



DETECTION OF COMMON (*invA*) GENE IN SALMONELLAE ISOLATED FROM POULTRY USING POLYMERASE CHAIN REACTION TECHNIQUE

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ABSTRACT

Salmonellosis is one of the most common and widely distributed foodborne diseases, and its presence in poultry and poultry products is a global public health problem. Therefore, the present study was conducted to isolate *Salmonella* from internal organs, fecal matter and eggs of freshly dead, diseased living and apparently healthy chickens and ducks in Dakahlia governorate (Egypt). A total of 400 samples were collected as follows: 280 chickens, 20 chicken eggs, 89 ducks and 11 duck eggs. The samples were examined bacteriologically and serotyped. Forty five samples (11.25%) were found to be positive for Salmonellosis. Ten strains were detected (*S. Kentucky*, *S. Skansen*, *S. Typhimurium*, *S. Wingrove*, *S. Agona*, *S. Tananarive*, *S. Newport*, *S. Inganda*, *S. Enteritidis* and *S. Labadi*). Untyped salmonellae were detected. The isolated *Salmonella* was sensitive to gentamycin, ciprofloxacin, colistin sulphate, doxycyclin hydrochloride and amoxicillin. Polymerase chain reaction (PCR) for detection common gene (*invA*) was applied to all isolated strains and showed positive amplification of 284 bp fragments.

Keywords: *Salmonella*, *invA* gene, PCR

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1. INTRODUCTION

Salmonella is gram negative, non spore-forming, usually motile, facultative anaerobic bacilli belong to the family *Enterobacteriaceae*. Infection with *Salmonella* may or may not lead to fatal Salmonellosis [1]. Avian salmonellosis is an important disease causing serious impediment to the development of poultry industry especially in developing countries of Asia and Africa. Since no "effective" immunoprophylactic measures are available for the disease uptill now, strict biosecurity is the only alternative to preclude the disease [2]. Polymerase chain reaction (PCR) is molecular biology technique which has taken up an increasingly significant space in the field of laboratory diagnostics, allowing

the detection of various pathogens such as *salmonella* species in different kind of food. PCR can reduce the time required to detect and identify the agent with high specificity and sensitivity [3]. The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with a potential diagnostic application [4].

2. MATERIALS AND METHODS

2.1. Samples collection

A total number of 400 samples from chickens and ducks were collected as follows: 280 chicken samples (freshly dead, diseased living and apparently healthy birds), 20 chicken eggs, 89 ducks (freshly

dead, diseased living birds and apparently healthy) and 11 duck eggs were obtained from different farms, markets, backyards located in Dakahlia Governorate under aseptic condition in ice box and transferred to the laboratory.

2.2. Bacteriological examination

Cultivation and isolation of *Salmonella*: It was done according to ISO 6579 [5] by pre-enrichment of the collected samples in Buffered Peptone Water as 1:10 dilution and then incubated aerobically at 37°C for 18 hours. 0.1 ml was transferred to a tube containing 10 ml of the Rappaport Vassiliadis Soy broth and then incubated at 41.5°C for 24 hours. One ml of the pre-enrichment culture were also transferred to a tube containing 10 ml of the Muller–Kauffmann tetrathionate/novobiocin broth and then incubated at 37°C for 24 hours. From the enrichment culture, 10 µl were inoculated onto the surface of Xylose Lysine Deoxycholate (XLD), Hektoen Enteric, Brilliant Green, *Salmonella-Shigella* and MacConkey's agar plates then incubated at 37°C for 24 hours. The plates containing characteristic colonies of *Salmonella* were selected and the gram staining test was performed. Each colony showing typical colonial appearance were subjected to biochemical identification and examined for hydrolysis of urea, H₂S production, lysine decarboxylation, indole test, methyl red test, Voges Preskauer test and citrate utilization.

2.3. Serological typing of *Salmonella* organism:

The isolates that were preliminarily identified biochemically as [6] *Salmonella* were subjected to serological identification according to Kauffman-White Scheme for determination of somatic (O) and flagellar (H) antigens.

2.4. Antibiotic susceptibility testing:

Determination of the susceptibility of the isolated salmonellae to antibiotic discs was adopted using the disc diffusion technique [7]. The discs that used for *Salmonella* were

oxytetracyclin, ciprofloxacin, enrofloxacin, ampicillin, amoxicillin, gentamycin, neomycin, colistin sulphate, chloramphenicol and doxycycline hydrochloride.

2.5. Confirming the identification of isolated strains using the Polymerase chain reaction (PCR) technique:

Extraction of bacterial DNA by QIAamp®DNA Mini Kit (Cat. No. 51304 Qiagen) and specific primers for *Salmonella* organism was used according to [8]. Sequence of forward primer (*invA*) was GTGAAATTATCGCCACGTTTCGGGCA A) and reverse primer was TCATCGCACCGTCAAAGGAACC).

DNA samples were amplified in a total of 25 µl as the following: 12.5µl of PCR master mix, 1µl of forward primer, 1µl of reverse primer, 4.5µl of PCR grade water and 6 µl of the template. The PCR was performed under the following conditions (primary denaturation: 94°C / 5 min., secondary denaturation: 94°C / 30 sec., annealing: 55°C / 30 sec., extension: 72°C / 30 sec., No. of cycles: 35 and final extension: 72°C / 10 min. Aliquots of amplified PCR products were electrophoresed in 1.5% agarose gel. The samples and a 100 bp DNA ladder were loaded in the wells in amount of 8µl of sample. A current of 80 V for 1 hour was passed on the medi horizontal electrophoresis unit. Specific amplicons were observed under ultraviolet transillumination compared with the marker. The gel was photographed by a gel documentation system and the data were analyzed.

3. RESULTS

3.1. Result of cultural, morphological and biochemical characters of the isolated salmonellae:

Salmonella on XLD appeared as smooth colonies with black center. On brilliant green agar it changed the color of the medium to red/pink, while on *Salmonella*-

Shigella agar it appeared pale colored colonies indicated non lactose fermenting with or without black centers. On Hektone enteric agar, it produced deep blue colored colonies and on MacConkey's agar it appeared as pale, colorless smooth, transparent and raised colonies. The staining characters appeared as Gram negative, non spore forming short rod shaped. The results of biochemical identification of the isolated *Salmonella* are shown in Table 1.

3.2. Prevalence of *Salmonella* isolation from different samples:

400 examined samples that were represented as 280 samples from chickens, 89 samples from ducks, 20 samples from chicken eggs and 11 samples from duck eggs. Forty five samples were found to be positive for *Salmonella* from a total number of 400 examined samples with an incidence of 11.25%.

3.3. Prevalence of salmonellae recovered from internal organs, fecal matter and eggs of different types of flocks:

30 samples (10.71%), 11 (12.36%), 3 (15%) and 1 (9.09%) were found to be positive from chicken (internal organs and fecal matter), duck (internal organs and fecal matter), chicken eggs and duck eggs, respectively (Table 2).

3.4. Results of serotyping of the isolated salmonellae.

Ten strains were detected (*S. Kentucky*, *S. Skansen*, *S. Typhimurium*, *S. Wingrove*, *S. Agona*, *S. Tananarive*, *S. Newport*, *S. Inganda*, *S. Enteritidis* and *S. Labadi*) also untyped salmonellae strains were detected.

3.5. Results of serotyping of the isolated *Salmonella* from chickens internal organs and eggs.

Four *S. Agona*, two *S. Wingrove*, three *S. Tananarive*, twelve *S. Typhimurium*, two *S. Newport*, one *S. Enteritidis*, two *S. Labadi* and four un typed *Salmonella* were isolated from chickens internal organs with a

percentage of (13.33%), (6.67%), (10%), (40%), (6.67%), (3.33%), (6.67%) and (13.33%), respectively. But in chicken eggs two *S. Typhimurium* and one *S. Enteritidis* were isolated from chicken eggs with a percentage of (66.67%) and (33.33%) respectively (Table 3).

3.6. Results of serotyping of the isolated *Salmonella* from duck internal organs and eggs.

Two *S. Skansen*, four *S. Typhimurium*, two *S. Kentucky*, two *S. Inganda* and one untyped *Salmonella* were isolated from ducks internal organs with a percentage of (18.18%), (36.36%), (18.18%), (18.18%) and (9.1%) respectively. But one *S. Typhimurium* was isolated from ducks eggs with a percentage of (100%) (Table 4).

3.7. Results of the sensitivity tests for the isolated salmonellae

All salmonellae were sensitive to gentamycin, ciprofloxacin, colistin sulphate, doxycyclin hydrochloride and amoxicillin. All examined salmonellae were sensitive to chloramphenicol and ampicillin except *S. Tananarive* and *S. Inganda*, respectively. All salmonellae were sensitive to neomycin except *S. Skansen* and some untyped salmonellae. On the other hand, *S. Typhimurium*, *S. Kentucky*, *S. Agona* and *S. Wingrove* were resistant to enrofloxacin and other salmonellae were sensitive to enrofloxacin. *S. Typhimurium*, *S. Enteritidis* and some untyped salmonellae were resistant to oxy-tetracycline and other salmonellae were sensitive to oxy-tetracycline.

3.8. Detection of common gene of *Salmonella* (*invA*) using polymerase chain reaction (PCR):

All *Salmonella* serovars in this study showed positive amplification of 284 bp fragment specific for the *invA* gene (common gene) with total percentage (100%) from examined samples (from chicken and duck) (photo no. 1 and 2).

Detection of common (*inv A*) gene in salmonellae isolated from poultry using PCR

Table (1) Results of biochemical identification of the isolated salmonellae using standard laboratory tests.

Type of media	Result of biochemical identification
Urea agar	Negative result - the color of urea agar was yellow.
Triple sugar iron agar	Positive result - alkaline slant (red), acid butt (yellow) with H ₂ S and gas production.
Lysine Iron Agar	Positive result - Deep purple (alkaline) slant and alkaline butt, No gas production, no H ₂ S production
Simmon's Citrate	Positive result – Blue color.
Indole reaction	Negative result - Yellow ring.
Methyl Red test	Positive result - Red color at the surface.
Voges- reaction	Proskauer Negative result - No bright red color.

Table (2) prevalence of salmonellae recovered from (internal organs, fecal matter) and eggs of different types of flocks.

Type of samples	Number of examined samples	Number of positive samples	%	Number of negative samples	%
Chicken (internal organs, fecal matter)	280	30	10.71%	250	89.29%
Duck (internal organs, fecal matter)	89	11	12.36%	78	87.64%
Chicken eggs	20	3	15%	17	85%
Duck eggs	11	1	9.09%	10	90.91%

Table (3): Results of serotyping of the isolated *Salmonella* from chickens internal organs and eggs.

Type of the isolated strains	Number and percentage (internal organs)	Number and percentage (eggs)
<i>S. Agona</i>	4 (13.33%)	0 (0%)
<i>S. Wingrove</i>	2 (6.67%)	0 (0%)
<i>S. Tananarive</i>	3 (10%)	0 (0%)
<i>S. Typhimurium</i>	12 (40%)	2 (66.67%)
<i>S. Newport</i>	2 (6.67%)	0 (0%)
<i>S. Enteritidis</i>	1 (3.33%)	1 (33.33%)
<i>S. Labadi</i>	2 (6.67%)	0 (0%)
Untyped <i>Salmonella</i>	4 (13.33%)	0 (0%)
Total	30 (100%)	3 (100%)

Table (4) Results of serotyping of the isolated *Salmonella* from duck internal organs and eggs.

Type of the isolated strains	Number and percentage (from internal organs)	Number and percentage (from eggs)
<i>S. Skansen</i>	2 (18.18%)	0 (0%)
<i>S. Typhimurium</i>	4 (36.36%)	1 (100%)
<i>S. Kentucky</i>	2 (18.18%)	0 (0%)
<i>S. Inganda</i>	2 (18.18%)	0 (0%)
Untyped <i>Salmonella</i>	1 (9.1%)	0 (0%)
Total	11 (100%)	1 (100%)

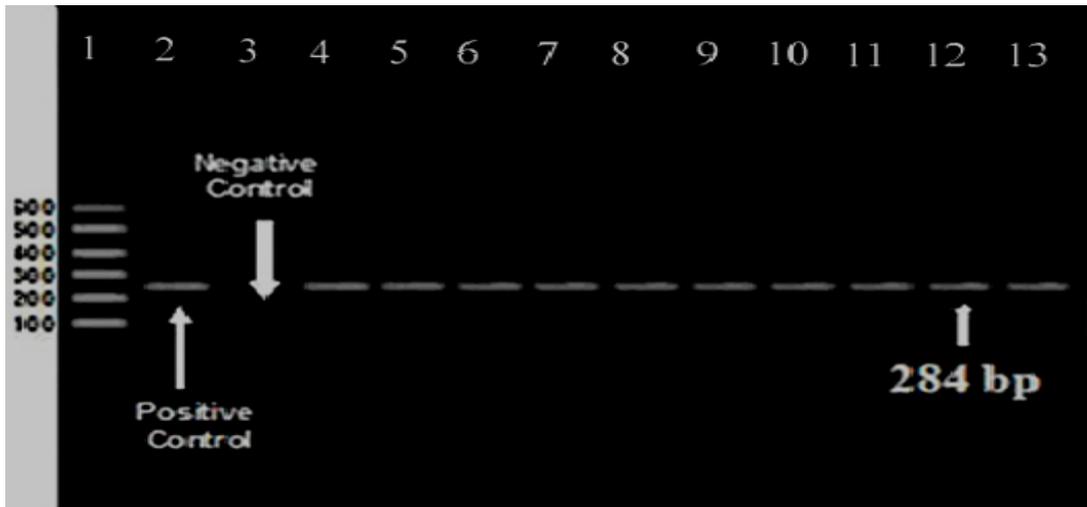


Photo No. (1) PCR result using primer of *invA* gene in chicken samples.

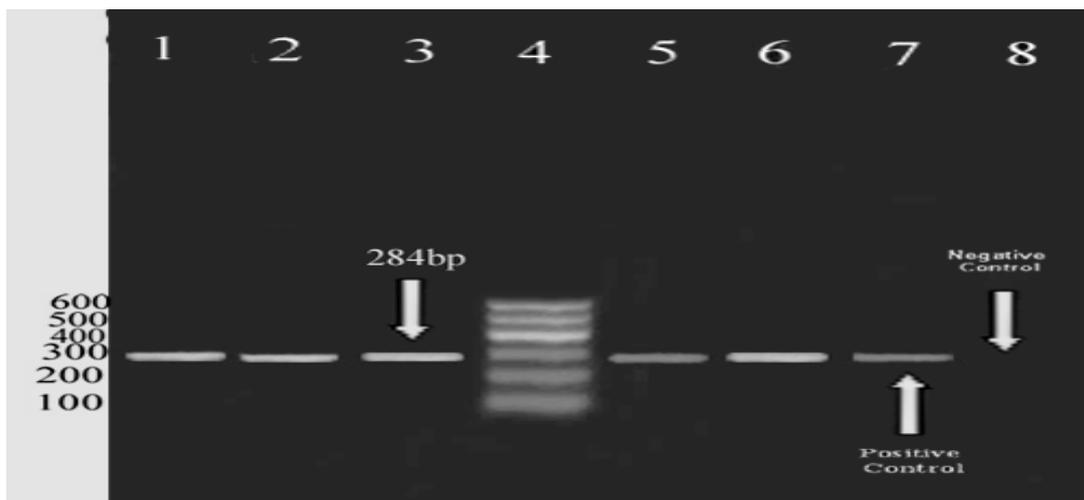


Photo No. (2) PCR result using primer of *invA* gene in duck samples.

4. DISCUSSION

In this study, 45 samples out of 400 samples from chickens and ducks were found to be positive to *Salmonella* (11.25 %). A total of 30 samples were found to be positive from chicken (internal organs and fecal matter) with a percentage of (10.71 %), while in duck 11 samples from (internal organs and fecal matter) were found to be positive with a percentage of (12.36 %), and this result was nearly in coordinating with some researchers such that an incidence of *Salmonella* from a total 70 samples was (11.42%) [9]. Also, Five hundred sixty-nine *Salmonella* was isolated from 4745 samples with incidence (11.99%) from poultry, poultry products [10].

In table (3), the isolated salmonellae from chicken internal organs and chicken eggs were: four *S. Agona*, two *S. Wingrove*, three *S. Tananarive*, twelve *S. Typhimurium*, two *S. Newport*, one *S. Enteritidis*, two *S. Labadi* and four un typed *Salmonella* with a percentage of (13.33%), (6.67%), (10%), (40%), (6.67%), (3.33%), (6.67%) and (13.33%), respectively. However, in chicken eggs two *S. Typhimurium* and one *S. Enteritidis* were isolated from chicken eggs with a percentage of (66.67%) and (33.33%), respectively. The predominant serotypes of *Salmonella* were *S. Typhimurium* and *S. Enteritidis* which agree with previous study [11]. While another study revealed that 57 (9.90%) were positive for *Salmonella*, and the most prevalent serotypes were *Salmonella Typhimurium* (40.35%) and *Salmonella Newport* (26.31%) [12].

In table (4) two *S. Skansen*, four *S. Typhimurium*, two *S. Kentucky*, two *S. Inganda* and one untyped *Salmonella* were isolated from ducks internal organs with a percentage of (18.18%), (36.36%), (18.18%), (18.18%) and (9.1%), respectively. However one *S. Typhimurium* was isolated from ducks eggs with a percentage of (100%), and these results differ from a study that examined 160 samples from ducks and *Salmonella*

isolated with percentage of 3.3%, and it's serotyping yielded three different serovars including *Salmonella Typhimurium*, *Salmonella Derby* and *Salmonella Enteritidis* [13].

All *Salmonella* strains were sensitive to gentamycin and this was agreed with a study reported that (99.3%) isolates were sensitive to gentamycin [14] but on the contrary, study reported that the isolates were highly resistant to ampicillin, chloramphenicol, gentamycin, trimethoprim, tetracycline, and sulfamethoxazole [15]. All isolated *Salmonella* strains were sensitive to amoxicillin. Whereas about 93.3% of isolated *Salmonella* strains were sensitive to amoxicillin [16].

In this study, PCR assay was carried out for the detection of the *invA* gene from isolated strains has revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of a 284 bp PCR amplified fragment which agrees with a study performed and recorded the same results [17]. Amplification of *invA* gene now has been recognized as an international standard for detection of *Salmonella* genus [18]. The *invA* gene encodes a protein in the inner membrane of bacteria, which is necessary for invasion to the epithelial cells of the host [19].

5. REFERENCES

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الكشف عن الجين المشترك في السالمونيلا المعزولة من الدواجن بتقنيته تفاعل البلمرة المتسلسل

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اقسم البكتريولوجي والمناعة والفطريات- كلية الطب البيطري- جامعة بنها² قسم البكتريولوجي والفطريات والمناعة - كلية الطب البيطري- جامعة الزقازيق³ وحدة السيرولوجي - معهد بحوث صحة الحيوان- الدقي - جيزة⁴ المعمل الفرعي بالمنصورة - معهد بحوث صحة الحيوان- الدقي - جيزة.

الملخص العربي

يتسبب ميكروب السالمونيلا في خسارة اقتصادية هائلة في صناعة الدواجن ولذلك فقد تم فحص 400 عينة (300 عينة من الدجاج وبيض الدجاج و100 عينة من البط وبيض البط) من مصادر مختلفه في محافظة الدقهلية. تم اخذ العينات من (الكبد ، الطحال ، القلب) بالاضافه الى الزرق. تم عزل 45 عترة من السالمونيلا من 400 عينة للدواجن بنسبة 11.25% (30 عترة من الدجاج و3 عترات من بيض الدجاج و11 عترة من البط وعترة واحده من بيض البط المجمع من المنازل والأسواق) وتصنيف هذه العترات وجد انها سالمونيلا أجونا- سالمونيلا وين جروف- سالمونيلا تيفيميوريم- سالمونيلا تانانريفى- سالمونيلا نيوبورت- سالمونيلا انتريتيدس - سالمونيلا سكانسن- سالمونيلا كنتاكي- سالمونيلا انجاندا- سالمونيلا لابادى و سالمونيلا غير مصنفة . تم اجراء اختبار الحساسيه للمضادات الحيوية المختلفه للعترات المعزولة. وقد تم ايضا اجراء اختبار تفاعل البلمرة المتسلسل باستخدام البادئ العام للكشف عن جين *invA* وقد تبين تواجده بجميع المعزولات.

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