EVALUATION OF THE ISOLATION AND DETECTION METHODS FOR *SALMONELLA* SPP. FROM EGG SHELL CONTAMINATION USING MULTIPLEX PCR.

Israa Adnan Ibraheam Al-Baghdady*, Ashwak Bassim Jassim**
Zainab Khudher Ahmed***
*College of Science for women,University of Babil,Babil.Iraq
**Genetic engineering and biotechnology for postgraduate studies ,University of Baghdad,Baghdad,Iraq
***College of Nursing,University of Babil.Babil, Iraq

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ABSTRACT

A total of seventy four egg samples were collected from different markets sources, to investigated for *Salmonella*spp. the bacteria was isolated and identified using culturing on selective media, in addition to, biochemical and serotyping by monovalent antisera. Multiplex Polymerase Chain Reaction(PCR) detection of the*invA; and DH* genes was used for conformation of the identification of the *Salmonella* spp.. Twenty eight samples from seventy four eggs provide positive results with PCR as *Salmonella* spp. depending on *InvA* gene which is the target for the identification at the genus level.

INTRODUCTION

Salmonella spp.considered among the leading causes of community acquired bacterial gastroenteritis worldwide and the second leading cause of bacterial foodborne illness(1).Salmonellosis are associated with the consumption of contaminated products such as poultry, meat, eggs, milk, seafood (2). Transmission is usually derived from fecal contamination on the egg shell,it also includes contamination through environmental vectors, such as farmers, pets and rodents.

Therefore, development of rapid and sensitive methodsfor detection of *Salmonella* directly from different samples may have a significant impacton the disease burden caused by this pathogen. Toolsdeveloped for such purposes could help both in preventing thespread of outbreaks and in diagnosis (3). Traditional methodsfor *Salmonella* identification are based on cultures using selective media and characterization of suspected colonies by biochemical and serological tests,these methods are generally time-consuming, therefore, a rapid method is necessary for the identification of *Salmonella* (4).

Molecular technique is alternative methods for *Salmonella* detection and typing,Polymerase Chain Reaction has become a potentially powerful tools in microbiological diagnostics due to its simplicity, rapidity, reproducibility, and accuracy.

The aim of this study was to investigate the contamination of table eggs with *Salmonella* speciesby specific multiplex PCR assay.

MATERIALS AND METHODS

Egg samples

Seventy four egg samples were collected from different markets sources,to investigated for *Salmonella*using sterile cotton swabs to remove over entire surface area of the eggshell , two swabs were used for each sample.

Bacteriological Examination and identification:-

1-First swab was added to peptone broth, incubated for 24 h at 37°C.

2- Enriched in tetrathionate broth for 24 h at 37°C, streaking on selective media XLD and SS agarfor 24h at 37°Cto isolate the suspected colonies of *Salmonella.*

3-Suspected colonies were selected and further biochemical tests with API 20E and Mini API were done.

4- The isolates were confirmed as *Salmonella* by biochemical tests, send to reference laboratory /Central health public laboratory (CHPL) for serological conformation.

Molecular Identification of *Salmonella* spp.:-

1- DNA EXTRACTION:-

Total genomic DNA was isolated directly from 2ned swab which input in 200 *m*l distilled water in eppendroff tube,extraction carried out by using genomic DNA kit (Gene aid) according to(5).

2- PCR:-

PCR used for detection of the*invA;and DH* genes for conformation the identification of the *Salmonella*spp., according to(5).These primers synthesized by Cinna gen company (Table 1).

Table (1) : The sequence and concentration of forward and reverse primers oftheinvA; and DH genes.

Primer type	Primer size	Product size
(InvA gene) InvA F InvA R	 CGAGCAGCCGCTTAGTATTGAG CCATCAAATTAGCGGAGGCTTC	881bp
(Flic-d gene) Dh F Dh R	GCTTAATGTCCAAGATGCCTAC GAGCAACGCCAGTACCATCTG	587bp

PCR reaction was conducted in 100 µl of reaction mixture containing 50 µl of green master mix,5 µl of each primer,10 µl DNA template and 30 µl of deionized water (Table 2).

Table (2): The mixture of conventional PCR working solution for detection of the*invA; and DH* genes in *Salmonella* spp.

Working solution	
Water	30 µl
Forward primer	5 µl
Reverse primer	5 µl
DNA	10 µl
Master mix	50 µl
Final volume	100 µl

Amplification was conducted using a master cycler eppendroff programmed with 30 cycler for Initial denaturation 95°C for 2 min. ,Denaturation for 94°C 30 sec., Anneling64°C 30 sec.,Extention72°C 30 sec. and final Extention 72 °C 5min. (Table 3).

Table (3): PCR program forfragment*invA; and DH genes* amplification by the conventional methods.

Thermocycler conditions	Temperature ($^{\circ}C$)	Time (min)
Initial denaturation	95°C	2min.
Denturation	94°C	25sec.
Primmer annealing	64°C	30sec.
Primmer extension	72°C	30 min.
Final extend	72°C	5 min.
Cycles number : 30 cycle		

Gel Electrophoresis:-PCR products and the ladder marker were resolved by electrophoresis on 2% w/v agarose gels.

RESULTS AND DISCUSSION

1- Egg analysis:-

Examination of egg samples:-

A total of seventy four egg samples were tested by conventional culture method for detection of *Salmonella* spp.,thirty one samples were positive using culturing method table (2).Depend on morphology, round pale colony with black center on XLD, SS agar..The outcome of biochemical tests clarified thatall isolates fermented glucose not lactose appeared as red surface and yellow bottom of KIA slant with gas and H^2O formation for their further conformation API20E were used also in diagnosis according to (6) and (7).

Table (2): Results of *Salmonella* spp.detected by using culture methods and PCR method from different egg samples.

No. of total samples	No. of positive samples		No. of negative samples
74	31(41.8%)		43 (58%)
	28 samples were PCR Positive(37.8%)	31 samples were culture positive (41.8%)	

Molecular level and PCR technique :-

All samples of egg were detected for contamination by *Salmonella* with PCR ,twenty eight samples from seventy four eggs provide positive results with PCR as *salmonella* spp. depending on *InvA* gene which is the target for the diagnosis at the genus level, which is located on the pathogenicity island 1 of *Salmonella* spp.it is essential for invasion of epithelial cells (8).All isolates formed PCR products of the expected size ,Dh primer for the detection of *Flic- d* gene give positive results in all *Salmonella* isolateswhich is present in over 100 *Salmonella*serovar fig.(1)which, encode for the synthesis ofH (flagellar) antigens one of the three antigens responsible for the basis of classification for *Salmonella* by Kauffman–White scheme (9).

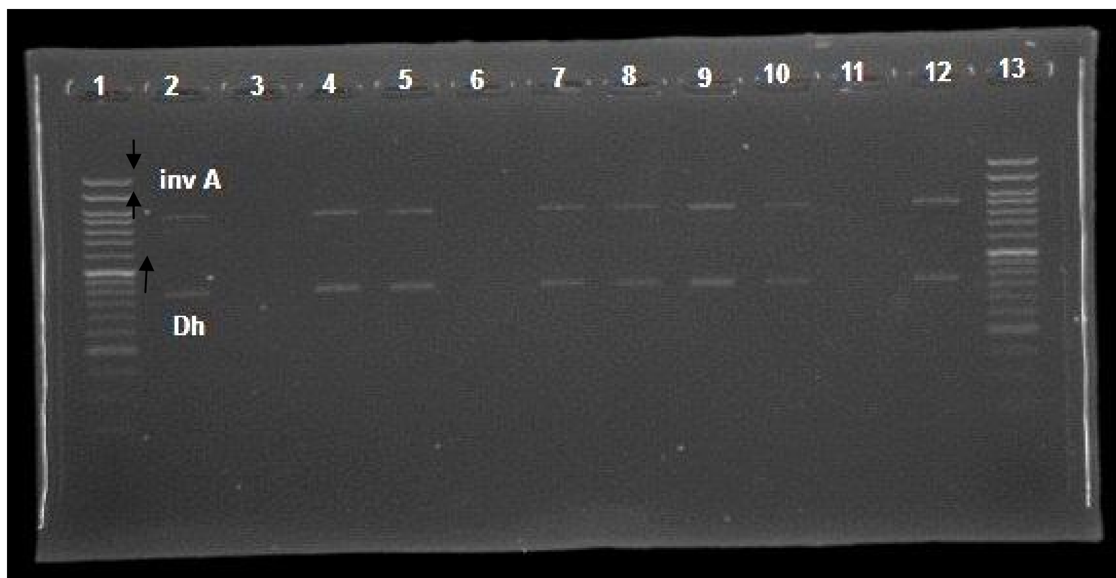

Fig.(1): agarose gel electrophoresis of PCR product. PCR was carried out with DNA obtained from *Salmonella spp.*(line1 and line 13 are marker 50bp), line 2 was positive control, line 3 was negative control, line 4,5,7-10, 12 were positive for *Salmonella spp.* .

Multiplex PCR can be used as quality control method for detect egg contamination from different sources.*Salmonella* in eggs constitute a public health warning, can colonize the ovaries of hens and contaminate the internal contents of eggs(10) .

استخدام السلسلة المتبلمره المتعددةالثنائية لتقييم وتشخيص التلوث بجرثومة السالمونيلافي قشرة البيض

إسراء عدنان ابراهيم * ، أشواق باسم جاسم **، زينب خضر احمد ***

*كلية التربيه للبنات ،جامعة بابل ،بابل ،العراق.
**معهد الهندسه الوراثيه والتقانات الاحيائيه وجامعة بغداد ،بغداد ،العراق.
كلية التمريض، جامعة بابل ،بابل ،العراق.

الخلاصة

جمعت أربعه وسبعين عينه من البيض من اسواق مختلفة لغرض فحص السالمونيلا وتم عزل البكتريا وشخصت باستخدام الزرع على وسط زرعي انتقائي بالإضافة الى الفحوصات الكيموحيويه والسيرولوجيه. أستخدمت تقنية السلسلة المتبلمره المتعددة الثنائية بالاعتماد على تشخيص كل من جينات *invA; DH* لغرض تأكيد تشخيص بكتريا السالمونيلا.أعطت ثمانية وعشرون عينه من مجموع اربعه وسبعين نتائج موجبه اعتمادا على تقنية البلمره المتسلسلة على أنها بكتريا السالمونيلا اعتمادا على جين *InvA* الذي يعد مهما لتشخيصها على مستوى النوع.

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