DETECTION OF COMMON (*inv* A) GENE IN SALMONELLAE ISOLATED FROM POULTRY USING POLYMERASE CHAIN RECTION TECHNIQUE

Ashraf, A. Abd El Tawwab [a], Ahmed, M. Ammar [b], Aisha, R. Ali [c], Fatma, I. El Hofy [a] and Mohammed, E. E. Sayed Ahmed [d]

[a] Bacteriology, Immunology and Mycology Dep., Fac. of Vet. Med., Benha Univ., Egypt. [b] Bacteriology, Mycology and Immunology Dep., Fac. of Vet. Med., Zagazig Univ., Egypt. [c] Serology Unit -Animal Health Research Institute, Dokki – Giza, Egypt. [d] Animal Health Research Institute (Mansoura branch) Dokki – Giza, Egypt.

A B S T R A C T

Salmonellosis is one of the most common and widely distributed foodborne diseases, and it's 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 (*inv*A) was applied to all isolated strains and showed positive amplification of 284 bp fragments.

Keywords: *Salmonella*, *inv*A gene, PCR

(BVMJ-25 [2]: 70 -77, 2013)

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 *inv*A 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 37ºC for 18 hours. 0.1 ml was transferred to a tube containing 10 ml of the Rappaport Vassiliadis Soy broth and then incubated at 41.5ºC 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 37ºC 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 37ºC 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, H_2S 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, chloramphnicol 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 (*inv*A) was GTGAAATTATCGCCACGTTCGGGCA 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.* Kentukey, *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 *inv*A gene (common gene) with total percentage (100%) from examined samples (from chicken and duck) (photo no. 1 and 2).

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_2S and gas production.
Lysine Iron Agar	Positive result - Deep purple (alkaline) slant and alkaline butt, No gas production, no H_2S 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- Proskauer reaction	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)
S. Agona	4 (13.33%)	0 (0%)
S. Wingrove	2 (6.67%)	0 (0%)
S. Tananarive	3 (10%)	0 (0%)
S. Typhimurium	12 (40%)	2 (66.67%)
S. Newport	2 (6.67%)	0 (0%)
S. Enteritidis	1 (3.33%)	1 (33.33%)
S. Labadi	2 (6.67%)	0 (0%)
Untyped *Salmonella*	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)
S. Skansen	2 (18.18%)	0 (0%)
S.Typhimurium	4 (36.36%)	1 (100%)
S. Kentucky	2 (18.18%)	0 (0%)
S. Inganda	2 (18.18%)	0 (0%)
Untyped *Salmonella*	1 (9.1%)	0 (0%)
Total	11 (100%)	1 (100%)

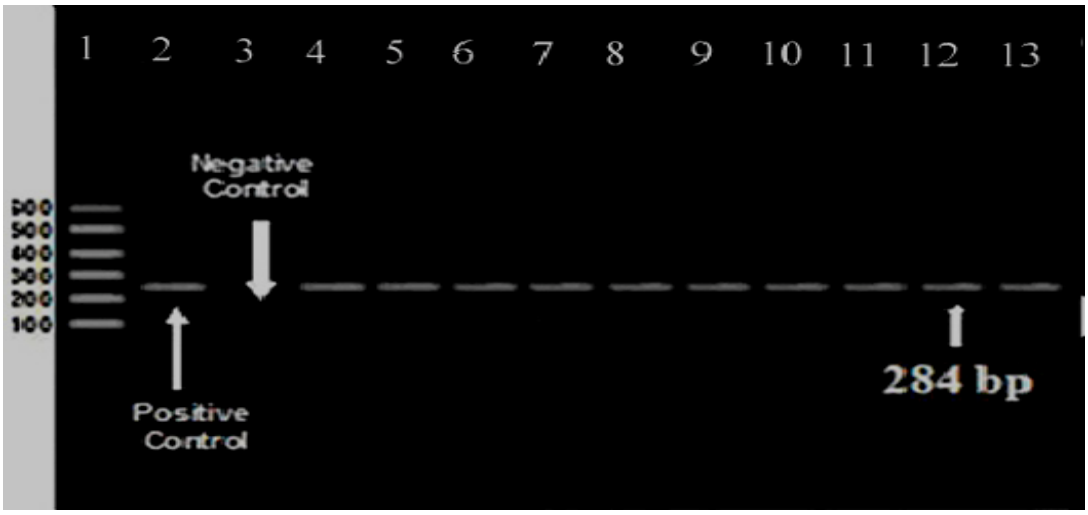

Photo No. (1) PCR result using primer of *inv*A gene in chicken samples.

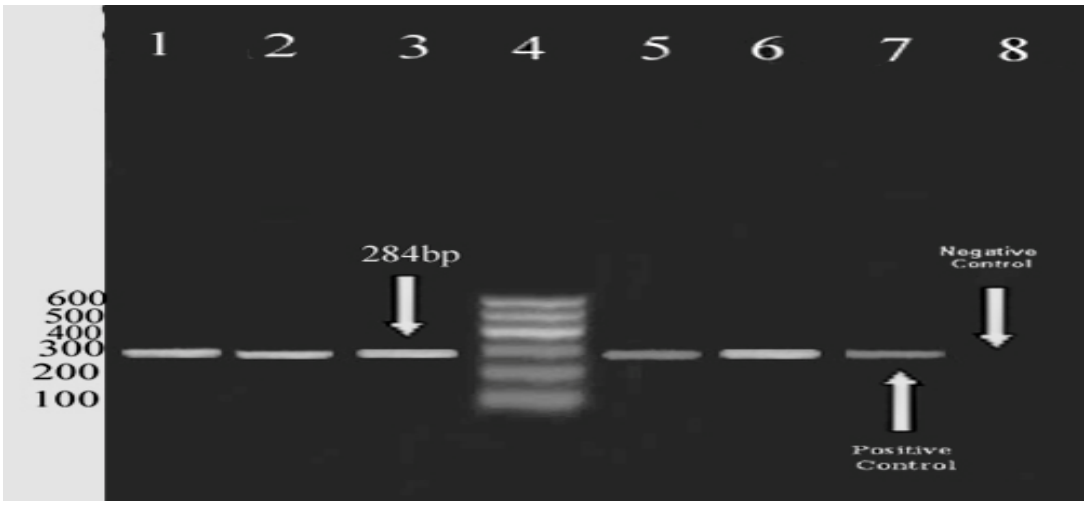

Photo No. (2) PCR result using primer of *inv*A gene in duck samples.

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, trimethoprime, 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 *inv*A 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 *inv*A gene now has been recognized as an international standard for detection of *Salmonella* genus [18]. The *inv*A 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

1. Ekperigin, H.E., Nagaraja, K.V. 1998. Microbial food borne pathogens. *Salmonella*.Vet Clin North Am Food Animal Practice. 14(1):17-29.
2. Rajagopal, R., Mini, M., Ramanathan, R. 2013. Outbreaks of salmonellosis in three different poultry farms of Kerala, India. Asian, Pac, J, 3(6):496-500
3. Santos, L.R., Nascimento, V.P., Oliveira, S.D., Flores, M.L., Pontes, A.P., Ribeiro, A.R., Salle, C.T.P. and Lopes, R.F.F. 2001. Polymerase chain reaction (PCR) for the detection of *Salmonella* in artificially inoculated chicken meat,

Review Inst. Medicine trop. S. Paulo, 43 (5):247-250.
4. Jamshidi, A., Bassami, M., Afshari-Nic, S. 2009. Identification of *salmonella* species and *Salmonella* Typhimurium by a multiplex PCRbased assay from poultry carcasses in Mashhad- Iran. Int. J. Vet. Res. 3:43– 48.
5. ISO 6579 2002. Microbiology of food and animal feeding stuffs- horizontal method for the detection of *salmonella* species. International standard. (4thedition).
6. Kauffmann, F. 1973. Serological diagnosis of *salmonella* species. Kaufmann White Scheme, Copenhagen, Denmark.
7. Finegold, S. M., Martin, E. T. 1982. Diagnostic microbiology. 6th Ed., The C.V. Mosby Company, St. Louis, Toronto, London.
8. Oliveira, S.D., Rodenbusch, C.R., Cé, M.C., Rocha, S.L.S., Canal, C.W. 2003. Evaluation of selective and non-selective enrichment PCR procedures for *Salmonella* detection. Lett .Appl . Microbiol. 36(4):217-221.
9. Hossain, M., Chowdhury, E., Islam, M., Haider, M., Hossain, M. 2006. Avian *Salmonella* Infection: Isolation and identification of organisms and histopathological study. Bang. J. Vet. Med. 4 (1): 07 – 12.
10. Roy, P., Dhillon, A.S., Lauerman, L.H., Schaberg, D.M., Bandli, D., Johnson, S. 2002. Results of *Salmonella* isolation from poultry products, environment, and other characteristics. Avian Dis., 13:793 – 803.
11. Abd-El-Rahman, M. A., Moussa, H. M. 2000. Bacteriological and histopathological studies on *Salmonella* isolates from ducks in North Sinai. Egyptian Journal of Agricultural Research. 78(1):15-24.
12. Abdellah, C., Fouzia, R., Abdelkader, C., Bencheikh, S., Mouloud, Z. 2009. Prevalence and anti-microbial susceptibility of *Salmonella* isolates from chicken carcasses and giblets in Meknès, Morocco, African. Journal of Microbiology Research. 3(5):215 – 219.
13. Asawy, A. M. E., El-Latif, M. M. A. 2010. Some bacteriological and serological studies on enteritis in ducks. Assiut Veterinary Medical Journal. 56:239-249.
14. Boris, H., Borka, S., Gordan, K., Fani, K. 2012. Antimicrobial resistance and serotyping of *Salmonella enterica* subsp. *enterica* isolated from poultry in Croatia. Vet. arhive 82: 371-381.
15. Yah, S.C., Eghafona, N.O. 2007. Plasmids: A vehicle for rapid transfer of antibiotic resistance markers of *salmonella* species in animals. J. Amer. Sci., 3(4):86-92.
16. Taddele**,** M.H., Rathore, R., Dhama**,** K. 2012**.** Antibiogram Assay of *Salmonella* Gallinarum and Other *Salmonella enterica* Serovars of Poultry Origin in India**.** Asian Journal of Animal and Veterinary Advances, 7:309-317.
17. Dione, M.M., Saha, D., Mohammed, N.I., Adegbola, R.A., Ieven, M., Antonio, M. 2011. Antimicrobial resistance and virulence genes of non-typhoidal *Salmonella* isolates in The Gambia and Senegal. J. Infect. Dev. Ctries, 5:765-775.
18. Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. 12:7046 – 52.
19. Darwin, K., Miller, V. 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. Clin Microbiol Rev. 12:405 – 428.

الكشف عن الجين المشترك فى السالمونيلا المعزوله من الدواجن بتقنيه تفاعل البلمرة المتسلسل

أشرف عواد عبد التواب [1]، احمد محمد عمار[2]، عائشه رجب على[3]، فاطمة ابراهيم الحوفى[1]، محمد ابراهيم ابراهيم سيد احمد[4]

[1]قسم البكتريولوجي والمناعة والفطريات- كلية الطب البيطري- جامعة بنها.[2] قسم البكتريولوجي والفطريات والمناعة - كلية الطب البيطري- جامعة الزقازيق.[3] وحدة السيرولوجى – معهد بحوث صحة الحيوان- الدقي – جيزة. [4] المعمل الفرعي بالمنصورة – معهد بحوث صحة الحيوان- الدقي – جيزة.

الملخص العربي

يتسبب ميكروب السالمونيلا فى خسارة اقتصادية هائله فى صناعة الدواجن ولذلك فقد تم فحص 400 عينه (300 عينه من الدجاج وبيض الدجاج و100 عينه من البط وبيض البط) من مصادر مختلفه فى محافظة الدقهلية. تم اخذ العينات من (الكبد ، الطحال ، القلب) بالاضافه الى الزرق. تم عزل 45عتره من السالمونيلا من 400 عينه للدواجن بنسبة 11.25٪ (30 عتره من الدجاج و3 عترات من بيض الدجاج و11 عتره من البط وعتره واحده من بيض البط المجمع من المنازل والأسواق) وبتصنيف هذه العترات وجد انها سالمونيلا أجونا- سالمونيلا وين جروف- سالمونيلا تيفيميوريم- سالمونيلا تانانريفى- سالمونيلا نيوبورت- سالمونيلا انتريتيدس - سالمونيلا سكانسن- سالمونيلا كنتاكى- سالمونيلا انجاندا- سالمونيلا لابادى و سالمونيلا غير مصنفه . 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.

(مجلة بنها للعلوم الطبية البيطرية: عدد 25(2):70-77, ديسمبر 2013)