmente media. Harvested cells were subjected to TZ lysing solution. DNA lysates were purified using pellet paint. The fluorescent pellets were resuspended in 50µl of milli-Q water and 10µl was added to optimized PCR reaction mixture as described in Figure 7. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments. Inserted is the Gel electrophoresis of PCR amplification products representing a different number of cells added initially to the ground beef (Lanes: a, 50; b, 100; c, 150; d, 200 cells / 10 g). Controls without Taq polymerase (Lane f) and without DNA (Lane e) were performed. Row 1 and Row 2 were identical.

Extraction of Target Cells from Ground Beef Samples: When homogenates of unseeded frozen ground beef, prepared with and without enrichment media, were plated on VRBA plates and incubated at 37°C for 2 days, *E. coli* 0157:H7 colonies were not detected. PCR assay showed no evidence of target bacteria, indicating that frozen ground beef samples were free from dead and viable *E. coli* 0157:H7. These results also confirmed that the freeze and thaw process was conducted successfully. A recovery of 75% ± 4 was obtained after passing the homogenate through the coffee filter. Recovery decreased to 65% ± 3 after centrifuging the resuspended pellets at 1000 rpm for 2 min. The final pellet, which was used for PCR assay, resulted in a recovery of 57% ± 6. These data represents the mean values of the percent recovery and standard errors derived from the initial data of eleven different experiments.

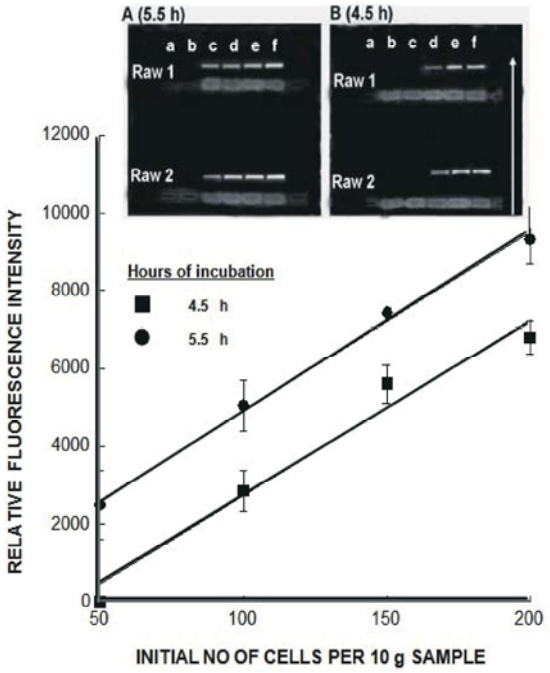

Fig. 8: Detection of *E. coli* 0157:H7 (SLT-2 templates) in ground beef after an incubation periods of 4.5 and 5.5 hours in TSB+ at 37°C. Ground beef was seeded with different initial number of cells. After differential centrifugation, pellets were resuspended in TSB+ pre-enriccmente media. Harvested cells were subjected to TZ lysing solution. DNA lysates were purified using pellet paint. The fluorescent pellets were resuspended in 50µl of milli-Q water and 10µl was added to optimized PCR reaction mixture as described in Figure 8. Graphs represent the mean values of amplified DNA resulting from integrated peak areas which represent a typical agarose gel with standard deviations derived from three different experiments. Inserted is the Gel electrophoresis of PCR amplification products representing a different number of cells added initially to the ground beef (Lanes: c, 50; d, 100; e, 150; f, 200 cells/ 10 g). Controls without Taq polymerase (Lane a) and without DNA (Lane b) were performed. Row 1 and Row 2 were identical.

Enrichment of Bacterial Cells: Bacterial cells were isolated as described above and then enriched in 30 ml of Tryptic Soy Broth plus 0.5% dextrose incubated at 37°C for different intervals of time (3.5, 4.5 and 5.5 h). Enrichment was performed in order to revive injured cells and increase the number of target microorganisms thereby increasing the sensitivity of the PCR assay.

An increasing linear relationship occurred when the final cell numbers obtained after 3.5 and 4.5 hours of incubation were plotted against the initial cell numbers on a semi log paper, while a near linear increasing relationship occurred after 5.5 hours of incubation (data is not present). Injury to the specific pathogen of interest can enhance problems of normal flora interference. Many treatments used in food processing such as heat, cold, drying, freezing, osmotic activity, chemical additives and preservatives and other factors can sublethally injure bacterial cells [22, 23]. Heat-injured *E. coli* O157:H7 cannot undergo repair and form colonies on selective media, such as Sorbitol MacConkey agar (SMAC) or xylose lysine decarboxylase agar (XLD), respectively, because the selective agents or dyes in these selective agars can inhibit the repair of heat-injured pathogens [33, 34, 35]. Significant differences between SMAC and tryptic soy agar (TSA; nonselective medium) for recovery of injured microorganisms have been observed [35, 36, 37].

The Detectable Limits of *E. coli* O157:H7 in Ground Beef: A linear relationship occurred between the initial number of *E. coli* 0157:H7 added to 10 g ground beef samples and the relative integrated peak areas of PCR analysis products resulting from the amplification of SLT-1 and SLT-2 target sequences (purified with pellet paint) and isolated from enrichment media (Figures 7 and 8). SLT-1 and SLT-2 DNA templates purified with pellet paint prior to PCR were successfully detected when cells were isolated from 10 g ground beef seeded with 100 and 50 cells respectively and subjected to enrichment in TSB+ at 37°C for 5.5 hours (Figures 7 and 8). The detectable limit of SLT-2 decreased to 100 cells / 10 g of seeded ground beef after an incubation time of 4.5 hours in TSB+ at 37°C (Figure 8).

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