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A Comparison between Two Kinds of Primers for Detection of *Salmonella* in Artificial Contamination Beef Meat in Iraq and Confirm the Results by Sequencing Alignment

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***Abstract* – Two primer was compared against each other for the maximum efficiency of detecting salmonella species using PCR assay in Iraq, the first one adopted from the research of Rahn [3] which is targeting the *InvA* (284 bp) genein all *salmonella* species. The second adopted from the research of Jitrapakdee [2], which is targeting a snap of 199 bp, were success to detecting all 52 *salmonella* serovars prevalent in Thailand. Beef meat sample were bought from retail market in Baghdad and undergone an artificial contamination with *salmonella* dilutions ($4x10^5$, $4x10^3$, $4x10^1$, $4x10^{-1}$ cell/ml) with enrichment period for (0, 6, 18 and 24) hour in 37oC respectively. PCR test was sensitive for all dilution concentrations of artificial contamination under all enrichment periods for the two primers. However, bands obtained from the primer of Rahn [3] were more obvious than bands of the primer of Jitrapakdee [2] and 24 hours as an enrichment period look like an adequate period to obtain a clear enough band for the two primers. All samples of bacteria have sequencing ID gb|CP011394.1 and 98% identities, score 243 with wild type of *Salmonella enterica* from the Gene Bank that leads to changes in amino acid from Aspartic acid (D) to Serine (S).**

***Keywords* – *Salmonella*, *InvA*, Artificial Contamination, McFarland.**

I. INTRODUCTION

Food-borne transmission of salmonellosis is of increasing concern to many countries, including Iraq. This problem has brought attention to the need for a rapid and sensitive detection assay for *salmonella* in food. The conventional culture methods of detecting *salmonella* are time consuming (4-5 days), labor intensive and costly. Alternative methods have been developed including enzyme-linked immunoassay, monoclonal antibody-based assay and DNA hybridization; some assays still require a prolonged enrichment period whilst others suffer from low sensitivity which has limited their acceptance[2].

Polymerase chain reaction (PCR) provides a means to circumvent these problems by permitting enzyme replication of a preselected region of DNA and thus gives a highly sensitive and specific detection procedure. The PCR technique has been used effectively to detect many food-borne bacteria including *salmonella*[3] by amplifying a sequence with the *Inv A* gene of *S.typhimurium* as a means of detecting *salmonella* but a low level of sensitivity and a few false-negative results were observed. Reference [2] have developed a PCR-based assay for the detection of *Salmonella* by amplifying a *salmonella* specific fragment using primers derived from a DNA clone constructed from *S. Weltevreden* chromosome. They were success to detecting all 52 *salmonella* serovars prevalent in Thailand. This study is to compare the effect and sensitivity of two kinds of primers (Rahn primer and Jitrapakdee primer) for detection the *salmonella* contamination by applying them on artificial contamination beef meat in Iraq.

II. MATERIALS AND METHODS

This study was conducted in the Collage of Agriculture/ University of Baghdad with cooperation from Biotechnical Researches Center/ Annahrain University for PCR tests.

Bacteria Culture

An isolated culture of non-typhoid *salmonella* was obtained from the Veterinary Collage / University of Kofa in betri dish streaked onto nutrient agar. The culture was isolated according to the method cited in [4]. Bacteria identity was confirm by culturing it in *salmonella shigella* agar (SSagar) and incubating for two days in 37^{o}C, differentiated transparent colonies with black center on SS agar surface was obtained. Then bacteria was cultured again in ten betri dish with nutrient agar at 37^{o}C for two days until harvesting it with buffered peptone water (BPW), to get an adequate amount.

To confirm the identity of bacteria the traditional isolation method was conducted. 25g from each sample was added to 225 ml of buffer peptone water, all samples were incubated for 36 hour. One ml of each culture was transferred to Rappaport-vassiliadis broth and selenite F broth, then, incubated at 42^{o} C and 37^{o}C for 24 and 48 hour of incubation respectively. One loopful from each of enriched broth was streaked onto plates of *salmonella shigella* (SS) agar and xylose lysine deoxycholate (XLD) agar, incubated at 37^{o}C for 24 hour. The plates were examined for the presence of typical colonies of *salmonella* transparent colonies with black center in SS agar and red colonies with black center in (XLD) agar. Approximately 5 days are required to identify the identity of the bacteria strain without serological tests [4].

Preparing of McFarland standard solution

According to [7], to estimate the gram-negative bacteria concentration in a suspension, McFarland standards can be used to visual approximation. McFarland Standards are generally ranged from 0.5 to5 or to 10 or more, according to need. The standards were made by preparing a 1%

solution of anhydrous $BaCl_2$ and a 1% solution of H_2SO_4 mixed in the proportions listed in the Table (1). They should be stored in the dark, at 20-25°C, for a period less than approximately six months.

Artificial Contamination

After the harvesting of bacterial culture, the bacteria was suspended in buffered peptone water 2% which was prepared by dissolving 20g of peptone (LAB140) into one liter of deionized distilled water and heating for 121°C and leaving it to cool. Then, 10g of phosphate buffer saline (PBS) was added to keep pH in optimum range (7.0-7.2) during the experimental period. By adding BPW gradually to a random aliquot (may be 5-10 ml) of the liquid of suspended bacterial cells with comparison with

Table 1: McFarland Standard Solution.

McFarland Scale	Cell (x10^{8}) /ml	1% $BaCl_2$/1% H_2SO_4 (ml)
0.5	**1.5**	**0.05/9.95**
1	**3**	**0.1/9.9**
2	**6**	**0.2/9.8**
3	**9**	**0.3/9.7**
4	**12**	**0.4/9.6**
5	**15**	**0.5/9.5**

(Sutton, 2011).

McFarland scale number 1 (3x10^{8} Cell/ml) optically, an initial concentration (3x10^{8} C/ml) from suspended bacterial liquid was obtain. An aliquot of 10 ml of this initial liquid was adjusted again by diluting it with 30 ml (3x10) of clear 2% BPW to obtain the first demanded dilution (1x10^{8} C/ml). The procedure continues by diluting 1ml of the last solution with 100ml (1x10^{2}) of clear 1% BPW to get (1x10^{6}) cell per ml of liquid media, by repeating this process two time more, there will be four separated demanded dilutions (10^{8}, 10^{6}, 10^{4}, 10^{2} cell/ml) ready for use.

By following the issues described in [6], four samples of minced beef meat were purchased from locally markets in Baghdad, 25 gram from each sample were weighted carefully and inoculated with 1ml of one of the dilutions respectively. After waiting for a while to insure the penetration of the solution inside meat tissue, 225ml of 1% BPW was added to the samples, homogenized carefully for 30 seconds. Because the final 225 ml of liquid media was added, the new concentrations will be approximately (4x10^{5}, 4x10^{3}, 4x10^{1}, 4x10^{-1} cell/ml).Four aliquots from each solution were taken to undergo pre-enrichment step for (0, 6, 18 and 24) hour in 37°C respectively. After that, they were frozen until PCR assay would be performed.

DNA Extraction

Table 2:Geneaid, GBB 100 kit Component.

Component	Amount (ml)
Gram + Buffer	30 ml
GT Buffer	30 ml
GB Buffer	40 ml
W1 Buffer	45 ml
Wash Buffer (Absolute ethanol should be added)	25 ml (100)
Elution Buffer	30 ml
GD column	100 peaces
2mlcollection tube	200 peaces

(Geneaid, 2015)

DNA of *salmonella ssp* were extracted directly from the suspended meat sample using mini gDNA Bacteria kit [1](table2).

The procedure was as follows:

1- Aliquots of 1 ml from all *salmonella* artificial contamination solutions and/or from all natural contamination solutions were transferred to a micro-centrifuge tube for DNA extraction. Centrifuged at 15000 rpm for 1minute, supernatant was discarded. 200µl of GT buffer was added, shaken vigorously to re-suspend the billets by using pipette, then incubated at room temperature for 5 minutes.

2- 200µl of GB buffer was added to the sample, shaken vigorously, Incubated at 60° C for at least 10 minutes with inversion from side to side every 3 minutes.

3- 200µl of absolute ethanolwas added to the sample, mixed well immediately by pipette. The mixture then was transferred to GD column and centrifuged at 15000 rpm for 2minutes then, contents of the collection tube were discarded and replace with new collection tube.

4- 400µl of W1buffer is added to the GD column. Centrifuged at 15000rpm for 30 second and the flow-through is discarded. Then, 600µl of wash buffer was added (absolute ethanol was added before that to wash buffer) to the GD column. It was centrifuge at 15000rpm for 30 second, the flow-through was discarded again, and then centrifuged at 15000 rpm once more for 3minutes.

5- Dried GD column is transferred to a new 1.5ml micro-centrifuge tube. 100µl of pre-heated was added (Elution buffer should be prepare before, by heating it to 60°C), wait for 3minutes to allow elution buffer to be absorbed, centrifuged at 15000rpm for 30 second to elute the purified DNA. The eluted genomic DNA is stable and can be used directly, or stored in freezing condition for later analysis.

PCR Assay

Primers

The two primers were applied; the first was adopted from Rahn [3] for the gene *invA* in *salmonella ssp*. The second is a primer for a deferential snap of DNA adopted from Jitrapakdee [2]respectively;lyophilized primers were dissolved in a sterile distilled water to give a final concentration of 10 pmol/µl according to ALFA. Both illustrated in table (3).

Reaction set up

The optimization of amplification was performed as follows; Two target sequences of the genomic *salmonella ssp* DNA coding for *invA* [3]), and the differential DNA snap [2] were demanded. Multiplex PCR was performed with Bioneer (AccuPower PCR PreMix) master mix in 25µl as a total volume table (4).

PCR

PCR-amplification was done via using The amplification reactions carried out in a PCR thermal cycler. Then, the productswere stained by ethidium bromide and photographed. Cycling conditions are presented in table (5).

Agarose Gel Electrophoresis

Agarose gel was used to separate DNA fragment after PCR or to confirm total DNA existing after extraction. 1% or 1.5% agarose concentrations (w/v) were used respectively. Agarose gel was dissolved in (1X) TBE buffer and heated until liquid become clear again, allowed to cool down reaching to 50-60^{o} C. then the gel would be poured in casting tray with the wells comb positioned in one end of the tray. After cooling down to the room temperature, comb would be removed carefully and the gel replaced into electrophoresis chamber. Samples of total DNA were mixed with loading dye 3/6 (v/v) and loaded by small pipette into the gel wells. No loading dye was used in checking steps of PCR products, because the Master Mix reaction buffer contains a dye already.

Table 3: Primers sequences.

Reference	Sequences 5‘- 3’		No of Bases
Rahn*et al.*, (1992)	F	GTG AAA TTA TCG CCA CGT TCG GGC AA	26bp
	R	TCA TCG CAC CGT CAA AGG AAC C	22bp
Jitrapakdee *et al*,. (1995)	F	GAT CAT CCA TTC GGC ATT AAA CA	23bp
	R	CTC AGC GAC GGA AGG GTA AAT C	22bp

Table 4: Component of PCR reaction:

Components			**Volume (µl)**
Total DNA			1.5
Primers: 1µl for each primer			4
Master mix	Top DNA polymerase	1U	5
	Each dNTP (dATP, dGTP, dTTP)	250µM	
	TrisHCl (pH9.0)	10mM	
	KCl	30mM	
	$MgCl_2$	1,5mM	
	Stabilizer and tracking dye		
Distilled			14.5
Total volume			25

Table 5: The cycling conditions.

Step	**Temperature ^{o} C**	**Time**	**No. of Cycle**
Denaturation 1	94^{o}C	3 min.	1 cycle
Denaturation 2	94^{o}C	1 min.	35 cycles
Annealing	53^{o}C	1 min.	
Extension	72^{o}C	1 min.	
Final extension	72^{o}C	5 min.	1cycle

The gel was entirely drawn in TBE buffer solution, and electrophoresis was run for 1hour to check total genomic DNA and 1.5 hour for PCR products analysis at 5V/cm (approximately 70 volt per one run). Agarose gels were stained with ethidium bromide 0.5g/ml for 30 minutes. DNA bands were visualized under U.V transilluminator at 365 nm wavelength. A gel documentation system was used to document the observed bands[5]. A 100bp DNA ladder from BIONEER was used as a marker for PCR products.

Sequence alignment

Sequencing of *invA* gene and differential DNA snap was performed by national instrumentation center for environmental management (nicem). Online at (http://.snu.ac.kr/main/?en_skin=index.html).

Biotechnology lab, machine is DNA sequencer 3730XL, Applied Bio system), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program.

III. RESULTS AND DISCUSSION

PCR test

PCR test was sensitive for all dilution concentrations of artificial contamination (4x10^{5}, 4x10^{3}, 4x10^{1}, 4x10^{-1} cell/ml) under an enrichment periods of 6, 18 and 24 hour respectively and for the two primers of [3] and [2] (fig. 1 and 2). However, bands obtained from the primer of Rahn [3] were more obvious than bands of the primer of Jitrapakdee [2]. So, studies which depends on [3] will bemoreprecise specially as an indicator upon natural contamination. On the other hand, 24 hours as an enrichment period look like an adequate period to obtain a clear enough band for the two primers.

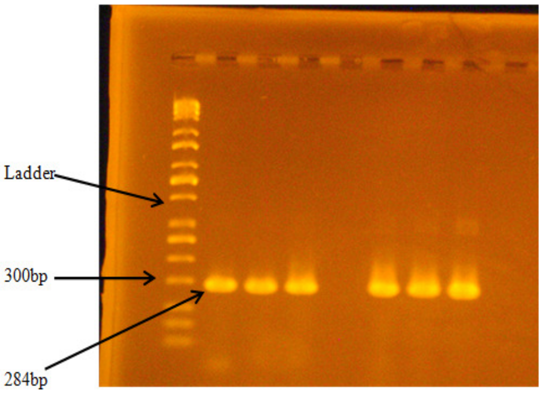

Fig.1. Agarose gel electrophoresis for detection of Rahn [3] primer from *Salmonella spp* artificial contamination local meat sample (284 bp), 24 hour enrichment period, directly extraction of *Salmonella ssp* DNA. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide. Lane:1 (M:100bp ladder); Lane: 1, 2,3,4, 5,6, (PCR product).

The reason behind the lake of clearness in the bands which belong to the primer of Jitrapakdee[2] is mentioned by the same research. The primer of Jitrapakdee [2]was designed to cover about 52 serovars of *salmonella,* while the primer of Rahn[3] was designed to cover more than 200 *salmonella* serovars [2] and [3]. Therefore, the opportunity of the primer to find an adequate amount of DNA to make more amplification steps in the same time is bigger for the first one.

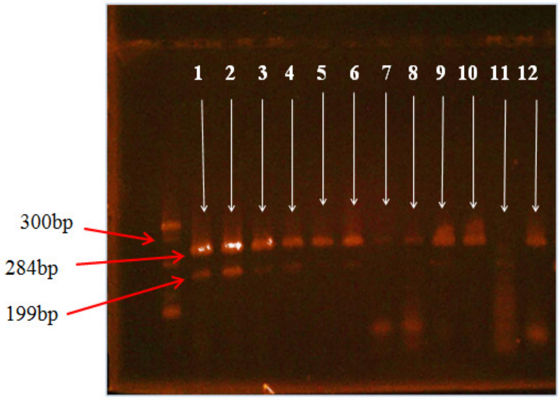

Fig.2. Agarose gel electrophoresis for detection of Rahn [3] (284bp) and Jitrapakdee[2] (199bp) primers from *salmonella spp* artificial contamination local meat sample , directly extraction of *Salmonella ssp* DNA. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide. Lane:1 (M:100bp ladder); Lane: 1,2,3 are 4x10^{5} cell/ml *Salmonella ssp* artificial contamination 24, 18 and 6 hour enrichment period respectively. 4,5,6 are 4x10^{3} cell/ml *Salmonella ssp* artificial contamination 24, 18 and 6 hour enrichment period respectively. 7,8,9 are 4x10^{1} cell/ml *salmonella ssp* artificial contamination 24, 18 and 6 hour enrichment period respectively. 10, 11, 12 are 4x10^{-1} cell/ml *Salmonella*ssp artificial contamination 24, 18 and 6 hour enrichment period respectively.

Sequencing

Sequencing of invasion protein A gene and phosphatase gene were performed by machine is AB13730XL, Applied Biosystem, Macrogen company, USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program, which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program. Sequencing of coding regions of the amplified product of invasion protein A gene and phosphatase gene for detection of any polymorphism within these sequences related to classify of bacteria as shown in fig. (3 and 5). The results were compared with data obtained from Gene Bank published BLAST program which is available at the NCBI online. The polymorphism G995114A and A995114G that we observed within the *Salmonella enterica* subspp of *Enterica* serovar *Enteritidis* of invasion protein A gene,are shown in (fig.3).Which appears that all sample of bacteria have sequencing ID gb|CP011394.1 and 98% identities, score 243 with wild type of *Salmonella enterica*from the Gene Bank, change cod from GAC> AGC, It is a missense polymorphism (Transversion) that leads to changes in amino acid from Aspartic acid (D) to Serine (S), shown in (fig.4).

Sequencing ID in gene bank, score, expect and compatibility of DNA sequences obtained shown as table (4).

In total, bacteria had three-transition polymorphism, +3206910 C\T, 3206913 T\C and 3207056 C\ T,and one transversion T\A 3206912.Which results change of codon from GTT>GAC (Valine (V) / Aspartic acid (D),and ACC>ACT (Threonine (T) / Threonine (T) and GCC>GCT (Alanine (A)/ Alanine (A) and compatibility 98%, score 300 with the wild type of gene bank under sequencing ID gb|CP011394.1|_shown Fig. (5 and6) and Table (6and 7).

1F. 284

*Salmonella enterica*subsp. *enterica*serovar*Enteritidis* str. 18569, complete genome
Sequence ID: gb|CP011394.1|

	Score	Expect	Identities	Gaps	Strand
	449 bits(243)	8e-23	252/256(98%)	2/256(0%)	Plus/Plus

type III secretion system protein InvA

```
Query  3       TATTGGCGA-AGCCTGGCGGTGGGTTTTGTTGTCTTCTCTATTGTCACCGTGGTCCAGTG  61
               ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct994873  TATTGGCGATAGCCTGGCGGTGGGTTTTGTTGTCTTCTCTATTGTCACCGTGGTCCAGT-  994931

Query  62      TTATCGTTATTACCAAAGGTTCAGAACGCGTCGCGGAAGTCGCGGCCCGATTTTCTCTGG  121
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  994932  TTATCGTTATTACCAAAGGTTCAGAACGCGTCGCGGAAGTCGCGGCCCGATTTTCTCTGG  994991

Query  122     ATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTATTATTGATG  181
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  994992  ATGGTATGCCCGGTAAACAGATGAGTATTGATGCCGATTTGAAGGCCGGTATTATTGATG  995051

Query  182     CGGATGCTGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCCT  241
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  995052  CGGATGCTGCGCGCGAACGGCGAAGCGTACTGGAAAGGGAAAGCCAGCTTTACGGTTCCT  995111

Query  242     TTAGCGGTGCGATGAA  257
               ||   |||||||||||
Sbjct  995112  TTGACGGTGCGATGAA  995127
```

Fig.3. Sequencing of sense flanking the partialinvasion protein Agene, for *Salmonella* as compared with standard *Salmonella enterica subsp. enterica*, obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

invasion protein A, partial [*Salmonella enterica subsp. enterica*]
Sequence ID: gb|AID54913.1|

Score	Expect	Method	Identities	Positives	Gaps	Frame
128 bits(321)	8e-36	Compositional matrix adjust.	64/65(98%)	64/65(98%)	0/65(0%)	+2

```
sample 62   IVITKGSERVAEVAARFSLDGMPGKQMSIDADLKAGIIDADAARERRSVLERESQLYGSF  241


ncbi   33   IVITKGSERVAEVAARFSLDGMPGKQMSIDADLKAGIIDADAARERRSVLERESQLYGSF  92


sample   242SGAMK   256
ncbi   93    DGAMK   97
```

Fig.4. Amino acid sequence of the translated invasion protein A gene, the character in group is the site of polymorphism with change from Seine to aspartic acid.

Salmonella enterica subsp. *enterica*serovar*Enteritidis* str. 18569, complete genome
Sequence ID: gb|CP011394.1|

Score	Expect	Identities	Gaps	Strand
271 bits(300)	2e-69	156/160(98%)	0/160(0%)	Plus/Plus

UMP phosphatase

```
Query   13        GAAGAGACTGACATCGTCGGCGACAACCTGCGCACCGACATTCTGGCGGGGTTCCAGGCC   72
                  |||||||| |  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct3206901   GAAGAGACCGTTATCGTCGGCGACAACCTGCGCACCGACATTCTGGCGGGGTTCCAGGCC   3206960


Query   73        GGTCTGGAGACCATTCTGGTGCTTTCAGGCGTATCAACAATCAATGATATTGACAGTATG   132
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   3206961   GGTCTGGAGACCATTCTGGTGCTTTCAGGCGTATCAACAATCAATGATATTGACAGTATG   3207020


Query   133       CCGTTCCGCCCAAGCTGGATTTACCCTTCCGTCGCTGAGA   172
                  ||||||||||||||||||||||||||||||||||| ||||
Sbjct   3207021   CCGTTCCGCCCAAGCTGGATTTACCCTTCCGTCGCCGAGA   3207060
```

Fig.5. Sequencing of sense flanking the partialphosphatase gene, for *Salmonella* as compared with standard *Salmonella enterica subsp. enterica*, obtained from Gene Bank. Query represents of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

Phosphatase NagD, partial [*Salmonella enterica* subsp. *enterica*serovar Urbana str. R8-2977]
Sequence ID: gb|EHD05976.1|

Score	Expect	Method	Identities	Positives	Gaps	Frame
104 bits(259)	2e-27	Compositional matrix adjust.	51/52(98%)	51/52(98%)	0/52(0%)	+1

```
Query   1     ETDIVGDNLRTDILAGFQAGLETILVLSGVSTINDIDSMPFRPSWIYPSVAE   156


Sbjct   14    ETVIVGDNLRTDILAGFQAGLETILVLSGVSTINDIDSMPFRPSWIYPSVAE   65
```

Fig.6. Amino acid sequence of the translated Phosphatase gene, the character in group is the site of polymorphism with change from valine to aspartic acid.

Table 8: Sequencing ID in gene bank, score, expect and compatibility of DNA sequences obtained.

	Type of Gene	**Bacteria**	**Sequence ID**	**Score**	**Expect**	**Identities**	**No. nucleotide**
1	invasion protein A	Salmonella entericasubsp	gb|CP011394.1|	449	8e-123	**98%**	994873-995127
2	Phosphatase	Salmonella entericasubsp	gb|CP011394.1|	128	2e-69	**98%**	3206901**-**3207060

Table 7: Types of mutations detected in partialinvasion protein A and Phosphatase gene of *Salmonella enterica*. Query represent of sample; Sbject represent of database of National Center Biotechnology Information (NCBI).

Type of mutation	**Predicted effect**	**Amino acid change**	**Nucleotide change**	**Location of gene bank**	**Name of gene**
Transition	**Missense**	**Serine (S) / Aspartic acid (D)**	**GAC>AGC**	**93**	**invasion protein A**
Transversion **Transition**	**Missense**	**Valine (V) / Aspartic acid (D)**	**GTT>GAC**	**15**	**Phosphatase**
Transition	**Silent**	**Threonine (T) / Threonine (T)**	**ACC>ACT**	**16**	**Phosphatase**
Transition	**Silent**	**Alanine (A)/ Alanine (A)**	**GCC>GCT**	**64**	**Phosphatase**

IV. CONCLUSION

- PCR test was sensitive for all dilution concentrations of artificial contamination (4x105, 4x103, 4x101, 4x10-1 cell/ml) under an enrichment periods of 6, 18 and 24 hour respectively and for the two primers of Rahn et al. (1992) and Jitrapakdee et al. (1995).
- Bands obtained from the primer of Rahn et al. (1992) were more obvious than bands of the primer of Jitrapakdee et al. (1995).
- 24 hours as an enrichment period look like an adequate period to obtain a clear enough band for the two primers.
- All sample of bacteria have sequencing ID gb|CP011394.1 and 98% identities, score 243 with wild type of *Salmonella enterica* from the Gene Bank,that leads to changes in amino acid from Aspartic acid (D) to Serine (S).

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