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Microfluidic Chip for Direct Detection of *E. coli* O157:H7 in Ground Beef via Anti-Digoxigenin Immuno-PCR Assay

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Abstract: Microfluidic chip was successfully fabricated from polydimethylsiloxane (PDMS) and used for direct detection of *E.coli* O157:H7 in ground beef via anti-digoxigenin immuno-PCR. The PDMS channels were functionalized with 100 µg/ml streptavidin at room temperature for 6 min. A recovery of 60% ± 3.4 was obtained when bacterial cells were extracted from seeded ground beef using differential centrifugation. ProMag™ bind-IT™ microspheres were effectively used to capture 66% ± 4.2 of bacterial cells from ground beef extract. Biotinylated PCR amplicons of *E. coli* O157:H7 intimin gene were detected using anti-digoxigenin labeled with FITC. This chip allowed sensitive and rapid detection of a 5 cells/g ground beef while agarose gel electrophoreses failed to detect 50 cells/g ground beef. These microchannels offer pathways to eliminate the time-consuming and messy slab gel process and generate much more reproducible and high quality data.

Abbreviations: Intimin gene (eaeA) · Shiga toxins (stx1 and stx2) · Streptavidin (STA) · Biotinylated PCR amplicons labeled with digoxigenin (dual-labeled amplicons) · Polydimethylsiloxane (PDMS) · Anti-digoxigenin immuno-PCR (anti-dig-IPCR) · Mean grey Values (MGVs)

Key words: *E. coli* O157:H7 · Immuno-PCR · Streptavidin · Biotinylated primers · Anti-digoxigenin · Microfliuidics · Polydimethylsiloxane (PDMS) · Magnetic beads · Ground beef

INTRODUCTION

For more than two decades *E. coli* O157:H7, a toxin-producing food and waterborne bacterial pathogen, has been associated with large outbreaks of gastrointestinal illness in many parts of the world [1-4]. *E. coli* O157:H7 has been recognized as the most common enterohaemorrhagic *E. coli* (EHEC) serotype in North America contributing to more than 75,000 human infections [5] and 17 outbreaks [1] per year. In 2006, Centers for Disease Control and Prevention (CDC) reported that 199 people were infected by the outbreak of *E. coli* O157:H7 in 26 states at the United States of America. Moreover, 46 outbreaks occurred in Canada during the years 1999 and 2000 resulting in more than 1000 cases of human illness and six deaths [2]. The majority of foodborne illness outbreaks associated with *E. coli* O157:H7 have involved meat products, especially ground beef [6]. Molecular methods, particularly PCR, have proven useful as screening tests for *E. coli* O157:H7 and for confirmatory identification of putative isolates [7-9], but is limited in part by the presence of PCR inhibitors in complex biological materials, such as meat product. PCR-based assays identify *E. coli* O157:H7 by amplifying the characteristic such as the sequences of shiga toxins (stx1 and stx2) genes [10, 11], the intimin gene (eaeA) involved in the attachment of bacteria to enterocytes [11] and rfb genes [12] that are involved to the biosynthesis of the O157 *E. coli* antigen (rfb O157).

PCR microchips have been successfully developed [13,14], using different microfluidic technologies [15, 16] for decentralized screening of pathogens in food, water and environment as well as for clinical diagnosis. However, PCR products can be off-line detected by all existing DNA detection methods. Agarose gel electrophoresis is the most widely used method for the detection of PCR products [17, 18] using intercalating dyes such as ethidium bromide and SYBR Green [19, 20].

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Two main advantages were reported for gel electrophoresis method: real-time detection and versatility [21, 20]. Nevertheless, indiscriminate binding is a major disadvantage since both specific and nonspecific PCR products can produce the same type of signal, therefore it is difficult to differentiate between them. Moreover, this technique is time-consuming and labor intensive. In order to avoid the drawbacks of off-line DNA detection methods, great efforts have been made to develop on-chip quantitative and/or qualitative PCR detection. However, to date, only a limited number of microfluidic devices, which are capable of automatically detecting the PCR product, have been reported. Fluorescence based real-time and end-point DNA detection is a powerful and important detection technique [21-24]. These methods have been developed to ensure whether the DNA template is successfully amplified [25, 26] and therefore used as a criteria for the PCR efficiency. However, since the sequence-nonspecific fluorescence dyes are often utilized for the on-chip real-time PCR systems, both specific and nonspecific products will produce the same fluorescence signals that are difficult to differentiate. In order to overcome these challenges, an off-line microchannels platform was fabricated to detect PCR amplicons via anti-dig-IPCR. We adopted the notion of PCR-ELISA, an established non-multiplex immunoassay that is capable of detecting *E. coli* O157 [27-29], with a modified procedure using anti-digoxigenin immuno-PCR (anti-dig-IPCR) in polydimethylsiloxane (PDMS) microfluidic chips. Generally, micro reaction microchannels allow rapid diffusion of reactants across the chamber because the character length is comparable to diffusion length [16]. In this study, rapid prototypical microchannels enabled fast diffusion of biomolecules from liquid phase to liquid-solid interface, potentially accelerating the process in the multistep heterogeneous assay for the detection of *E. coli* O157:H7 in ground beef, as part of a broader effort to reduce the potential health hazards posed by this pathogen.

MATERIAL AND METHODS

Microorganism and Routine Cultivation: E. coli O157:H7 strain EDL933 (obtained as a gift from Dr. Paul S. Cohen, Department of Cell and Molecular Biology, University of Rhode Island, USA) was cultured in 50 ml of tryptic soy broth (Difco, Detroit, Michigan) plus 0.5% glucose (TSB^+) 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 sterile saline (0.85% NaCl), Cell densities were determined by both plate counts using tryptic soy agar containing 0.5% glucose and the corresponding absorbance at 600nm.

Preparation of Ground Beef Samples: Ground beef containing 5% fat was purchased from a local retail store and 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 to inactivate any *E. coli* present and then stored at -20°C for future use. Aseptically, a sample was thawed and 50 g was added to 450 ml of sterile TSB^+ in 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. A 10-ml volume of filtrate was transferred into 100 ml TSB^+ in 250 ml baffled flasks. Flasks were then incubated for one day at 37°C. The growth was tested for *E.coli* 0157:H7 intimin (eaeA) gene using PCR.

Extraction of *E. coli* O157:H7 Cells from Ground Beef Samples: Aseptically, 0.2 ml of cell suspension containing (25, 2.5 x 10^2, 2.5 x 10^3, 2.5 x 10^4, 2.5 x 10^5, 2.5 x 10^6, 2.5 x 10^7 cells) in PBS was added to 50 g of thawed ground beef samples. The bacterial cells were allowed to bind for 15 min in a refrigerator at 5-8°C resulting in a final concentration of 0.5, 5.0, 50, 5 x 10^2, 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6 cells/g ground beef. Homogenate was prepared by stomaching 50 g of ground beef (5% fat) with 450 ml of PBS in a stomaching bag as described above. The homogenate 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 5 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.*

Promag^{tm} Bind-It^{tm} Magnetic Microsphere: ProMag^{TM} bind-IT^{TM} magnetic microspheres, 3.12 µm, (Bangs Laboratories, Inc. Fishers, IN, USA) were used to capture bacterial cells from pellets suspension. Magnetic beads were prepared as described by the manufacture with slight modification. A volume of 200 µl was dispensed in different five eppendorf tube (1.5 ml) and then placed on

magnetic separator (Invitrogen, CA, USA) for 30-60 seconds to allow complete separation of microspheres. Supernatant was carefully removed followed by washing the beads twice with 100µl coupling buffer (50mM MES, pH 5.2; 0.01% Tween® 20), using the magnetic separator. BacTrace anti-*E.coli* O157:H7 (KPL, Inc. MD, USA) was prepared in coupling buffer (1.0 mg/ml buffer). Washed beads were added to 200µl of anti-*E.coli* O157:H7, vortexed and then incubated for 30 minutes at room temperature using a rotator agitator (Dynal Biotech, INC, NY, USA). Unbound fragments of the antibody were removed using the magnetic separator and the beads were washed twice in 200µl sterile saline. A 1.0-ml pellets suspension was added to each eppendorf vial containing the antibody-coated beads, vortexed and then subjected to the rotator for 30 min at room temperature. Unbound materials were washed out using 1.0 ml saline. The complex of cells-beads combined from the five vials was suspended in 1.0 ml of sterile distilled water and 0.1 ml surface plated onto VRBA.

Preparation of Dual-Labeled Pcr Amplicons: Beads suspension (0.1 ml) was added to 0.1 ml of 2x TZ lysing solution [30] containing 2.0% Triton X-100 in 0.1 M Tris-HCl buffer plus 2.5 mg/ml sodium azide, pH 8.0. The mixture was placed in 1.5 ml eppendorf tube, heated in a water bath for 10 min and then cooled for 5 min in ice bath. The lysates were centrifuged at 10,000 g for 10 min at room temperature prior to PCR.

The primers selected to amplify the intimin (eaeA) gene segment were: Biotin-5'-GAGCACAATCGCTGTTGTTAGCGT-3' and 5'-TGTCGCTTGAACTGATTTCCTCT-3' to amplify 180-bp fragment nucleotides (424th bp to 603rd bp) of the intimin gene. The primers were synthesized and biotinylated by Integrated DNA Technology (Coralvilb, IA) and were designed for the published nucleotide sequence of intimin gene (GenBank no. U32312).

The PCR protocol described by Roche Applied Science, Mannheim, Germany was used. PCR was conducted by adding 10 µl of DNA samples isolated from different concentration of cell densities as described above to 90 µl of PCR mixture. The PCR mixture consisted of 2.5 U hot start polymerase (Promega), 1x PCR reaction buffer (Promega), 1 mM $MgCl_2$, digoxigenin labeling mix (Roche Applied Science) and 250 nM each primer in nuclease-free water (Integrated DNA Technology). The cycling conditions was conducted in a thermal cycler (Mastercycler ep Gradient S, Eppendorf, Westbury, NY) and consisted of initial denaturation at 95°C for 2 minutes

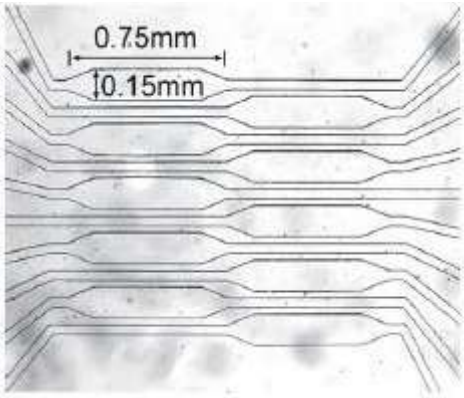

Fig. 1: Micrograph of the microchambers.

followed by 35 cycles with denaturation at 94 °C for 2 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 5 min. Controls consisted of all the ingredients of PCR mixture except the DNA template. PCR products (4 µl) was subjected to 2.0% agarose gel electrophoreses for 45 min containing 1 µg/ml of ethidium bromide in the electrophoresis buffer (0.2 M Tris base, 0.1 M sodium acetate, 0.01 M Na_2.EDTA, pH 7.8). Digital images were captured using a BIO-RAD Universal Hood Π ChemiDoc XRS molecular imager and Mitsubishi P93 printer.

Fabrication of PDMS Microchannels: To fabricate the microchannels, 100 µm-thick SU-8 2100 photoresist (Microchem, Newton, MA) was coated on a 4-inch silicon wafer. The masked photoresist was then patterned by exposing it to 365-nm collimated light, followed by post baking at 95°C for 30 minutes. It was then developed in SU-8 developer (Microchem, Newton, MA) and rinsed in SU-8 developer (Microchem) to form the SU-8 structure. PDMS pre-polymer and cross-linking reagent (Dow corning) were mixed at a ratio of 10: 1 by weight. The mixture was degassed and then poured onto the silicon wafer. After 15 minutes hardening at 80°C, elastomeric PDMS was peeled off from the wafer and holes were punched through at the ends of the microchannels. The peeled PDMS was then bonded to a flat PDMS pad by plasma treatment in oxygen environment, forming closed volume of microchannels. Figure 1 presents the micrograph of these microchannels. The microfluidic chip consisted of 10 parallel microchannels that consumed less than 1 µl PCR amplicons per channel.

Surface Functionalization of Native PDMS with STA: Functionalization of PDMS microchannels with streptavidin (STA) was conducted using adsorbed

biotinylated BSA to prime the native PDMS. To generate STA functionalized surface, microchannels were first subjected to 2 µg/ml of biotin-LC-BSA conjugate (Thermal Fisher Scientific Inc, Rockford, IL) and incubated overnight at 4°C. The microchannels were then washed once with Tris-buffered saline Tween-20 (TBST, 0.1 M Tris, 150 mM NaCl, 0.02% Tween-20, pH 7.4) subsequently followed by coating with STA. To maximize STA coating, the incubation time and STA concentrations were investigated. STA (Promega, Madison, WI) was introduced to the microchannels at a concentration of (200, 100, 50 and 25 µg/ml in TBST buffer) and then incubated for 10 minutes at room temperature. Biotinylated alkaline phosphatase (1.6 µg/ml in TBST buffer) obtained from (Promega, Madison, WI) was sequentially added and incubated for 5 minutes at room temperature. The excessive amount of unbound enzyme was washed off using TBST washing buffer followed by adding fluorescent diphosphate (FDP) in reaction buffer (0.1 M Tris, 10mM $MgCl_2$, 10mM glycine, pH 9.0) resulting in a strong fluorescent biocomplex (excitation/emission ~ 490 nm/514 nm). Fluorescent images were captured by a Zeiss axioplan II microscope attached to a 5x objective lens at the Genomic Sequencing Center, University of Rhode Island. Quantification of the fluorescent signals was carried out by calculating the mean gray value (MGV) of a 240 x 50 pixels area using Image J software (http://rsb.info.nih.gov/ij/). Effect of incubation time on the binding of STA was conducted as previously described except for adding 100 µg/ml STA to biotin-coated PDMS microchannels followed by different incubation periods (2, 4, 6 and 10 min) at room temperature. Functionalized PDMS chips were stored in the refrigerator until use.

Detection of Dual-Labeled PCR Amplicons via Anti-Digoxigenin Immuno-PCR: The complex biotin-DNA-dig fragments were purified by subjecting 60 µl of amplified products to PCR clean-up kit (Invitrogen, Carlsbad, California) and then reconstituted in 50 µl of TE buffer (Integrated DNA Technology). Purified dual-labeled DNA conjugates were introduced to the functionalized microchannels and then incubated for 10 min at room temperature followed by a washing step with PBS buffer to remove unbound PCR product. Controls were conducted by adding conjugate-free samples (PCR negative control). A 2-µl anti-dig antibody conjugated with FITC (Roche Applied Science) prepared in PBS buffer (40 µg/ml) was introduced to the microchannels and then incubated for 10 minutes at room temperature followed by a washing step with PBS buffer. As described in Figure 2, the signal-generating format on the biotin-BSA primed PDMS is built by sequential coupling of streptavidin (STA), biotin-dig-DNA conjugate and fluorescent labeled anti-dig antibody. Microscopic images were then captured and quantified as previously described.

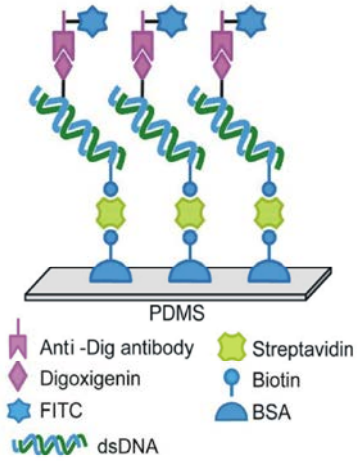

Fig. 2: Schematic representation of the detection assay.

RESULTS AND DISCUSSION

Extraction of E. Coli O157:H7 from Ground Beef Samples: When the filtrates of unseeded frozen ground beef were enriched in TSB^+ for one day at 37°C, PCR assay showed no evidence of target bacteria, indicating that frozen ground beef samples were free from dead and viable target cells. A recovery of 60% ± 3.4 was obtained after the extraction of target cells from ground beef with the aid of differential centrifugation and the use of coffee filters. 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. Magnetic beads were then successfully used to capture 66% ± 4.2 of target cells from pellets resulting in an overall recovery% of 40% ± 3.8. A proportional relationship occurred between the logarithm of cell number seeded into ground beef samples and the logarithm of cell number captured by antibody-coated beads (Figure 3).The use of the bind-IT surface offers stable coating, but without any damage to the tertiary structure of the protein resulting in a highly active and stable surface that offer significant improvements in the sensitivity and dynamic range of immunoassays. Magnetic bead-based methods provide

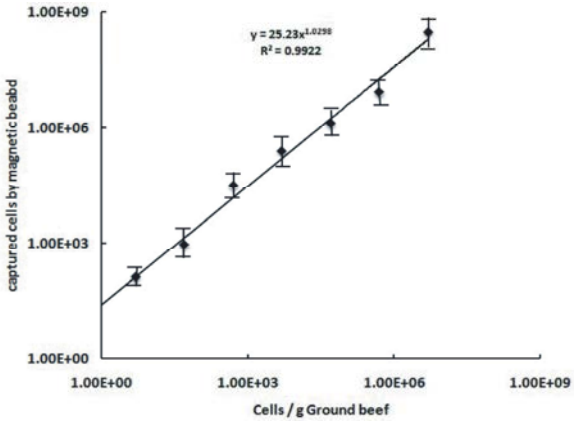

Fig. 3: The efficacy of cell capture using ProMag magnetic beads: ground beef samples were seeded with target cells (0.5, 5.0, 50, 5 x 10^2, 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6 cells/g) and then subjected to an extraction procedure using differential centrifugation. The final pellets were suspended in 5 ml of d. water and 1.0 ml was added to each eppendorf vial containing the antibody-coated beads, vortexed and then subjected to the rotator for 30 min at room temperature. Unbound materials were washed out using 1.0 ml saline. The complex of cells-beads combined from the five vials was suspended in 1.0 ml of sterile distilled water and 0.1 ml surface plated onto VRBA. The logarithm of cell number seeded into ground beef was plotted against the logarithm of cell number captured by antibody-coated beads. Data points represent three different experiments.

effective PCR inhibitor removal. The presence of PCR inhibitors slow down the PCR or reduce amplification efficiency through one or more of the following mechanisms: interference with the cell lysis step, degradation or capture of the nucleic acids, or inactivation of the thermostable DNA polymerase [31]

Functionalization of PDMS Microchannels: STA-biotin linkage was selected to capture DNA for the following advantages: STA-biotin interaction has showed a strong and remarkable affinity (K_d = 10^{-15} M) through a non-covalent bond, biotin can be conveniently incorporated into PCR amplicons, STA is structurally stable within a wide range of pH and temperature [32] and recombinant STA presents nearly neutral pI value [33] which protect solid phase from non-specific ionic interaction within charged proteins in nearly neutral pH buffer. Furthermore, STA is a tetrameric protein [33] that consists of four biotin binding subunits, suggesting that STA can decorate biotin-available surface while it maintains the capability of binding biotinylated amplicons at the rest of the unoccupied subunits. When different concentrations of STA (25, 50,100 and 200 µg/ml) were subjected to PDMS microchannels coated with biotin followed by adding biotinylated alkaline phosphatase, the enzyme assay resulted in an increase in the fluorescent intensity with the increase of STA concentration (Figure 4). Maximum immobilization of STA to biotin-coated PDMS microchannels occurred at a concentration of 200 µg/ml followed by 100 µg/ml. When 100 µg/ml STA was added to biotin-coated PDMS microchannels and incubated at

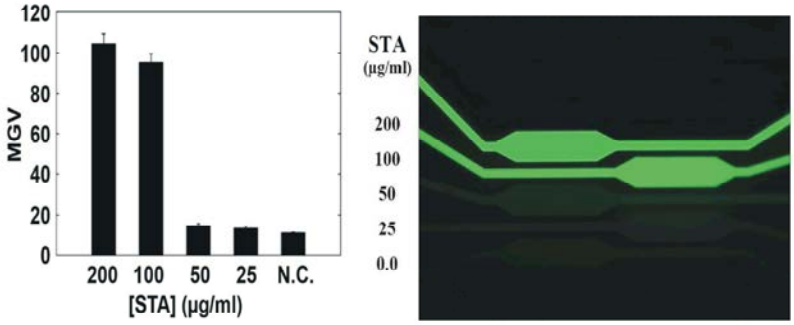

Fig. 4: Effect of STA concentration on the binding of STA to biotin-coated PDMS microchannels: Various concentrations of STA (200, 100, 50 and 25 µg/ml in TBST buffer) were introduced to the biotin-coated microchambers and incubated for 10 minutes at room temperature. After the removal of unbound STA, biotinylated alkaline phosphatase (1.6 µg/ml in TBST buffer) was then added and incubated for 5 minutes at room temperature. The excessive amount of unbound enzyme was washed off using TBST washing buffer followed by adding fluorescent diphosphate (FDP) in reaction buffer. Fluorescent images were captured by a Zeiss axioplan II microscope (excitation/emission ~ 490nm/514nm. Fluorescent signals were quantified by calculating the mean gray value (MGV) in each microchamber using Image J software. Par graphs represent the mean values driven from three different experiments.

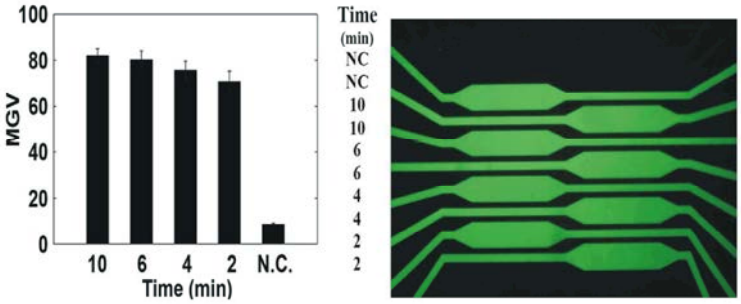

Fig. 5: Effect of incubation time on the binding of STA to biotin-coated PDMS microchannels, experiments were conducted as described in Figure4 except for adding 100 µg/ml STA to biotin-coated PDMS microchamber followed by different incubation periods at room temperature. Par graphs represent the mean values driven from three different experiments.

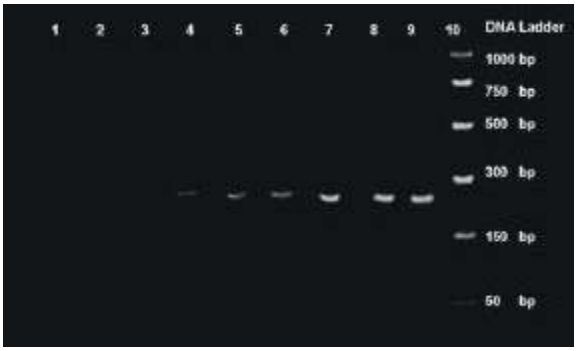

Fig. 6: Gel electrophoresis of amplified Biotinylated PCR amplicons labeled with digoxigenin (dual-labeled amplicons) of *E. coli* intimin gene. DNA lysates were obtained after lysing cells captured by beads using TZ lysing solution and then 10 µl was added to 90 µl of PCR mixture as described above. Thus, lanes 2 to 9 represent different concentrations (5, 5.0, 50, 5 x 10^2, 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6 cells/g ground beef) respectively. Lanes 1 and 10 represent non DNA templates and the DNA Ladder. Digital images of agarose gel were captured by BIO-RAD Universal Hood Π ChemiDoc™ XRS molecular imager. `

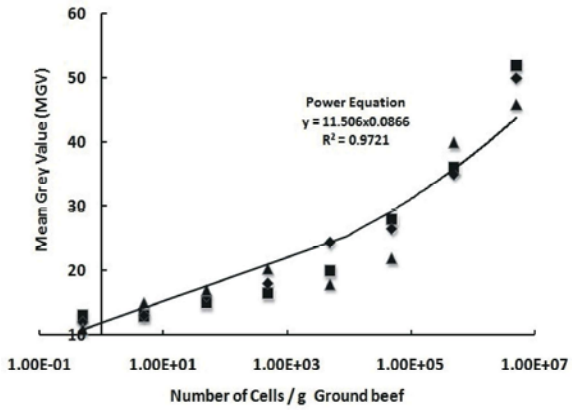

Fig. 7: Detection of dual-labeled PCR amplicons via Anti-Digoxigenin Immuno-PCR chip assay. Various concentrations of dual-labeled PCR amplicons resulting from different concentrations (5, 5.0, 50, 5 x 10^2, 5 x 10^3, 5 x 10^4, 5 x 10^5, 5 x 10^6 cells/g ground beef) were introduced to the microchamber coated with STA. Unbound amplicons were washed off, anti-dig labeled with FITC was added and then unbound anti-dig was washed off. Fluorescent signals were captured by a Zeiss axioplan II microscope and quantified using Image J software followed by measuring MGV. Data points represent the mean values driven from three different experiments.

room temperature for different periods (2, 4, 6 and 10 min), a slight increase in the binding capacity occurred with the increase of the incubation time (Figure 5). Therefore, a concentration of 100 µg/ml STA added to biotin-coated PDMS and incubated for 6 min at room temperature was selected for the optimal parameters for STA immobilization.

Detection of Dual-Labeled PCR Amplicons on Agarose Gel Electrophoreses: Digital images of agarose gel captured by BIO-RAD Universal 1Hood Π ChemiDoc XRS molecular imager confirmed that synthesized and biotinylated primers were successfully able to amplify a 180-bp fragment (nucleotides (424th bp to 603rd bp) of the intimin gene (Figure 6). Lanes 4 to 9 showed an increase in the concentration of intimin gene amplicons with the increase of DNA templates added to the PCR reaction mixture. However, no detectable bands were observed with amplicons obtained from DNA templates extracted from both 5 and 50 cells/g ground beef as shown in Figure6 (lanes 2 and 3 respectively).

Quantitative Detection of Dual-Labeled PCR Amplicons via Anti-Dig Immuno-PCR: We evaluated the sensitivity of anti-dig Immuno-PCR immunoassay in the detection of PCR amplicons, comparing it to the sensitivity of agarose gel electrophoreses. As described previously, dual-labeled PCR amplicons were captured on STA and were subsequently bound to fluorescent-labeled anti-dig. As shown in Figure 7, a proportional relationship occurred between the log scale of cell density seeded into ground beef and the mean grey values (MVSs) of fluorescent images captured by a Zeiss axioplan II microscope (excitation/emission ~ 490nm/514nm and analyzed by Image J software. This relationship generated a power equation of ($y=11.506\ X^{0.0866}$) and R^2 of 0.9721. The use of anti-dig Immuno-PCR allowed the detection of as low as 5cells/g ground beef while gel electrophoreses failed to detect 50 cells/g ground beef.

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