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Research article

Molecular detection of invA, ssaP in *Salmonella typhimurium* isolated from chicken in Al-Qadisiyah Province

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Abstract

Salmonella is considered the most important cause of foodborne diseases. Identification of Salmonella typhimurium evaluated using bacteriological assay followed by PCR technique. In this study, 40 intestinal content specimens of poultry were collected randomly from different farms of Al-Qadisiyah province. Outs of 40 samples obtained, 14 isolates (35 %) were detected as Salmonella typhimurium according to conventional bacteriological characteristics, the Vitek 2 system for identification and molecular assays. Two sets of primers were designed for detecting invA and ssaP genes. These genes potent the virulence of Salmonella typhimurium. The primers were made in this study by using NCBI\GenBank and design online. The primers were made by (Bioneer) company, Korea. Molecular assay of the isolates gives away specific PCR products of 677bp for the invA gene and 314bp for ssaP gene. The invA genes were amplified in 11 (78.5%) out of 14 isolates of Salmonella typhimurium, while ssaP genes were amplified in 10 (71.4%) out of 14 isolates of Salmonella typhimurium. The result of the study confirms the ability of these specific primers for detection of salmonella typhimurium in samples of chicken as well as the rapidly and sensitivity of the PCR method as a good tool for bacteriological identification.

Keywords: *Salmonella typhimurium*, Virulence factors, Poultry, PCR tech.

Introduction

Salmonella species is the most important pathogen in the world as source of food borne illness in chicken, it cause economic loos in poultry and poultry products industries (1). *Salmonella* caused about 80% of infections in human globally (2). Phylogenetic analysis of *Salmonella typhimurium* show the effects of different factors in the existence of *Salmonella* spp in animal, and cross-contamination among animals, feed and environment (3). Infections by *Salmonella* species in human caused by uncooked of meat poultry (4). However, *Salmonella* genus include two major species, Salmonella *enterica* subspecies *enterica* serovar *typhimurium* and *Salmonella bongori*. *Salmonella typhimurium* cause disease in human (5) (6). About 75-80% of human infection caused by beef, poultry meat and eggs which contaminated by *Salmonella* (3) (7). Almost, molecular detection of bacteria in is rapid, sensitive powerful tool in the bacteriological diagnostic compared to culture techniques. Therefore, determination of *Salmonella* in fecal material reduce time of disease diagnosis (8). Many of chromosomal virulence genes have been used to identify *salmonella* in fecal samples of poultry including invA and ssaP, these genes are target for PCR assay of *salmonell*a serovar (9) also, and these genes are clustered in the islands of the pathogenicity of *salmonella* species (10). The invA gene is virulent and encoded a protein found in the inner layer of membrane of the bacteria, this gene contains sequences unique found in all

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salmonella serovars, this gene is responsible for invasion of the bacteria in to epithelial cells of the host (11). ssaP contributes to attenuate of virulence also have been used to explain the importance of this gene at survival of the bacteria in the cells of host and at stages of infection (12). The goal of this research is to determination of *salmonella typhimurium* incidence in the chicken in Al-Qadisiyah Province by using conventional bacteriological methods including cultural and biochemical tests, also determination sequencing of invA and ssaP genes among the *salmonella* isolates by using specific PCR technique.

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 320

Sample collection:

Forty specimens of intestinal contents of chicken were collected randomly from different farms located in Al-Qadisiyah province.

Bacterial isolation:

Samples were transported to the laboratory of microbiology faculty of Veterinary Medicine\ University of Al-Qadisiyah for bacteriological assay. All specimens inoculated into Salmonella-Shigella agar at 37c for 24-48-hr, also all specimens were examined on XLD agar and of blood agar then incubated for 24 hours at 37C°. Then the isolates were activated by inoculated on-Salmonella CHROM agar and incubated at 37C° overnight. The biochemical reactions to identification of species of *Salmonella* such as catalase, oxidase, in dole production, urease and citrate utilization. Carried out according to (12). Also use of Vitek 2system for rapid identification of five isolates.

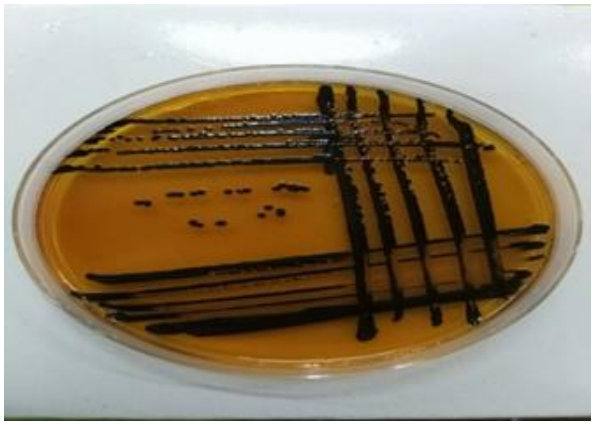

Figure (1): *Salmonella typhimurium* on Salmonella-Shigella agar

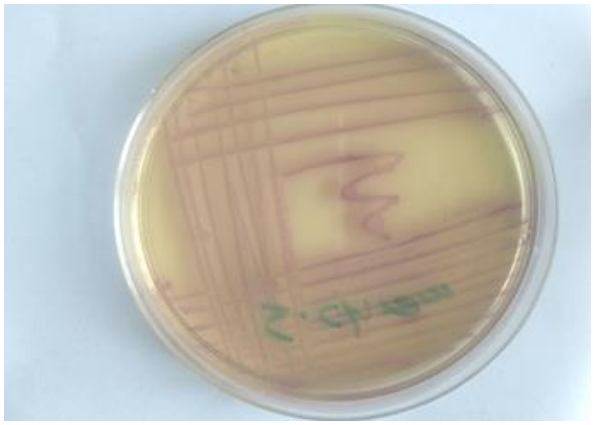

Figure (2): *Salmonella typhimurium* on-Salmonella CHROM agar

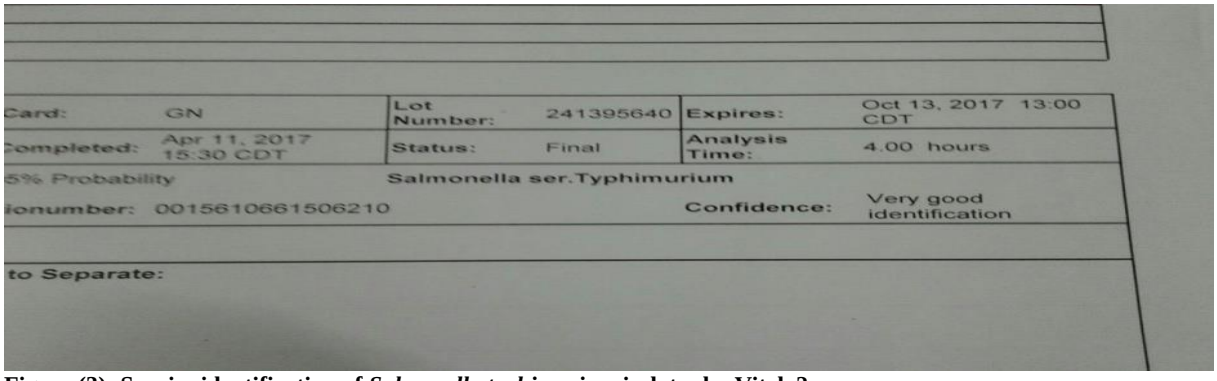

Figure (3): Species identification of *Salmonella typhimurium* isolates by Vitek 2

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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Bacterial DNA extraction and PCR Method

PCR technique was performed to determination of virulence factors genes (InvA and ssaP gene) in *Salmonella typhimurium* based using specific primers were design in current study as following steps:-

1-DNA extraction:

The bacterial isolates were subjected to bacterial nucleic acid extraction by using commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Gene aid Biotech Ltd. USA). The extraction method was done according to the manufacture instructions using gram-positive bacteria DNA Protocol extraction method by using (20 mg/ml) lysozyme buffer.

2-Nanodrop:

The extracted DNA was estimated by nanodrop device at 260/280nm, and then kept at deep freezer until used in PCR method.

3-Primers:

The PCR primers that used in this study for detection virulence factors genes were designed in this study using NCBI Gene sequence database and primer 3 plus design. These primers were provided from Bioneer Company, Korea as following table (1).

Table (1): PCR primers and their sequence and GenBank codes

Primer	Sequence		Amplicon	GenBank
invA	F	TCCTTTGACGGTGCGATGAA	677bp	M90846.1
	R	CTGTTATCGTCCAGGCCCTC		
	F	TGAGGGAAGTTGGGTTGC		
ssap	R	ACCCCATATGCAGCAACTGA	314bp	NC-003197.2

4-PCR master mix preparation:

The mix was prepared using (Accu-Power PCR-PreMix-Kit) master mix reagent and done according to company instructions as a following table (2).

Table (2) Company instruction of PCR master mix

Master mix	Volume
DNA template (10 ng/ µl)	5 µl
Forward primer (10 pmol)	1 µl
Reverse primer (10 pmol)	1 µl
PCR water	12 µl
Total volume	20

The PCR mix that revealed in table above placed in AccuPower PCR -PreMix that contain all other PCR components, which needed to reaction such as (Taq DNA polymerase, dNTPs, 10 PCR buffer). Then, all the PCR tubes transferred into vortex centrifuge for 3 minutes. Then transferred into thermocycler (MyGene, Bioneer. Korea).

5-PCR thermocycler conditions:

Table (3) PCR thermocycler conditions

PCR	Temp	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30 sec.	30 cycle
Annealing	60C	30 sec.	
Extension	72C	1min	
Final extension	72C	5 min	1
Hold	4C	Forever	-

6-PCR product analysis:

The PCR products were examined by electrophoresis in a 1% agarose gel using 1X TBE buffer, stained with ethidium bromide, and investigation under UV transilluminator.

Results

Firstly, detection of *Salmonella typhimurium* in current study first by cultural features By using conventional bacteriological methods: 14 out of the 40 chicken samples (35%) were culture positive for *salmonella* In present study, two primer sets were designed to detect the virulence factors of *Salmonella* species by using PCR, targeting the invA gene and ssaP. This research confirms the ability of the specific

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primer sets to detect the isolates as Salmonella. All isolates were subjected to specific gene invA positive by the predicted product a 677-bp DNA fragment. While to gene ssaP positive by the predicted products a 314-bp. The result shows that invA and ssaP genes were present in 78.5% (11\14), 71.4% (10\14) of the samples (figure1and figure 2).

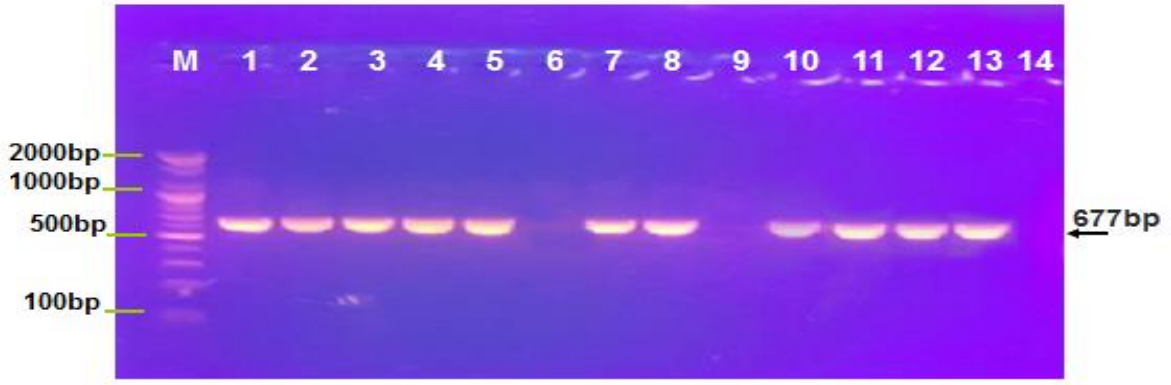

Figure (4): Image of Agarose gel electrophoresis showing analysis of product of the PCR for *invA* genes in *Salmonella typhimurium* isolates. M marker (2000-100bp), lane (1-14) isolates were positive in (677bp) product of PCR except the isolates (6, 9, 14) were negative.

Figure (5): Image of Agarose gel electrophoresis showing analysis of product of the PCR for *ssaP* genes in *Salmonella typhimurium* isolates. M marker (2000-100bp), lane (1-14) isolates were positive in (314bp) product of PCR except the isolates (3, 4, 5, 7) were negative.

Discussion

Recently, there are attempts to establish assay which specific and sensitive to reduce the time of identification of *Salmonella* species and their virulence genes from different samples, so the present study supports to confirm the identification of the *Salmonella* by the specific primers that are selected.(13)(14). Detection by conventional culture assay show 35% positive for *Salmonella typhimurium* in other countries studies have recorded the prevalence s of *Salmonella* spp in poultry samples with percentages of contamination range from 3% to 6% (15). Other researches from a total of 1125 samples from poultry, 22.2% were identified as *Salmonella typhimurium* (16).while (17) obtained 8.3% of poultry carcasses were found to be contaminated with *Salmonella*. InvA gene is essential for invasion of tissue for *Salmonella* virulence (18). In this study presence of invA gene in *Salmonella typhimurium* from chicken samples was 78.5%, while in several studies in different areas about invA genes confirmed that this gene record 100% of the Salmonella (19). The sequences of primer

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that are tested from the gene invA of *Salmonella typhimurium* have ability to detect rapidly *Salmonella* species rapidly in this study. Also (20) designated a study about invA gene was detected in all isolates of *Salmonella, also* this result agreed with those which obtained by (21) in south of Brazil. In this article, all bacteria from *Salmonella typhimurium* showed a 677bp amplicon; result from InvA gene pair of primer. While (17) reported 284-bp DNA fragment. In extant study presence of ssaP gene in *Salmonella, typhimurium* from chicken samples was 71.4%. The (12) detect *ssaP* gene in 100% of the samples from seafood associated *Salmonella* isolate in India. The differences as compared between current studies with previous researches may be due to cross contamination methods that used for detection sample collection as well as the origin of samples.

References

1-Amavisit P, Browning G F, Lightfood D, Anderson CS. Rapid PCR detection of Salmonella in horsefaecal samples. Vet. Microbiol. (2001); 79: 63-74.

2-Vieira A, AR Jensen, SM Pires, S Karlsmose, HC Wegener, DLF Wong (2009). WHO Global Foodborne Infections Network Country Databank-a resource to link human and non-human sources of *Salmonella*. Proceedings of the 12th Symposium. International Society for Veterinary Epidemiology and Economics, Durban, South Africa.

3-Mello RT, Guimarães AR, Mendonça EP, Coelho LR, Monteiro GP, Fonseca BB, Rossi DA. Identificação sorológica e relação filogenética de *Salmonella* spp. de origem suína. Pesq. Vet. Bras. (2011); 31(12):1039-1044.

4-Panisello PJ, Rooney R, quantick PC, Stanwell-Smith R. Application of foodborne disease outbreak data in the development and maintenance of HACCP system. Int. J. Food Microbiol. (2000); 59:221-234.

5-Faucher SP, Forest CH, Beland M, Daigle F. A novel PhoP-regulate*d* locus encoding the cytolysin *clyA* and the secreted invasin *taiA* of *Salmonella* enterica serovar Typhi is involved in virulence. Microbiology (2009); 155:477-488.

6-Desin TS, Lam PK, Koch B, Mickael C, Berberov E, Wisner AL, Townsend HG, Potter AA, Oster W. *Salmonella enterica* serovar Enteritidis SPI-1 is not essential, but facilitates rapid systemic spread in chickens. (2009); Infect. Immun. doi:10.1128/IAI.00039-09.

7-Hald T, Vose D, Wegener HC, Koupeev TA. Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. Risk Anal. (2004); 24:255-269.

8-Malorny B, J Hoorfar, C Bunge, R Helmuth, Multicenter validation of the analytical accuracy of Salmonella PCR: towards an international standard. Appl. Environ. Microbiol. (2003); 69: 290-296.

9-Jamshidi A, Bassami MR, Afshari-Nic S. Identification of Salmonella spp. and Salmonella typhimurium by a multiplex PCR-based assay from poultry carcasses in Mashhad- Iran Int.J.Vet.Res. (2009); 3, 1:43-48, 2009.

10-Daigle F. Typhi genes expressed during infection or involved in pathogenesis. J. Infect. Dev. Ctries. (2008); 2:431-437.

12-Bhowmick PP, Devananda D, Ruwandeepika HAD, Karunasagar I, Karunasagar I. Presence of *Salmonella* Pathogenicity Island 2 genes in seafood associated *Salmonella* serovars and the role of s*seC* gene in survival of *Salmonella enterica* serovar Weltevreden in epithelial cells. Microbiology (2011); 157:160-168.

13-Ferretti R, L Mannazzu, L Cocolin, G Comi, F Biochemica, 4: 19-21. Clementi, Twelve-hour PCR-based method for detection of Salmonella spp. In food. Appl. Environ. Microbiol., (2001); 74: 977-978.

14-Schneder A, C Gronewald, M Fandke, B Kurth, S Barkowski, K Berghof-ager. Real-time detection. Of the genus Salmonella with the Light Cycler system. Biochemica., (2002); 4: 19-21.

15-Zhao G, Ge B, De Villena J, Sudler R, Emily Yeh E, Zhao S, White DG, Wagner D, Jianghong Meng J. Prevalence of Campylobacter spp.,Escherichia coli, and Salmonella serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D. C., Area. Appl Environ Microbiol. (2001); 67: 5431- 5436.

16-Emaddi-Chashni SH, Hassanzadeh M, Bozorgmehri Fard MH, Mirzaie S. Characterization of the *Salmonella i*solates from backyard chickens in north of Iran, by serotyping, multiplex PCR and antibiotic resistance analysis. Arch. Razi Inst. (2009); 64:77-83.

17-Malmarugan Sh, Thenmozhi V Jonson R. Inv A gene specific PCR for detection of Salmonella from broilers..Tamilnadu Veterinary and Animal Scinces University, Vet. World, (2011); Vol.4 (12):562-564.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

18-Khan AA, Nawaz MS, Khan SA, Cerniglia CE. Detection of multidrug-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. FEMS Microbiol. Lett. (2000); 182:355-360.

19-Zahraei ST, Mahzoonae MR, Ashrafi A. Ampllification of invA gene of *Salmonella* by polymerase chain reaction (PCR) as a specific method for detection of *Salmonella.* J. Fac. Vet. Med. Univ. Tehran (2006); 61:195-199.

20-Ogunremi Susan Nadin-Davis, Andrée Ann Dupras, Imelda Gálvan Márquez, Katayoun Omidi, Louise Pope, John Devenish, Teresa Burke, Ray Allain, Daniel Leclair. Evaluation of a Multiplex PCR Assay for the Identification of *Salmonella* Serovars Enteritidis and *Typhimurium* Using Retail and Abattoir Samples. Journal of Food Protection: February (2017); Vol. 80, No. 2, pp. 295-301.

21-Karen A Borsoi, Thales Q Furian, Anderlis Borsoi, Hamilton LS Moras, Carlos T PSalle , Vladimir P Nascimento. Detection of virulenc e -associated gene in salmonella enteritidis isolates from chicken in south of Brazil, (2010); south of Brazil.