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Pre-enrichment effect on PCR Detection of *Salmonella* Enteritidis in artificially-contaminated raw chicken meat

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Abstract

Salmonella remains to be a major foodborne pathogen for animals and humans and is the leading cause of foodborne infections and outbreaks in various countries. *Salmonella* Enteritidis is one of the most frequently isolated serotypes in poultry and poultry products from human food poisoning cases. It can cause mild to acute gastroenteritis as well as other common food poisoning symptoms when infection takes place in human. Nucleic acid amplification technologies such as Polymerase Chain Reaction (PCR) is a tool that is rapid and sensitive for detection of bacterial pathogen. We report the successful detection of *S.* Enteritidis by PCR in raw chicken meat artificially-contaminated with serial concentration of *S.* Enteritidis using crude DNA extracts as DNA template. PCR primers, ENT-F and ENT-R targeted on *sdfI* gene were used to amplify DNA region unique to *S.* Enteritidis with crude DNA extract of the samples, yielded product with the size of 303 bp. These primers were specific to *S.* Enteritidis when tested by *in-silico* simulation against genome database of targeted bacterial species and confirmed in PCR as amplification bands were observed with *S.* Typhimurium, *S.* Polarum and *S.* Gallinarum. The established PCR can detect as few as 9.4 X 10^1 CFU/ml of inoculated *S.* Enteritidis concentration and proved that pre-enrichment effect have significant effect on PCR detection by increasing 1000-fold of the sensitivity limit compared to the non pre-enriched samples. The PCR technique indicated that it can be successfully coupled with pre-enrichment step to offer advantage in routine screening and surveillance of bacterial contamination in food samples.

Introduction

World-leading cases of salmonellosis are caused by *Salmonella enterica* subspecies enterica serovar Enteritidis or commonly called *S.* Enteritidis. Salmonellosis is often associated in human salmonellosis cases in USA (Strawn *et al.*, 2014), Europe (Thorns, 2000; Janecko *et al.*, 2014), and China and England (D'Aoust and Maurer, 2007). An estimated of 1.4 million cases of salmonellosis occur each year in the United States alone, of which a few was reported to the authorities (Lynch and Tauxe, 2009). Human infections epidemic caused by S. Enteritidis was observed during the last two decades of the 20th century (Scallan *et al.*, 2011) which caused human gastroenteritis. Extensive study conducted by Matheson *et al.* (2010) found that the diagnosis from *Salmonella enteritis* revealed that *S.* Enteritidis was the most frequently isolated serotypes which accounted more that 50% of the infection cases caused by *Salmonella*. In the most area of united states, *S.* Enteritidis infection is present in hens with estimation that one in every 20,000 eggs is internally contaminated leading to an economic loss of up to USD 1 billion annually (Barbour *et al.*, 2001). Shell eggs and egg containing products is strongly associated with *S.* Enteritidis (Patrick *et al.*, 2004; Howard *et al.*, 2012). Besides that, broiler chickens, raised for their meats and the chicken products were also reported to be contaminated with *S.* Enteritidis (Altekruse *et al.*, 2006). This foodborne pathogen is the most common causative agent of non-typhoidal salmonellosis in Malaysia (Ngoi and Thong, 2013) which commonly was isolated from food and animal sources (Thong *et al.*, 2011), retail poultry samples (Rusul *et al.*, 1996) with raw or undercooked poultry meat and eggs existed as a great threat for infection in humans (Gillespie *et al.*, 2005). The pathogen can cause mild to acute gastroenteritis as well as other common symptom of food poisoning when infection takes place in human body. Conventional culture methods for detection of *S.* Enteritidis are known for its laborious and time-consuming, which take

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4-7 days to obtain results. This has encourage the development and use of other techniques that offer faster result time with better sensitivity such as the one obtained with the amplification of nucleic acid. Polymerase Chain Reaction (PCR) is one of nucleic acid amplification technologies that has become increasingly important to permit rapid and sensitive detection tools for diagnostics of bacterial pathogen (Lungu *et al.*, 2012; Paião *et al*., 2013).

Thus, the purpose of this study was to establish a PCR protocol in order to detect *S.* Enteritidis in a simulated environment of artificially-infected chicken meat samples and to elucidate the effect of pre-enrichment on the PCR detection. In this study, we reported the detection of *S.* Enteritidis by PCR using ENT primers targeting at 303 bp of *sdfI* gene in raw chicken meat artificially-contaminated with various concentration of *S.* Enteritidis culture. Gene *sdfI* is a chromosomal region which related to the invasiveness and infection of poultry and eggs (Agron *et al.*, 2001) and the sequence of the ENT primers used in this study is specific to *S.* Enteritidis (Alvarez *et al*., 2004). Thus, ENT primers targeting this gene fragment was used in this study to determine its sensitivity towards Enteritidis serovar and its specificity with some important *Salmonella* serovar.

Materials and Methods

Bacterial strains and culture conditions

Reference culture of *S.* Enteritidis used in this study was isolated locally from chicken meat. Culture from ATCC, *Salmonella* Typhimurium ATTC™ 53648, *Salmonella* Pullorum ATTC™ 10398 and *Salmonella* Gallinarum ATTC™ 9184 were used as reference serovar strains for ENT primers specificity evaluation. All *Salmonella* serovar in this study were cultured for 24 h in TSB medium (Oxoid) and incubated at 37°C, 150 rpm. These serovars will be used for primer specificity tests. For artificial inoculation of chicken meat samples with *S.* Enteritidis, ten-fold serial dilutions of the overnight *S.* Enteritidis culture were prepared in buffered peptone water (BPW, Oxoid). To determine the number of cells for inoculation, 100 µL of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilution culture were spread-plated on tryptone soy agar (TSA, Oxoid) in triplicate and incubated at 37°C for 24 h. The numbers of colonies formed were recorded for CFU/mL calculation.

Inoculation of raw chicken meat samples

Chicken meat breasts were purchased from Giant hypermarket. Ten grams of each meat sample was weighed and transferred in a sterile 400 mL stomacher bag with filter (Bagfilter® 400 mL, Interscience) and inoculated with 100 µL of each *S.* Enteritidis dilutions. An uninoculated negative control was prepared by adding 100 µL of sterile BPW into corresponding sample.

Enrichment and crude DNA extraction

Following inoculation with *S.* Enteritidis culture, 90 ml of sterile BPW was added to each meat samples and homogenized at 230 rpm using stomacher (Stomacher®400 Circulator, Seward) for 1 min. Each homogenate was transferred to a sterile 250 mL Erlenmeyer flask and were separated into two groups, one without incubation and another one with incubation at 37°C for 24 h prior to performing extraction of the crude DNA from the respective liquid homogenates. Crude DNA extraction was performed on each homogenate and on each *Salmonella* strain of pure cultures according to boiling cell-lysis by Bäumler *et al.* (1997) but with a slight modification. A 1 mL portion of the homogenate and cultures was centrifuged at 15,000 g for 4 min. The pellet was resuspended in 500 µL sterile distilled water and vortexed vigorously. The cell suspension was boiled for 10 min, immediately chilled on ice for 10 min and centrifuged again at 15,000 g for 4 min. The supernatant containing crude DNA was transferred into a new 1.5 mL tube and 5 µL was used as DNA template in PCR.

Primers and PCR amplification

Primers, ENT-F and ENT-R (Alvarez *et al*., 2004) for PCR were chosen to be used in this study after evaluation of its serovar specificity was carried out by *in silico* PCR amplification program (insilico.ehu.es) against all *Salmonella* genome database and additionally by BLAST program (ncbi.nlm.nih.gov). Primers sequence used in this study are ENT-F, 5'-TGTGTTTTATCTGATGCAAGAGG-3' and ENT-R, 5'-GAACTACGTTCGTTCTTCTGG-3', obtained from *sdfI* gene sequence with a theoretical PCR product of 303 bp. A total reaction volume of 25 µL of PCR mixture using 1X PCR master mix (Dreamtaq polymerase, Thermoscientific) containing 2 mM $MgCl_2$, 0.025U/µL Taq DNA polymerase and 0.2 mM of each dNTP; 0.5 µM of each forward and reverse ENT primer, 5 µl of crude DNA template and nuclease-free water adjusted to a total volume of 25 µl. PCR reaction was performed in a thermocycler (DNA Dyad, BioRad). The thermocyler was programmed by preheated 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C and final extension for 4 min at 72°C. A 5 µL of PCR product was taken for analysis by electrophoresis on

2% agarose gel and stained with 0.3 µg/mL ethidium bromide for visualization of the amplicons under UV light gel documentation system (Alpha Imager®, Alpha Innotech).

Results and Discussion

The concentration of *S.* Enteritidis for artificial inoculation in raw chicken samples was determined by plate count method on the TSA. Plate 10^{-6} dilution was selected for plate counting as the colonies grown on the plate was ranging between 25-250 colonies which are recommended as countable range according to US Food and Drug Administration Bacterial Analytical Manual (BAM). The *S.* Enteritidis stock culture concentration was calculated to be 9.4 X 10^8 CFU/mL. Based on the *in silico* PCR amplification results (Figure 1), the ENT primers generated theoretical product of 303 bp (lane 6) with *S.* Enteritidis when tested with all (27) strains of *Salmonella* genome database. Additionally, BLAST result showed that both ENT-F and ENT-R primers have 100% sequence similarity to *Salmonella enterica* subsp. *enterica* serovar Enteritidis accession no. AF370707.1, AF370716.1 and AM933172.1. Based on the *in silico* and BLAST results, the ENT primers were predictedly specific to the *sdfI* gene of *S.* Enteritidis bacteria. In the actual PCR reaction, we demonstrated that samples from *S.* Enteritidis crude DNA was successfully amplified, generating a specific 303 bp product and no amplification was observed from the selected *Salmonella* serovars; *S.* Typhimurium, *S.* Pollarum and *S.* Gallinarum. This findings of specificity is similar to the PCR result conducted by Alvarez *et al*. (2004) and proven the accuracy prediction of *in silico* PCR amplification and BLAST tools which aid in the primer sequences evaluation. One of the critical points of the primer sequence for a specific target organism is the primers sequence to be amplified must be unique to the targeted serovar and must not present any homology with other organisms. The PCR results verified that the sequences targeted by ENT primers was only present in *S.* Enteritidis serovar which confirmed the specificity of the ENT primers against the particular serovar (Figure 2).

In this study, comparison of PCR results was performed between crude DNA template from samples spiked with serial concentration of *S.* Enteritidis cells and homogenized in BPW which taken immediately without 24 h incubation (Figure 3) and pre-enriched samples homogenate after 24 h incubation (Figure 4). The results summary from Table 1 shows that without pre-enrichment step, the PCR has limit of

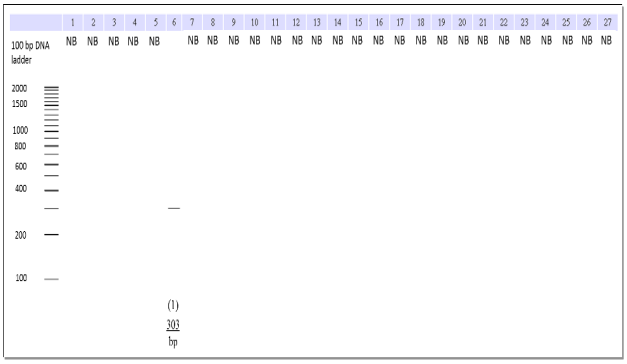

Figure 1. In silico PCR amplification of ENT primers against strains of *Salmonella* spp. NB : No bands

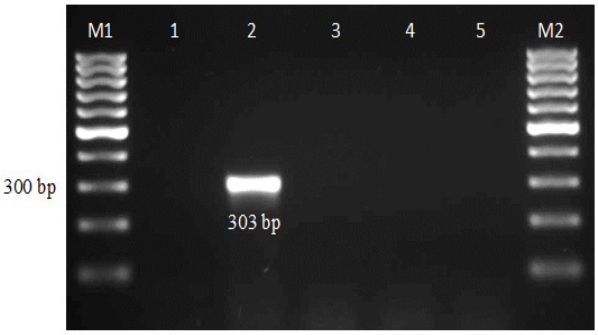

Figure 2. PCR Specificity of ENT primers with crude DNA from selected *Salmonella* serovars. Lane M1 & M2: 100 bp DNA ladder (Fermentas), 1: Negative control, 2: *S.* Enteritidis, 3: *S.* Typhimurium, 4: *S.* Pollarum and 5: *S.* Gallinarum

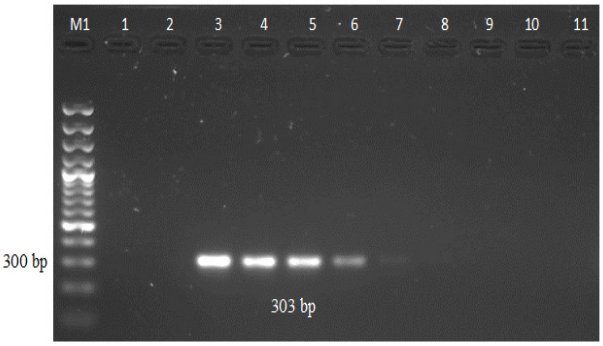

Figure 3. PCR Sensitivity for *S.* Enteritidis detection in chicken meat samples homogenate (without 24 h incubation). Lane M1: 100 bp DNA ladder plus (Fermentas), 1 : Negative control (no DNA template) 2: Negative control (spiked with sterile BPW), 3 to 9: Raw chicken meat samples inoculated with *S.* Enteritidis culture; 3: 9.4 X 10^8 CFU/mL, 4: 9.4 X 10^7 CFU/mL, 5: 9.4 X 10^6 CFU/mL, 6: 9.4 X 10^5 CFU/mL, 7: 9.4 X 10^4 CFU/mL, 8: 9.4 X 10^3 CFU/mL, 9: 9.4 X 10^2 CFU/mL, 10: 9.4 X 10^1 CFU/mL and 11: 9.4 X 10^0 CFU/mL *S.* Enteritidis culture

sensitivity at 9.4 X 10^4 CFU/mL as a faint band was observed at lane 7 (Figure 3). Cells concentration that is lower than this could not be amplified. On the contrary, with the pre-enrichment step, the limit of sensitivity has increased to 9.4 X 10^1 CFU/mL of the initial cells concentration inoculated into the chicken

Table 1. Comparison of PCR detection in chicken samples artificially-contaminated with serial cells concentration with *S*. Enteritidis between samples homogenate in BPW without 24 h incubation and pre-enriched samples after 24 h incubation at 37°C. ('-' : negative PCR result and '+' : positive PCR result)

Homogenate samples and Inoculated cells concentrations	Negative control (no DNA template)	Negative control (spiked with BPW)	**Spiked-cells concentration (CFU/mL), (9.4 X a CFU/mL) Whereby, a is the exponential value below:**								
			10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
Non-incubated samples without 24 h incubation	-	-	-	-	-	-	+	+	+	+	+
Pre-enriched samples after 24 h incubation	-	-	-	+	+	+	+	+	+	+	+
Lane	1	2	3	4	5	6	7	8	9	10	11

meat samples. The limit of sensitivity of the PCR was found to be more sensitive compared to previous PCR performed by Paião *et al*. (2013) at 10^2 CFU/mL and also ELISA method performed by Brooks *et al*. (2012) at 10^5 to 10^6 CFU/mL of *S*. Enteritidis culture.

The inclusion of the pre-enrichment step has tremendously gain the PCR sensitivity to 1000-fold of the cells concentration present in the artificially-inoculated samples and in accordance to the result by Myint *et al.* (2006). According to ISO 6579, food sampling to detect *Salmonella* necessitates the pre-enrichment of samples in BPW in order to recover the possible sub-lethally injured cells from the effect of food processing (Suo and Wang, 2013) and this is agreed by the study previously conducted by Schelin *et al*. (2014). In conclusion of the pre-enrichment step grants advantages over the direct extraction of crude DNA, since the BPW that serves as the pre-enrichment broth are relatively inexpensive, increase the number of bacterial cells to overcome the mixed micro flora in the samples and possibly dilute substances in food samples such as organic compounds, lipids, polysaccharides, protease pectin, and metal ions which could inhibit PCR reaction (Schrader *et al*., 2012).

Conclusions

The ENT primers had successfully amplified the targeted fragment of *sdfI* gene by PCR yielding 303 bp product in this study. These primer were specific to *S*. Enteritidis and is highly sensitive when coupled with pre-enrichment of samples in BPW which allow detection as low as 9.4 X 10^1 CFU/mL of inoculated *S.* Enteritidis in 25 g of pre-enriched chicken meat samples. It is anticipated that low number of targeted cells present in food samples can be detected using this technique which indicate its good applicability in diagnostic routines. The high sensitivity as well as the reduction in time and labour makes the PCR assay as an excellent alternative to conventional culture methods for the purpose of diagnostics, surveillance and generally to improve food safety.

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