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Rapid detection and identification of poultry *Salmonella* serotypes using multiplex PCR assay

Lujain Dh. Al-Khayat [1]*; Emad J.Khammas [2]; Ruqaya M.Ali[1]; Bashaer A. Al-Owaini[1] and Layth M. Salih Abdulrasool [1]

[1] Veterinary Directorate –Department of Central Veterinary laboratory & research / Baghdad; [2] College of Veterinary Medicine – University of Baghdad

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***Corresponding author:**
Email address:
lujain12345@yahoo.com

Abstract

Recently, rapid multiplex PCR assay has been used widely worldwide to identify and screen *Salmonella* and their most important serovars in the poultry industry without the need of the serological examination. This study designed to determine different *Salmonella* serotypes that isolated from chicken using multiplex PCR assay. Layer and broiler chicken internal organs including: liver, bile, spleen, heart, yolk sac, ceca, joint, ovary and oviduct were used to isolate *Salmonella sp.* Sixty (60) *Salmonella* isolates were subjected to amplification of *inv*A gene (invasion gene) for *Salmonellae sp.*; *fli*C gene (flageller filament protein) for *Salmonella typhimurium* and *sef*A gene (fimberial gene) for *Salmonella enteritidis* and *Salmonella gallinerum – pullorum*. Each primer pairs was optimize individually to ensure that each amplicon had the correct size. Then, Salmonella isolates passed to amplification by use three sets of primers *inv*A, *fli*C and *sef*A simultaneously in order to detect the genus *Salmonella* and their types in single reaction tube. The results of this study showed that all *Salmonella* isolates were positive for *inv*A gene amplified sequence. Moreover, the serotypes of *Salmonella typhimurium* and *Salmonella enteritidis* were identified by the presence of the specific amplified products to *fli*C gene for *S. typhimurium* and *sef*A gene for *S. enteritidis* and *Salmonella gallinerum-pullorum*. In conclusion, this study approved that applying multiplex PCR assay revealed the same sensitivity and specificity of uniplex PCR. Moreover, this technique was easy, reliable and save time and cost. The authors recommend to implement the combination between, routine multiplex PCR test and traditional culture methods to approach the effective and more accurate profile for the prevalence of *Salmonella* in flocks of poultry in Iraq.

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Introduction

Salmonella considers as the most important Gram-negative rod-shaped bacteria that belongs to the family of *Entrobacteriacea*. There are more than 2,500 known serotypes of *Salmonella enterica* (Ibrahim *et al.,* 2013). Avian salmonellosis mainly caused by two groups of *Salmonella*, the non-motile *Salmonella sp*. (*Salmonella gallinarum* and *Salmonella pullorum*) that cause fowl typhoid and pullorum disease respectively, and the motile *Salmonella sp.* (paratyphoid group) that cause paratyphoid infection and have zoonotic significance (Hossain *et al.,* 2006). *Salmonella* serotyping is a necessary first step in any epidemiological investigation (Shimizu *et al.,* 2014). The serotyping by the traditional methods is time-consuming, requires well-trained technicians and uses large amounts of high-quality sera (Helmy *et al.,* 2009). Shimizu *et al.,* (2014) mentioned that traditional serotyping requires at least three antibody- antigen reactions to identify *Salmonella* serovars. Moreover, it often requires many further tests for accurate serotyping (Shimizu *et al.,* 2014). Therefore, the development of a rapid and sensitive method to identify *Salmonella sp.* and their serovars is desirable (Paião *et al.,* 2013). Molecular biology based techniques including PCR assays have been reported for the rapid, specific, and sensitive detection of microorganisms in different clinical samples (Faik *et al.,* 2014). Multiplex PCR provides a way to fast and accurate detection of *Salmonellae* and their most important serovars through amplification of the specific target genes simultaneously in one reaction (Helmy *et al.,* 2009). Review of literature resulted scarce information regarding the rapid identification of *Salmonella sp.* using multiplex PCR assay in Iraq. So, this study designed to determine and identify different *Salmonella* serotypes of broiler and layer chickens using multiplex PCR assay.

Materials and methods

Sample collecting

A total of (577) samples collected randomly from layer and broiler chicken farms (different ages) in Baghdad governorate. The sample of each bird collected using aseptic technique and kept in a sterile plastic container. All samples put in a cold box and transfer immediately to the laboratory for bacterial isolation.

Bacterial isolation and identification

All samples were aseptically cultured into non- selective broth (peptone water broth) and incubated at 37oC for 24 hours. Consequently, all samples were grown on selective broth (selenite F broth) (Himedia), and incubated at 37oC for 24 hours. Later on, a loopful of each broth was streaked on the surface of the following selective medium: Salmonella and Shigella agar (Himedia), Xylose Lysine Desoxycholate Agar (Himedia), Brilliant green agar (LAB), chromogenic agar (Conda) and MacConkey agar plates (Oxoid). All cultures incubated at 37oC for 24 hrs. according to the methods described previously (OIE, 2012). All bacterial isolates were stained by Gram's stain (according to the manufacturer's instruction) to determine their staining characteristics and to ensure the purity of the culture. Biochemical identification was made for all isolated bacteria including sugar fermentation tests such as dextrose, glucose, sucrose, lactose, mannitol and maltose. The biochemical tests including oxidase, catalase, urease, TSI, indole,

lysine iron agar and simmone's citrate test that also done according to (Quinne *et al.*, 2004). The analytical profile Index (API) system (Liofilchem, Italy) was also done for further confirmation of the presumptive *Salmonella* isolates.

Molecular identification

The DNA extraction kit (Genaid, Korea) was used to extract DNA from each enriched bacterial culture. Consequently, the purified DNA used as a template for the PCR assay. Accordingly, the primers designed for the PCR investigation, and the sequence of primers used in this study is presented in (Table. 1).

Table. 1: Shows the sequence of primers that used for detection of *Salmonella* and their serovars.

Primer Sequences	Corresponding gene	Expected Product size	References
F: GTGAAATTATCGCCACGTTCGGGCAA	*inv*A gene *(Salmonella* spp).	284 bp	(Oliveira *et al*.,2002; Malorny *et al*.,2003)
R: TCATCGCACCGTCAAAGGAACC			
F: CGGTGTTGCCCAGGTTGGTAAT	*fli*C gene *(S. typhimurium)*	620 bp	(Oliveira *et al*.,2002; Anumolu and Lakkineni.,2012)
R: ACTGGTAAAGATGGCT			
F: GCGTAAATCAGCATCTGCAGTAGC	*sef*A gene *(S. enteritidis and Salmonella gallinerum–pullorum)*	488 bp	(Oliveira *et al*.,2002; Oliveira *et al*., 2003)
R: GATACTGCTGAACGTAGAAGG			

The DNA samples amplified by uniplex PCR reaction in a total of 25 µl (for each primer separately) as the following: 12.5µl of PCR master mix (Promega, USA), 5 µl of a template, 0.5µl of forward primer, 0,5µl of reverse primer and 6.5µl of PCR grade water. While, DNA samples amplified for multiplex PCR reaction in a total of 50 µl and as the following: 1µl of forward primer (from each of *inv*A, *fli*C and *sef*A), 1µl of reverse primer (from each of *inv*A, *fli*C and *sef*A), 0.2 1x Q-solution, 25µl of multiplex PCR master mix (Qiagen, USA), 5 µl of the template and 13.8 µl of PCR-free water. Both uniplex and multiplex PCR were performed according to the following procedures: primary denaturation: 95˚C / 2 min, secondary denaturation: 94˚C / 1 min, annealing at 58˚C / 1 min, extension at 72 ˚C 1 min, No. of cycles: 35 and the final extension at 72˚C / 10 min. Subsequently, all aliquots of amplified PCR products were electrophoresed on 1% agarose gel. A 5µl of 100 bp DNA ladder ((KAPA, South Africa) and the samples were filled in the wells. Later on, a current of 60 V was passed for 1 hour on the media in the horizontal electrophoresis unit. Eventually, the specific amplicons were observed under ultraviolet transillumination compared with the marker. A gel documentation system was used to photograph the samples, and the data were analyzed.

Results and Discussion

Totally 60 out of 577 (60/577) samples were positive for *Salmonella sp.* . All suspected *Salmonella* cultures that subjected to uniplex PCR amplification resulted in a product of approximately the molecular size 284 bp fragments of *inv*A gene (100%). These fragments were specific for all members of *Salmonella* species (Figure.1 A & B).

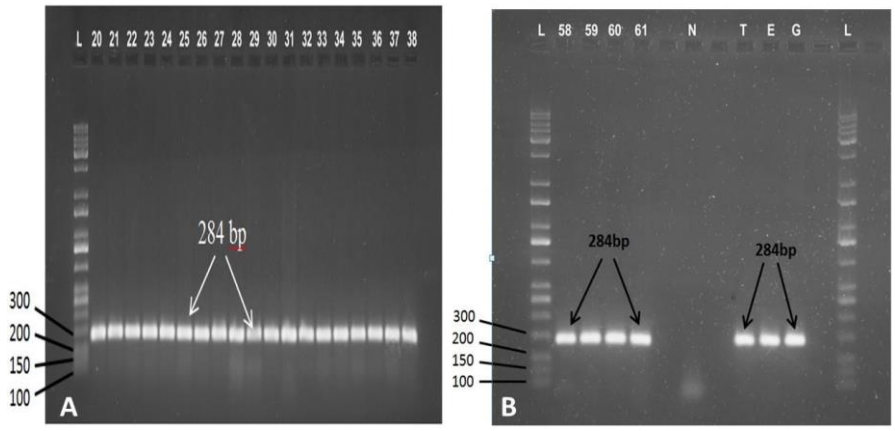

Figure.1: A) Agarose gel electrophoresis for amplification of *inv*A gene (284bp) of *Salmonella sp*, lane 20-38 positive results as *Salmonella sp.* (L): 100 bp marker. **B)** PCR amplification of 284 bp fragments of invasion (*inv*A) gene. Lanes 58, 59, 60 and 61 show positive results (*Salmonella spp.)*. Lane L shows PCR markers. Lane N: negative control. Lane T: positive control for *Salmonella typhimurium.* Lane E: positive control for *Salmonella enteritidis.* Lane G: positive control for *Salmonella gallinarum*.

These results are compatible with the observations reported previously (Abd El-Ghany *et al.,* 2012; Oliveira *et al.,* 2003), who found that *inv*A gene was able to identify all the examined *Salmonella* serovars. Also, these findings are in agreement (Abd El Tawwab *et al.,* 2013), who approved that the *inv*A gene was present in 100% of the poultry (chickens and ducks) *Salmonella* isolates. Moreover, this gene was identified by the presence of a 284 bp PCR amplified fragment. Accordingly, the primer sequences were selected from the gene *inv*A that encodes the protein in the inner membrane of bacteria and responsible for the invasion of the epithelial cells of the host. Previous studies approved that the ability of *Salmonella*-specific primers to detect *Salmonella* species were rapid and accurate (Shanmugasamy *et al.,* 2011 and Karmi, 2013). Meanwhile, these primers have been recognized as an international standard for detection of the genus of *Salmonella* (Malorny *et al.,* 2003).

Uniplex PCR amplification of DNA showed 13 out of 60 *Salmonella* isolates were revealed a specific product of approximate molecular size 620 bp fragments of the *fli*C gene, which considered as the specific fragments for *Salmonella typhimurium* species. All *Salmonella typhimurium* isolates and positive control strains that generated a product of molecular size 620 bp fragments of *fli*C gene were analyzed by agarose gel electrophoresis and represented in Figure. (2).

This result is compatible with the results reported previously by other researchers (Oliveira *et al.,* 2003; Moussa *et al.,* 2010; Anumolu and Lakkineni, 2012), who reported that *fli*C gene was able to identify *Salmonella typhimurium* serovar by amplify 620 bp fragments and present in all standard *Salmonella typhimurium* strains. This finding is in agreement with (Nader *et al.,* 2015), who showed, the *fli*C gene (flagellar filament protein) that was able to identify *Salmonella typhimurium* in contaminated food.

The results of this study also revealed that 20 out of 60 were *Salmonella enteritidis* by uniplex PCR amplification and generated a product of approximate molecular size 488

bp fragments of *sef*A gene specific for *Salmonellae enteritidis* species. The band size that detected in all *Salmonella enteritidis* isolates and analyzed by agarose gel electrophoresis is presented in Figure (3 A & B). Moreover, the positive control strains of *Salmonella enteritidis* and *Salmonella gallinarum* also amplified and generated a product of molecular size 488 bp fragments of *sef*A gene. This result is also in agreement with the result that obtained by (Oliveira *et al.*, 2003) and Moussa *et al.*, 2010), who applied a similar *sef*A primer sequence and amplified a single band of 488 bp to identify *Salmonella enteritidis* from poultry derived samples. The results of the present study are also compatible with previous studies (Murugkar *et al.*, 2003 and Borges *et al.*, 2013), who reported the presence of *sef*A gene (fimbrial gene) in 100% of poultry *Salmonella enteritidis* and it can be considered as a target gene to identify the serovar *Salmonella enteritidis* by PCR (Amini *et al.*, 2010).

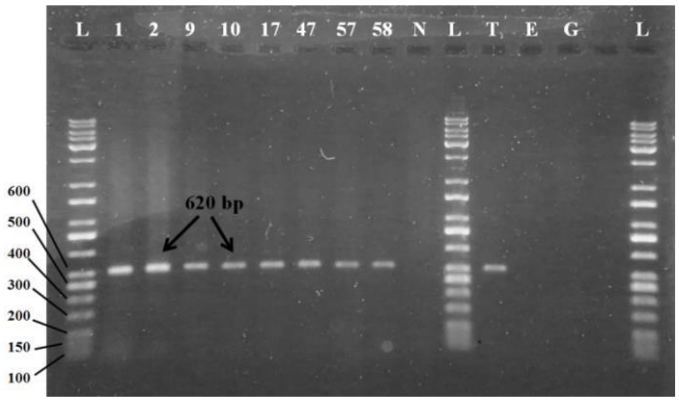

Figure. 2: PCR amplification of 620 bp fragments of *fli*C gene (flagellar filament protein). Lanes 1, 2, 9, 10, 17, 47, 57 and 58 show positive results (*Salmonella typhimurium*). Lane L shows PCR markers. Lane N: negative control. Lane T: positive control for *Salmonella typhimurium*. Lane E: positive control for *Salmonella enteritidis*. Lane G: positive control for *Salmonella gallinarum*.

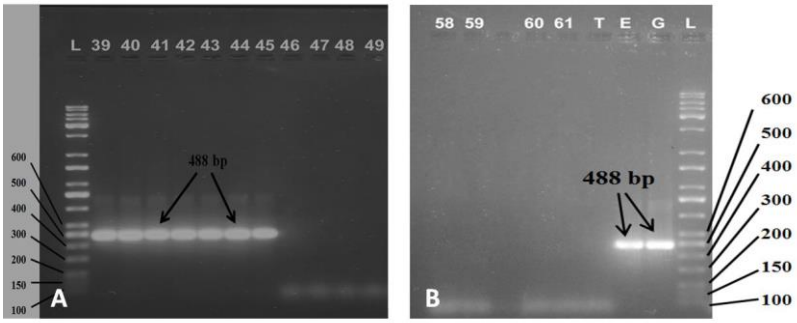

Figure.3: A) PCR amplification of 488 bp fragments of *sef*A gene (fimberial gene). Lanes (39- 45) show positive results (*Salmonella enteritidis*), while lanes (46-49) show negative results. Lane L shows PCR markers. **B)** PCR amplification of 488 bp fragments of `A gene (fimberial gene). Lanes 58, 59, 60 and 61 show negative results. Lane L. shows PCR markers. Lane N: negative control. Lane T: positive control for *Salmonella typhimurium*. Lane E: positive control for *Salmonella enteritidis*. Lane G: positive control for *Salmonella gallinarum*.

The optimization of each primer pairs was done separately to ensure that each amplicon has the correct size of *Salmonella sp.* that passed to amplification using three sets of primers *inv*A, *fli*C and *sef*A simultaneously, in a single reaction tube to detect the genus of *Salmonella* and their types (Figure 4). The results of the present study also revealed that multiplex PCR assay has the same sensitivity and specificity of uniplex PCR, as well as, it is easy and save time and cost. The results of this study are also compatible with the results reported by (Malkawi and Gharaibeh, 2004; Kumar, 2012; Chagas *et al.*, 2013; Nourmohamadi and Shokrollahi, 2014). It is also compatible with El Jakee *et al.*, (2016), who showed that multiplex PCR was faster, highly specific and sensitive assay compared to conventional PCR in detection of different *S. enterica* serovars.
In conclusion, this study approved that multiplex PCR able to amplify multiple genes in one PCR reaction. Moreover, it can detect *Salmonella* and their serotypes at the same specificity and sensitivity within a much shorter time and less cost than traditional PCR assays.

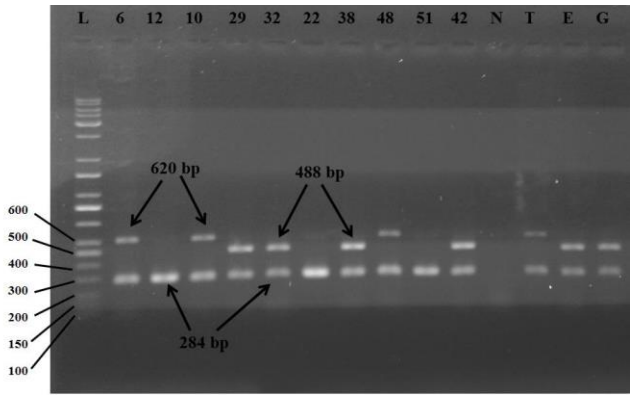

Figure. 4: Multiplex PCR with three pairs of primers for the genus *Salmonella, Salmonella typhimurium, Salmonella enteritidis* and *Salmonella gallinerum - pullorum* isolated from poultry. The 284 bp bands produced by *inv*A gene(specific for the genus *Salmonella*) , the 620 bp bands produced by *fli*C gene (specific for *Salmonella typhimurium*) and the 488 bp bands produced by *sef*A gene (specific for *Salmonella enteritidis* and *Salmonella gallinerum - pullorum*) respectively. L: marker (100 bp). N: negative control. T: positive control for *Salmonella typhimurium.* E: positive control for *Salmonella enteritidis.* G: positive control for *Salmonella gallinarum.*

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