



Estimation of limit of detection of *Salmonella typhimurium* in artificially contaminated chicken meat by cultured-based and polymerase chain reaction techniques

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Abstract

The objective of this study was to develop Polymerase Chain Reaction (PCR) procedure for detection of *Salmonella Typhimurium* in artificially contaminated chicken meat. The experiments were conducted with various dilutions of *Salmonella Typhimurium* reference the American Type Culture Collection ATCC (ATCC13311TM 4.4*10⁷) High concentration 4.4*10³ Colony Forming Units (CFU)/ml, low concentration 4.4*10² CFU/ml, very low concentration 4.4*10¹ CFU/ml inoculated in chicken meat, in order to determine limits of detection (LOD), optimum incubation times 18 to 20 hours of pre-enrichment in Buffered Peptone Water (BPW 1%). Hence, cultural methods and DNA extraction were performed according to kits instruction. The microbiological cultural test was capable to detect 1.76 CFU/mL, whereas PCR examination was able to detect 0.18 CFU/ml of initial dilution of *Salmonella Typhimurium* inoculated in chicken meat. Interestingly, the results were achieved in a less time period than that of classical culture. The PCR technique is beneficial in the methodology for detection of *Salmonella* in chicken meat.

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Introduction

Salmonella is one of the most important pathogen that can infect human through ingestion of contaminated food with a sub-classification of more than 2500 serovars including *Typhimurium* related to foodborne salmonellosis in human (1,2). This infection can be harsh particularly in immunosuppressive people like youngest or elderly people. It is estimated that the possible infection dose in the healthy individuals is very low and between 10⁵ to 10⁷ colonies of the bacterium (3). In many different developing countries, the numbers of food-borne infection cases are increasing. Almost all of them connected with the consumption of contaminated chicken products. In a survey performed by the Centre of Diagnostics and Research in Avian Pathology (CDPA) on a total of 1300 carcasses of chicken that tested for *salmonella* showing that the percentage of contaminated chicken meat was more than 17% (4,5). In addition, other

prospective study has confirmed that the contamination rate of this pathogen was about 10% (6). It is crucial to take actions for prevention of getting this foodborne infection based on standard quality control and detection techniques, so that improvement of food safety technologies related to salmonellosis. Nowadays, the cultured-based detection procedure is still used as a main way for the detection of *salmonella* in food products. In addition, *salmonella* should not be detected in 25g of food samples according to a number of governmental standard regulations and recommendations (7). However, this kind of techniques could be time consuming and not sensitive enough to detect low-abundance of the level of contamination (8). New methods have been introduced to get better results and get more advantages than traditional ways. PCR is a major molecular biology assay and considered as a novel and sensitive technique in the field of laboratory diagnostics (9). This new technology allows detecting of various food-

borne pathogens including *salmonella* in very low bountiful (10,11). However, the PCR assay has some drawback as false-positive may occur. Nevertheless, PCR can minimize the time for detection and identification of food-borne pathogens in food products. It is well known that the *InvA* Protein A gene (*InvA*) has a unique sequence encoding for *salmonella* spp. (12). Therefore, a pair of primers was used in the current study that specific for detection of the gene *InvA*.

The aim of the present study is to determine the LOD₅₀ (contamination level at which 50% of the samples are found positive) of *Salmonella Typhimurium* in artificially contaminated chicken meat and to compare the detection limits from the results of both cultured-based traditional and PCR assays.

Materials and methods

Bacterial strains and inoculation of food matrix

Aliquots of *Salmonella Typhimurium* ATCC strain 13311 CAT. No. 7E21 and *Escherichia coli* (*E. coli*) ATCC strain 51813 were used as reference strains in this study. *Salmonella Typhimurium* and the non-*Salmonella* bacterial strains information used in this study. These strains were cultivated into Tryptone Soya Broth (TSB, HIMEDIA, India) at 37°C for 24 hours for confluent growth.

Sample preparation

One hundred twenty samples of chicken meat were used in this research for artificial contamination. From each sample, 25 grams were weighed in sterile bags (Seward Medical Stomacher® 400 sterile bags). To this, 225 mL of BPW 1% were added and homogenized in a Stomacher (Laboratory Blender Stomacher 400, Seward Medical, UK) for about 30 seconds. This mixture was then inoculated with 1mL of high concentration of bacterial load 4.4×10^3 CFU/ml and low concentration of bacterial load 4.4×10^2 CFU/ml and very low concentration of bacteria 4.4×10^1 CFU/ml of dilution of bacterial strains (13).

Culture method

After 18 hrs. of pre-enrichment in Buffered Peptone water (BPW), isolation of *Salmonella Typhimurium* was conducted according to 6579-1:2017 procedure (13,14).

Briefly, 0.1 mL from the pre-enrichment solution was transferred separately into two different tubes containing 10 mL of Rappaport-Vassiliadis broth (Merck, USA) and into 1.0 mL of Mueller Kauffman Tetrathionate Novobiocin Broth Base for selective enrichment and incubated at 41.5°C and 37°C for 21 hrs. respectively. One loopful of broth cultures were streaked onto Xylose Lysine Deoxycholate (XLD) agar (Lab-M, UK) and CHROMagar (Oxoid, UK) and incubated for 21 hrs. at 37°C. Purification of colonies was done on nutrient agar. In addition, the suspected colonies were further confirmed by using biochemical and serological tests according to ISO 6579-1:2017.

DNA extraction by lysis buffer

The extraction of DNA from *S. Typhimurium* was adapted from a previous study (15). One ml of broth from the pre-enrichment were transferred into a tube and centrifuged at 10000 rpm for 5 min and the supernatant was discarded. The pellet was suspended in 500 mL of lysis buffer (containing 0.1 Mm Ethylenediaminetetraacetic acid (EDTA), 50 Mm Tris-Hcl, PH 8.0, 1% TRITON-X-100, 0.5% Tween-20) and vortexed for 10 second. The samples were placed on a thermal block (Multi-Blok Heater, Baxter, and EUA) at 95°C for 10 min. The mixture was centrifuged for 20s at 5000 rpm and the supernatant was stored at -18°C for 10 minutes. Finally, the PCR assay was done at the same day of extraction.

PCR procedure

PCR was carried out according to kit manufacture instructions. The procedure was adopted from a previous work that studied on more than 600 strains of *salmonella* in chicken (15). The thermal cycler that used in the current study was set in an Applied Bio-systems ABI GeneAmp PCR System 9700 thermal cycler. Samples were denatured at 94°C for 5 min as pre-holding step. Thirty-five cycles of amplification were run for 30s at 94°C, 30 s at 54°C and 30 s at 72°C, with the final extension continuing for 7 min. Twenty-five microliter aliquots of the reaction mixtures were electrophoresed through 1.5% agarose gel and fragments were revealed by staining with DNA safe dye (16) (Table 1).

Table 1: Primers that are used in this verification procedure for *S. typhimurium*

<i>S. typhimurium</i> primer	Length	Primer sequence 5' → 3'
<i>InvA</i> forward	22 bp	CGG TGG TTT TAA GCG TAC TCT T
<i>InvA</i> reverse	21 bp	CGA ATA TGC TCC ACA AGG TTA

Statistical analysis

For the performance of a qualitative method, sensitivity, specificity and LOD₅₀ were calculated. LOD₅₀ was calculated using a program in Excel as described in ISO that is freely available on the internet (17). The calculation

of sensitivity and specificity (%) is described below. Sensitivity= $(N+/N) \times 100$, where *N+* is the number of positive results at a low/high contamination level of the pathogen and *N* is the total number of analyzed samples at this level. Specificity= $(N-/N) \times 100$, where *N-* is the number

of negative results at blank level and *N* is the total number of analyzed samples at this level.

Results

Culture methods

It can be seen that this method was not able to recover very low concentration contaminated chicken (i.e less than 1 CFU/ml). However, this technique was able to detect 38 samples out of 40 (95%) from low concentration and 40 out of 40 (100%) from high concentration (10-50 CFU/ml). There were no false positives recorded in this experiment (Table 2). When inoculated on XLD agars, the different concentration showed the different recovery concentration growth of the pathogen (Figure 1).

Molecular detection

The sensitivity of the primers is 100% when the load of the pathogen in more than 1-5 CFU/ml in a sample and 75% when there is less than 1 CFU/ml (Table 3). The specificity of primer pairs used in the current study showed through the results of the PCR technique in detection of particular organisms in chicken. The specificity of the primers was 100% as the negative samples were non-detectable. All the samples that inoculated with high concentration of bacterial load were detectable by this particular set of primers. In addition, all the positive samples were analyzed and confirmed as bands by using gel electrophoresis from a composition of 1.5% of agarose. According to the position of this gene and detection primers, the band should be appearing around 284 bp (Figure 2).

Table 2: Cultured-based method results show the sample number and inoculation contamination level of *S. typhimurium*

No	Chicken samples	Contamination level	Positive reference	Negative reference
1	40	Less than 1 CFU/ mL	0/40 (0.0%)	Not detected
2	40	1-5 CFU/ mL	38/40 (95%)	Not detected
3	40	10-50 CFU/ mL	40/40 (100%)	Not detected

Table 3: PCR results show the sample number and inoculation contamination level of *S. typhimurium*

No.	Chicken samples	Contamination level	Positive reference	Negative reference
1	40	Less than 1 CFU/ mL	31/40 (75%)	Not detected
2	40	1-5 CFU/ mL	40/40 (100%)	Not detected
3	40	10-50 CFU/ mL	40/40 (100%)	Not detected

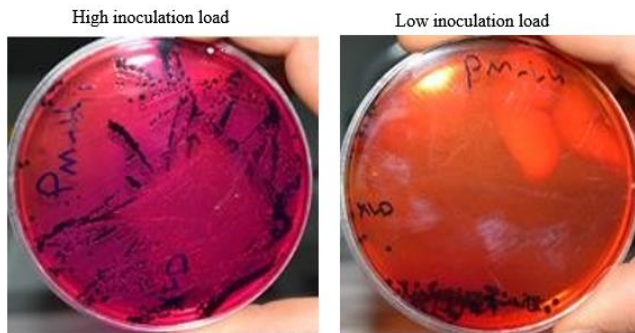


Figure 1: High and low bacterial inoculations on XLD agars. Typical colonies are red with black centers.

Limit of detection

Estimation the detection limit of *S. typhimurium* was determined according to previous interlaboratory data that are available online. In that program, the contamination level of each test portion of the samples has to be provided in CFU/g or CFU/ml. Simply, the lower inoculation levels of each test samples were divided by the total amount of test portion in a tube. In fact, the current study used 25 g of the sample plus 225 ml of BPW and the total amount in a

bottle was 250 g. hence, low inoculation level for the traditional method and PCR assay that were detectable was 4.4×10^2 CFU/ml and 4.4×10^1 CFU/ml, respectively. If these numbers divided by 250, there will be 1.76 and 0.18 log CFU/ml for cultured-based traditional and PCR methodologies, respectively.

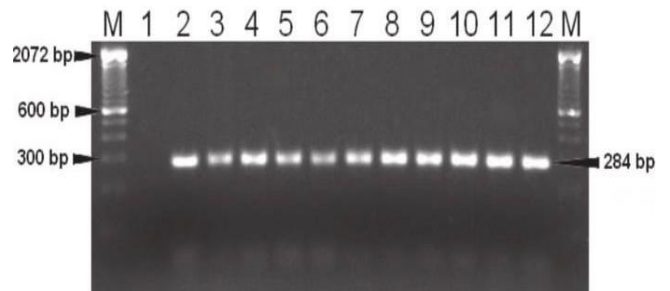


Figure 2: Gel electrophoresis for detection of *InvA* gene at 284 bp encoding *Salmonella Typhimurium* in artificially contaminated chicken meat. Image of PCR product running on 1.5 % of agarose gel for 90 minutes at 120V. M: DNA marker, Lane 1: Negative control, Lanes 2-12: Artificially positive samples.

Discussion

According to the ISO 6579-1:2017, the detection limit is 0.22-0.72 log CFU/ml for chicken meat (18). In the current research, the LOD of 0.39 log CFU/ml was scored for artificially contaminated chicken meat which is very close to the reference method. AENOR 16140, laboratory validation has stated that reference method for cultural growth with limit of detection of 0.40-1.02 CFU/g (19). In this particular aspect, these results are in accordance with those from AENOR 16140, laboratory validation method for detection of *Salmonella*. The findings of the study showed more than 95% of artificially contaminated sample were detected for the presence of low bacterial concentration in range of 1-5 CFU/ml. Not surprisingly, all samples were detected for the higher load of bacterial concentration in conventional methods with the detection limit of 100%. In the current findings, a pair of primers was applied to detect *salmonella typhimurium* serovar simultaneously using conventional PCR. The pair of the primers used to find *Salmonella* at genus level codifies the gene *InvA* that is widely distributed in *Salmonella* spp. (5,20,21). In addition, the reference procedure of the ISO numbered 6579-1:2017 used as a complementary test to PCR for detection the limit of *salmonella* conducted for all the examined samples.

These assays were established using artificially contaminated chicken meat samples after 16 hours of pre-enrichment and using the bacterial DNA extraction kit. In the current study, the PCR assay was able to detect a cell suspension of 1 CFU/ml (about 1 CFU per reaction) with a probability of 75% and a cell suspension of 10¹ CFU/ml (5 CFU per reaction) with a probability of 100%. These findings are in agreement with a previous research (22) where it found (5 CFU per reaction) with a probability of 70% and a cell suspension of (50 CFU per reaction/ml) with a probability of 100%. In terms of pre-enrichment step, the duration was approximately 16 hrs. and this was sufficient for the recovery and subsequent multiplication of sub-lethally injured *Salmonella* to be in detectable levels in the real-time PCR. A previous study has observed that the duration of 20 hrs. as a pre-enrichment step is sufficient for recovery of *Salmonella* spp. (22).

Conclusion

Fast detection of pathogens in food products is critical for the diagnosis of food poisoning and monitoring of food safety. Our studies represent the first report of using enrichment culture- based and PCR methods to detect LOD of artificially inoculated *Salmonella Typhimurium* in chicken in Duhok city of Iraq. The molecular detection method is efficient and reliable with higher sensitivity and specificity when compared to microbiological culture.

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Conflict of interest

Authors declare no conflict of interest.

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تقدير الحد الأدنى للكشف عن السالمونيلا التاييفوريوم في لحوم الدجاج الملوثة اصطناعياً باستخدام تقنية تفاعل سلسلة أنزيم البلمرة وبطرق تقليدية

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الخلاصة

كان الهدف من هذه الدراسة هو تطوير إجراء تفاعل سلسلة أنزيم البلمرة للكشف عن السالمونيلا التاييفوريوم في لحوم الدجاج الملوثة بشكل مصطنع ومقارنة النتائج مع الطرق التقليدية. تم إجراء الطرق التقليدية واستخراج الحمض النووي وفقاً لتعليمات شركات المنتجة للمواد المستعملة. كان الاختبار التحليل التقليدي الميكروبيولوجي قادراً على اكتشاف ١,٧٦ وحدة تشكيل المستعمرة من الجرثوم لكل مليلتر في حين كان فحص تفاعل السلسلة المتبلرة قادراً على اكتشاف ٠,١٨ وحدة تشكيل المستعمرة من الجرثوم لكل مليلتر من التخفيف الأولي للسالمونيلا التاييفوريوم المملح في لحم الدجاج. من المثير للاهتمام أن النتائج تم تحقيقها في فترة زمنية أقل من الطرق التقليدية الكلاسيكية. تقنية تفاعل السلسلة المتبلرة لها دور هامة ومفيدة في الكشف عن السالمونيلا في لحوم الدجاج.