Detection of invasion gene *invA* in *Salmonella spp*. Isolated from slaughtered cattle by PCR method

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Summary

The present study was carried out for the identification and molecular characterization of *Salmonella spp*. isolated from cattle at abattoir by biochemical, serotyping and virulence gene based polymerase chain reaction (PCR) techniques. Eleven *Salmonella* were isolated from cattle at abattoir, these isolates were cultured and biochemically characterized by double checking with a conventional method and by KB011 Hi Salmonella TM identification kit then confirmed by serotyping and testing for detection of the *invA* virulence gene by PCR by using a Salmonella-specific 506 bp *invA* gene amplicon. The biochemical and serotyping results revealed that the 11 isolates belonged to four serotypes, *S. enteritidis* was the predominant serotype,5 isolates (45.45%) followed by *S. newport* 3 (27.27%), *S. ohio*, 2 (18.18%) and *S. anatum*, 1(9.09%). The PCR technique confirmed that all Salmonella isolates carried the *invA* gene (DNA amplification showed one distinct band with molecular weight of 506 bp amplified fragment on electrophoresis in agarose gel).The PCR assay described herein was found to be a rapid and simple method to confirm the isolates as Salmonella.

Keywords: *Salmonella spp.*, *invA* gene, KB011 Hi Salmonella TM, Cattle.

Introduction

Salmonellosis is a direct occupational anthropo zoonotic disease of great economic and public health concern, which continue to be responsible for large numbers of infections in both humans and animals worldwide especially in developing and industrialized countries (1). Salmonella In humans is usually related to the consumption of contaminated foods, it has been isolated from many foodstuffs which include bovine meat (2 and 3). This pathogen has been widely isolated from cattle and the infected animals may carry these bacteria without any clinical symptoms, contaminated meat by salmonella may occur at abattoirs from the excretion of carrier animals, contaminated slaughterhouse equipment, floors and personnel in an abattoir (4 and 5).

The pathogenicity of *Salmonella spp*. is depending upon its ability to invade the cells that are normally nonphagocytic, the *invA* gene is essential for the process of invasion of epithelial cells by Salmonella (6). This gene of Salmonella contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application (7). Cultural techniques are universally recognized as the standard methods for the detection of bacterial pathogens, such as Salmonella in food stuffs (8). These techniques generally take longer time, while in vitro amplification of DNA PCR has become a potentially powerful alternative in microbiological diagnosis due to its rapidity and accuracy (9). There is an urgent need to strengthen the monitoring and surveillance of salmonellosis using suitable diagnostic tools so as to prevent and control its occurrence.

In Iraq, a broad range of Salmonella species has been isolated from the animals (10-13). Earlier studies have looked at the occurrence of this pathogen in foods of animal origin without necessarily determining their virulence characteristics. To our knowledge, no previous study has been done in Iraq to confirm the presence of *InvA* gene in *Salmonella spp*. isolated from cattle in abattoir. Therefore, this study was carried out to assess the presence of the *invA* virulence gene in salmonella isolated from slaughtered cattle by PCR.

Materials and Methods

The salmonella isolates used in this study and their sources have been described in details previously (13). These isolates were retested by assessing colony characterization, gram reaction, conducting the different biochemical tests by double checking with conventional methods (14) and by KB011 HiSalmonella TM identification kit (Hi-Media Laboratories- India). This kit composed from plastic strip which had twelve wells with sterile medium of different biochemical tests for identification of Salmonella species as follow Well 1-12 for MR test, Voges Proskauers test, Urease production, H_2S production, Citrate utilization, Lysine utilization, ONPG tests Lactose, Arabinose, Maltose, Sorbitol and Dulcitol respectively. Reagents supplied with kit: Methyl Red Indicator (I007); Barritt Reagent A (R029) and Barritt Reagent B (R030) for VP Test. This kit is a standardized colorimetric identification system utilizing conventional biochemical tests and carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization (15). Biochemically confirmed *Salmonella* isolates were re-cultured on Kligler Iron media for serotyping at the Central Public Health Laboratories, National Center of *Salmonella*, in Baghdad, Iraq.

Polymerase chain reaction (DNA extraction): To extract DNA for use in the molecular detection assay, bacteria were cultured on the XLD agar and one colony was selected and grown on nutrient agar. A colony was then selected and incubated in 5 ml nutrient broth, 1 ml of which was transferred into a 1.5 ml tube for centrifugation for 10 min at 18,000 rpm, the supernatant was discarded and the cell pellet was used for DNA extraction. The DNA of all isolates was extracted and purified using genomic DNA purification Kit (Genomic DNA Mini Kit, Geneaid.USA), according to the information of company. Primers used in this study were obtained from Bioneer, Korea. and were designed based on the sequence of the *invA* gene: Forward primer 5´ATGCCCGGTAAA CACATGAG´3 and reverse primer 5´CTCGCC TTTGCTGGTTTTAG´3 with an expected amplicon size of 506 bp. by using NCBI-Gene bank and primer 3 plus. These primers were prepared according to the information of company by dissolving each primer in 1000 µl of deionized distilled water.

Preparation of PCR Master Mix: PCR master mix reaction was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions (Table, 1). Reactions were carried out in a total volume of 20 µl containing: DNA polymerase 1U deoxynucleotide triphosphates (dNTPs) Bioneer, Korea) which include: 250 µM of each dATP, dGTP, dCTP, dTTP; 1.5mM of $MgCl_2$; 30 mM of KCl; 10 mM Tris-HCl (PH 9.0), 5 µl of template DNA, 1.5 µl of each forwarded and reversed primers and 12 µl of nuclease free water to complete the amplification mixture to 20 µl. Amplification PCR was performed in a DNA thermal cycler. After initial denaturation step for 5 min at 94°C, followed by 30 cycles of amplification were performed. Each cycle consisted of the following steps; 30 s at 94°C (denaturation), 30 s at 55°C (primer annealing) and 30 s at 72°C (extension) and 72°C for 7 min for final extension. Five microliters of total reaction mixture was loaded on a 1.5% agarose gel in comparison with a 100-bp molecular weight DNA marker and electrophoresed at 100V at 80 am for 60 min. The amplified DNA fragments were visualized by UV light illumination by staining with ethidium bromide. The PCR assay was done in the College of Veterinary Medicine/ University of Al- Qadissia.

Table, 1: Shows Components of PCR Master Mix Reaction.

PCR Master mix reaction components		Volume (1Rxn)	12Rxn , Rxn=No. of sample +1%(pipette error)
DNA template		5 µl	5 µl
Primer	F. primer	1.5 µl	18 µl
	R. primer	1.5 µl	18 µl
PCR water		12 µl	18 µl
Total volume		20 µl	

Results and Discussion

The present study was conducted for the identification and molecular characterization of *Salmonella spp*. isolated from cattle at abattoir by biochemical, serotyping and virulence gene based PCR techniques. The results of biochemical tests by double checking with conventional methods and by KB011 Hi SalmonellaTM Identification Kit showed that, all isolates gave reactions of *Salmonella spp*. they were positive for : Lysine utilization ,H_2S production, Citrate utilization, Methyl red, Arabinose, Sorbitol, Dulcitol and Maltose, and negative for: ONPG, Urease, Voges Proskauer's and Lactose (Fig. 1 and 2).

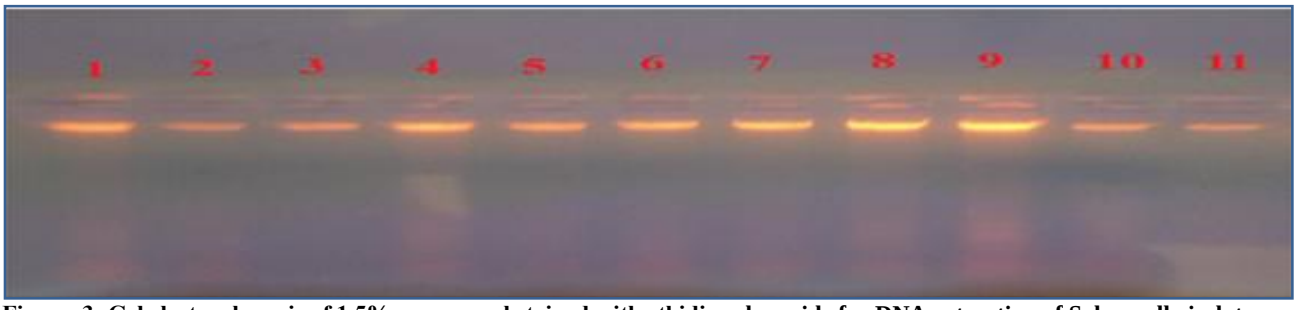
Figure, 1: KB011 HiSalmonella TM Identification Kit before inoculation

Figure, 2: KB011 HiSalmonella TM Identification Kit after inoculation

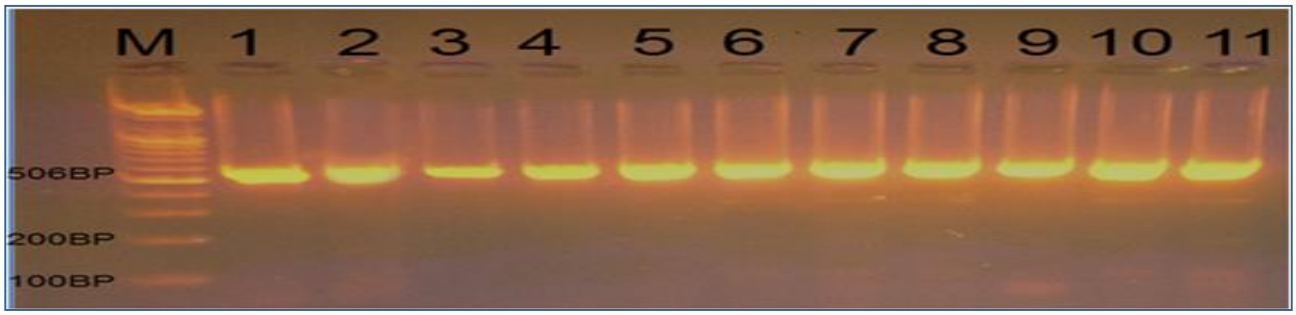
Currently, Salmonella is detected by standard bacteriological, biochemical and serological techniques. These techniques are generally time-consuming, tedious and expensive as they require hundreds of antisera as well as well-trained technicians (16 and 17). Many researchers underlined the importance and necessity of founding a more rapid and effective detection methods as a basis of control (18 and 19). Several rapid and sensitive methods have been developed for identification of Salmonella serotypes from clinical specimens (20). Salmonella specific PCR with primers for *inv*A is rapid, sensitive and specific for detection of *Salmonella* in many clinical samples (21 and 22). The results of biochemical and serological tests in this study revealed that the 11 isolates belonged to four different serotypes, S. enteritidis were the predominant serotypes, 5 isolates (45.45%) followed by S.newport 3 (27.27%), S. ohio 2 (18.18%) and S. anatum 1 (9.09%).

S. enteritidis is one of the most common serotypes of Salmonella spp. reported world-wide and causes an estimated 1 million cases of domestically acquired foodborne illness in humans annually in the United States (23).The long-term survival of Salmonella newport in manure or manure-amended soils indicates the potential risk of environmental spread and subsequent transmission to human and animals (24). Salmonella newport was isolated from faeces and gallbladder of slaughtered sheep in Duhok Abattoir, Iraq, (11). Three serotypes of Salmonella were detected in slaughtered animals and abattoir sewage in Zakho Abattoir, the isolated Salmonella serotypes were Salmonella hato 8 (66.66%), Salmonella anatum 3 (25%), and Salmonella enteritidis 1 (8.33%) (12). The DNA of all isolates was extracted and purified using genome DNA purification kit. The results were read by electrophoresis on 1.5 % agarose gel and exposed to U.V light in which the DNA

appeared as compact bands (Fig. 3). In PCR technique, all the Salmonella isolates amplified a 506 bp DNA amplicon, which suggested the presence of *invA* and further confirmed that all the isolates were Salmonella (Fig. 4).



Figure, 3: Gel electrophoresis of 1.5% agarose gel stained with ethidium bromide for DNA extraction of Salmonella isolates.



Figure, 4: PCR amplification of the invA gene in 11 Salmonella isolates. Lane 1-11 represents Salmonella isolates, lane M represent molecular weight marker, 100 bp ladder.

The PCR analysis demonstrated that all *Salmonella spp.* isolates carried the *invA* gene. These results closely agree with other studies reporting the detection of this gene in Salmonella isolates (7, 20, 25- 27), the *invA* gene is carried on a region of the bacterial chromosome known as the Salmonella pathogenicity island 1 (SPI1) and encodes a protein in the inner membrane of bacteria, which is essential for full virulence in salmonella and is thought to trigger internalization required for invasion of deeper tissue (22, 28 and 29). The amplification of invA gene is recognized as an international standard technique for the detection of salmonella genus and PCR assay using *invA* primers specific for salmonella considerably reduce the number of false positive and false negative results which commonly occur in diagnostic laboratories (30 and 31). Isolation of salmonella carrying invasion *invA* gene in the present study may indicate the poor sanitation of the environment under which animals are slaughtered and increases the burden of food borne infections in the people, and emphasizes the need to continuous education of the consumers on proper food handling and cooking practices to decrease the risk of transmission of salmonella and other foodborne pathogens from contaminated meat, and to strengthen the monitoring and surveillance by using suitable diagnostic tools to reduce the risk of invasive salmonellosis and control its occurrence. In conclusion, the results of the present study reveal that the application of PCR technique described is found to be highly sensitive, specific and less time consumption to confirm the isolates as Salmonella.

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الكشف عن جين الضراوة *InvA* في جراثيم السالمونيلا المعزولة من الأبقار المذبوحة بوساطة تفاعل تسلسل البلمرة في العراق

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الخلاصة

أجريت هذه الدراسة لتحديد وتوصيف جزيئي لأنواع السالمونيلا المعزولة من الأبقار في المجزرة بوساطة االفحوصات البايوكيميائية والتنميط المصلي و تفاعل تسلسل البلمرة. تم عزل 11 عزلة من الأبقارفي المجزرة ، زرعت هذه العزلات ووصفت بايوكيميائيا بالطريقتين التقليدية وباستخدام Salmonella Hi KB011) وتم تأكيدها بالتنميط المصلي وفحصت لتحديد جين الضراوة *InvA* بوساطة تفاعل تسلسل البلمرة. أوضحت نتائج الفحوصات البايوكيميائية والتنميط المصلي بأن الـ 11 عزلة تعود الى أربعة أنماط مصلية وان *S. enteritidis* هو النمط السائد وبنسبة 45.45 % تلاه *S. newport* بنسبة 27.27% و *S.ohio* بنسبة 18.18% و *S.anatum* بنسبة 9.09%. أثبتت تقنية تفاعل تسلسل البلمرة بأن جميع العزلات تحمل جين *InvA* وبينت حزمة واحدة ذات وزن جزيئي 506 bp على هلامة الأكاروز. أن هذه التقنية من الطرائق البسطة والسريعة لتأكيد جراثيم السالمونيلا.

الكلمات المفتاحية: السالمونيلا، جين *InvA* ، KB011 Hi Salmonella، الأبقار.