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IDENTIFICATION OF AVIAN SALMONELLOSIS IN AN INFECTED LAYER CHICKENS IN KERBALA GOVERNORATE, IRAQ

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Abstract

Salmonella enterica is a major threat facing the industrial chicken production globally. This study was conducted to determine the prevalence of *Salmonella enterica pullorum/gallinarum* and differentiate them from other serovars of *Salmonella enterica* in the dead chickens from poultry farms in Iraq. A total of 60 tissue samples (ovary and gall bladder) were collected from dead birds with suspected salmonellosis. Results indicated that 43.3% of dead chickens were infected by *Salmonella pullorum/gallinarum*, while 19.2% of population were affected by non-*Salmonella pullorum/gallinarum* infections. The results of non-*S. Pullorum/Gallinarum* isolates were distributed showed as 30% distribution. The PCR-based technique was focused on flhB gene and showed a deficiency in this region just in *Salmonella pullorum/gallinarum*, compared to other serovars serotypes of *Salmonella enterica*. This study concluded that one stepbiovar -specific multiplex PCR can be used in high precision to identify and distinguish *Salmonella Gallinarum/Pullorum* from other *Salmonella enterica* serovars types.

Key words: flhB gene, PCR, *Salmonella enterica*, *Salmonella pullorum/gallinarum*, PCR, flhB gene.

Introduction

The Salmonella genus is divided into the two species, *Salmonella enterica* and *Salmonella bongori* (Reeves and Stevenson, 1989; JCICSP, 2005). *Salmonella enterica* is divided into six subspecies including enterica subsp. (subsp. I), salamae subsp. (subsp. II), arizonae subsp. (subsp. III), diarizonae subsp. (subsp. IV), houtenae subsp. (subsp. VI) and indica subsp. (subsp. VII) (Tindall *et al*., 2005).

Salmonella infections are a zoonotic disease and according to the epidemiological patterns, infected birds are considered as reservoir of Salmonella. These bacteria remain in the intestinal lumen of all vertebral animals, spreading out and multiplying easily to the environment caring infection for other species (Turnbull, 1979).

Fowl typhoid (FT) and Pullorum disease (PD), caused by Gram negative bacteria, are considered to be primary and septicemic disease of chickens with poor mortality and high morbidity. The Clinical findings in chicken include loss of appetite, diarrhea, dehydration, ruffled feathers, pale and shrunken combs. The main clinical signs in mature birds are ruffled feathers, soft shell eggs, decreased egg production and fertility but multiple different serotypes of Salmonella are found in laying farms without exhibiting clinical sings (Guo *et al*., 2000; McWhorter and Chousalkar, 2018). The infection that is transmitted by contamination of eggs or hatcheries usually leads to death during the first few days of life (Berchieri *et al*., 2001). The transition between farms is due to poor biosecurity. There are several macroscopic and microscopic characteristics found in FT and PD including hepatitis, splenitis, enteritis, myocarditis, ventriculitis, pneumonia, arthritis, peritonitis and ophthalmic disorders, in laying hens saver from salpingitis and verities with abdominal ovulation and peritonitis in laying hens. An infection across the ovary and oviduct leading to an egg's injury later chicks that is one of the most important modes of transmissions of salmonellosis. Polymerase Chain Reaction (PCR) is an important tool for salmonellae detection and it gives rapid and definitive diagnosis of Salmonella serotypes infections in poultry (Guo *et al*., 2000). Some studies used PCR assay and distinguish *S. gallinarum* from *S. pullorum* by one-step PCR without using biochemical identification and serotyping (Xu *et al*., 2018).

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In Iraq, Mohammed, (2015) has isolated Salmonella spp. from broiler flocks suffered from synovitis and the clinical signs were characterized by enteritis, laminas with arthritis and emaciation that lead to economic losses due to mortality, medication cost and carcass contamination.

This study aimed to use PCR assay using primers targeting flhB gene to identify and discriminate *Salmonella pullorum/gallinarum* from other Salmonella serovars isolated from ovarian follicle and gallbladder of dead chickens with history of bacillary white diarrhea, decreased egg production, soft-shelled eggs and mortality.

Materials and Methods

Ethical approval

Ethical approval was obtained from Veterinary Medical Ethics in college of Veterinary Medicine, Karbala University, Iraq.

Sample collection and preparation

A total of 120 fresh ovarian follicles and gall bladders were collected from a different chicken flock previously suffered from bacillary white diarrhea, laid-egg deformities, soft-shelled eggs and decreased appetites in different flocks in Iraq. The abdomen and chest cavity were incised by sterile scissor and then 100 mg of fresh ovarian follicle mass and 100 µl of gallbladder contents were placed in 1.5 ml of micro-centrifuge tube labeled and immediately transferred by Ice-box to the microbiology laboratory in the college of Veterinary Medicine University of Karbala. This study was conducted from August to December, 2019.

DNA extraction

100 One hundred mg size of the congested ovarian follicle was sampled and sliced with a scalpel in a mortar with sterile distal water for grinding and then centrifuged at 3000 rpm for 10 min. Next, 50µl of gall bladder content and 50 µl of ovarian homogenized tissue sample were transferred into 1.5 ml tube using a sterile micropipette and spatula, respectively and then 200µl Buffer CL, 20µl Proteinase K and 5µl RNase were added. The Solution was added into sample tube and mixed with vortex and all steps of the DNA extraction kit (G-spinTM Total DNA Extraction Kit, South Korea) protocol were followed according to the manufacturer instructions.

Primer and PCR optimization

Primers used in this study were (forward: 52 -TTC GCG ACG AAT TTA AAG AGA GCG AAG-32) and (reverse: 52 -CAG CGT TTA AGC TGC CAG ACC CAG GCC-32), which amplify a 182-bp fragment of flhB (gene involved in flagellar biosynthetic protein) of *S. pullorum/gallinarum* and a 379-bp fragment of flhB of non-*S. pullorum/gallinarum*. According to the (Xiong *et al*., 2016), these primers are appropriate for one-step PCR assay for distinguishing *S. pullorum/gallinarum* from non- *S. pullorum/gallinarum*. These two pairs of primers were selected and evaluated for nucleotide similarity in regard to the BLAST algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov). One step-PCR assay was conducted in a final volume of 25 µl; 1µl of 5 mM of each primer and 100-300 ng of DNA template, 10X Taq Reaction Buffer containing 0.125 µl of Taq DNA Polymerase, 0.5 µl volume of 10 mM dNTPs. The PCR optimization was recorded as 95°C for 5 min followed by 35 cycles consisted of 95°C for 40s, 65°C for 45s and 72°C for 55s and final extension 72°C for 7 min, PCR products were visualized by 1.5% agarose gel electrophoresis (Intron, Korea).

Statistical analysis

The results were analyzed statistically using IBM SPSS Statistics software (version 23) by Chi square test at significant level of $p < 0.05$.

Results and Discussion

In this study, the overall all molecular assay of *S. pullorum/gallinarum* infections was 43.3% in dead chickens (Table 1), however, (Okwori *et al*., 2013) found two culture isolates (1.3%) of *S. gallinarum* among 150 samples collected from two poultry farms and (Bouzoubaa *et al*., 1992), found eight isolates of *S. pullorum/gallinarum* among 200 sample isolates in two regions of Morocco. The author relied on his research on the use of sero agglutination and micro agglutination test, one of the most common reasons due to found detection of *Salmonella* serovar *pullorum/gallinarum* isolates were was clearly confirmed the confirmation of presence of it its infection in the village's flocks Synchronized with which have intensive management and poor hygiene, as well as the Shedding secretions of these bacteria are considered as intermittent bacterium (De Oliveira *et al*., 2004). Our results indicated that *Salmonella* serovar *pullorum/gallinarum* had more prevalence in the

Table 1: Prevalence of *Salmonella pullorum/gallinarum* and other *Salmonella* serovars in organs sampled from chickens.

Bacterial isolates	Ovary No. (%)	Gallbladder No. (%)	Total No. (%)
S. pullorum/gallinarum	34 (56.6%)	18 (30 %)	52 (43.3%)
Non- *S. pullorum/gallinarum*	6 (10%)	26 (43.4%)	32(19.2%)
No PCR product	20 (33.3%)	16 (26.6%)	36(30%)
Total	60	60	120

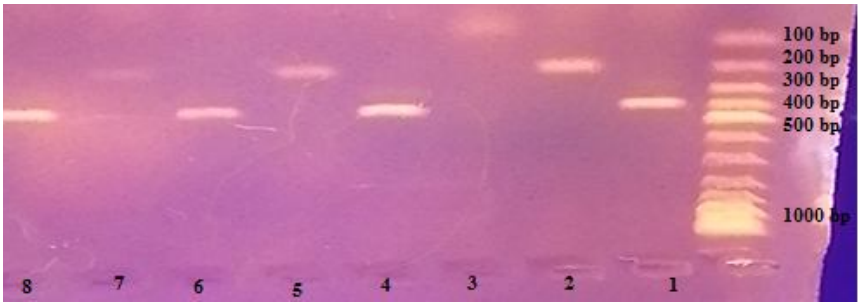

Fig. 1: PCR technique using genomic DNA from chicken ovary and gallbladder samples using specific primers targeting *flhB* gene. The PCR amplifies a product of 182 bp with *S. pullorum/gallinarum* in the 2, 5 and 7 lanes and non-*S. pullorum/gallinarum* in the 1, 4, 6 and 8 lanes which gave 379 bp.

ovarian cells (34/60, 56.6%) than gallbladder (18/60, 30%). The main characteristic of *Salmonella* serovar *pullorum* infection in the ovary of laying hens is the ability to persist asymptomatic for a long time in chickens (Wigley *et al*., 2001). The infection of *Salmonella* serovar *pullorum* take place during the sexual maturity phase by colonizing in both oviduct and ovary of immature hens leading to lay infected eggs (Oliveira *et al*., 2005). The *Salmonella* serovar *pullorum* have many mechanisms for eggs infection due to its pathogenicity and colonization in several different sites of the reproductive tract in different stage of maturity (Wigley *et al*., 2001). In addition, *S. pullorum/gallinarum* cause relatively high mortality among chickens and it is difficult to control this bacteria. These bacteria even in hatcheries and farms through biosecurity, cleaning and disinfection of facilities (Shivaprasad, 2000). On the other hand, the overall all molecular assay of non-*S. pullorum/gallinarum* infections in chickens was 19.2%. These results of non-*S. pullorum/gallinarum* isolates were distributed on two sides as 33.3% and 26.6% for in ovary and gallbladder, respectively. Allele specific PCR assay did not indicate the specific type of microbial strain among *Salmonella enterica,* as more than 2500 serotypes have been identified for it. Generally, most of studies indicate that the most frequent isolates are *Salmonella enterica* serotype *enteritidis* and *Salmonella enterica* serotype *typhimurium*. There are numerous possible explanatory theories for *S. enteritidis* and S. Typhimurium prevalence, including decreased immunity of birds against *S. pullorum*, *S. gallinarum* and *S. enteritidis* and after that spread through the flock and most birds in the farm (Bäumler *et al*., 2000). Birds can be infected by *S. entrica* through oral-fecal transmission (Foley and Lynne, 2008). At the same time, the newly hatched chicks also can be colonized through the nose or cloaca (Brito *et al*., 1995). Also, *Salmonella* spp. can be transmitted by vertical transmission via ovaries, oviducts, or infected eggs. These infections may become asymptomatic in adult birds and may cause a decrease in egg production with intra-abdominal ovulation and mortality (Kabir, 2010).

One step multiplex assay method is useful method to accurately identify and distinguish *Salmonella gallinarum/ pullorum* from other serovars of *S. enterica*. This PCR-based technique was focused on flhB gene and showed a lack of region just in *S. pullorum/ gallinarum* compared to other serovars (Xiong *et al*., 2016).

The primers have been developed for amplification (182bp) of flhB gene fragment for *S. pullorum/ gallinarum* and at the same time, (379bp) of flhB gene fragment for non-*S. pullorum/gallinarum* as shown in fig. 1.

One of the causes of avian salmonellosis in poultry farms is contaminated or dirty nest boxes which lead to affect the eggs. Vertical transmission of *Salmonella* spp. also occurred by colonization in oviduct and ovaries (Berchieri *et al*., 2001). At the same time, egg transmission may result from the ova contamination in following ovulation (Jordan *et al*., 1956). Egg transmission may result from contamination of of the ovum following ovulation, so the main method for vertical transmission through the localization of *S. pullorum* or *S. gallinarum* in the ovules before ovulation (Beach and Davis, 1927).

The ovarian lesions were characterized by soft ovary, discolored with cystic or nodular ova, regressed ovarian follicles, mild to severe congested ovary and in some cases, ovary becomes hemorrhagic. Finally, intra-abdominal ovulation may take place leading to peritonitis and decreased eggs production as shown in fig. 2 and 3. It

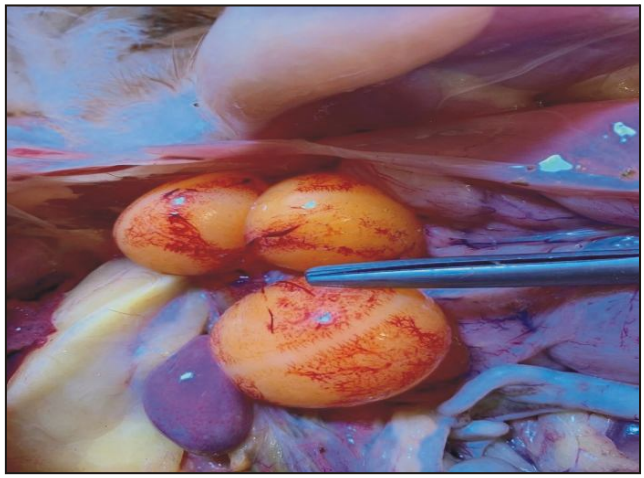

Fig. 2: *Salmonella pullorum/gallinarum* infections characterized by congested ovary, regressed ovarian follicles, and splenomegaly).

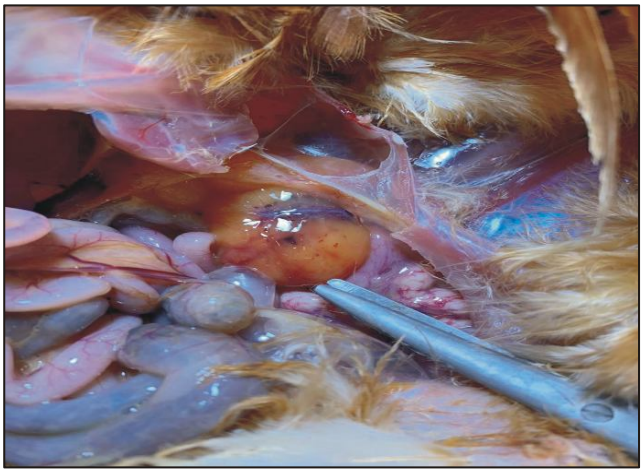

Fig. 3: *Salmonella pullorum/gallinarum* infections with ovarian lesion characterized by soft and hemorrhagic ovary with cystic or nodular ova and regressed ovarian follicles.

also may lead to distended oviduct and containing serous exudate. Consequently, infected hens lay soft-shell and dirty eggs covered by diarrhea. The spleen of infected birds was characterized by enlargement (splenomegaly) and discoloration. (Erbeck *et al.*, 1993; Shivaprasad, 2000).

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References

Bäumler, A.J., B.M. Hargis and R.M. Tsolis (2000). Tracing the origins of *Salmonella* outbreaks. *Science*, **287(5450):** 50-52. DOI: https://doi.org/10.1126/science.287.5450.50.

Beach, J.R. and D.E. Davis (1927). Acute infection in chicks and chronic infection of the ovaries of hens caused by the fowl typhoid organisms. *Hilgardia*, **2:** 411-424. DOI:10.3733/hilg.v02n12p411.

Berchieri, Jr, A., C.K. Murphy, K. Marston and P.A. Barrow (2001). Observations on the persistence and vertical transmission of *Salmonella enterica* serovars *pullorum* and *gallinarum* in chickens: effect of bacterial and host genetic background. *Avian Pathology*, **30(3):** 221-231. DOI: 10.1080/03079450120054631.

Bouzoubaa, K., K. Lemainguer and J.G. Bell (1992).Village chickens as a reservoir of *Salmonella pullorum* and *Salmonella gallinarum* in Morocco. *Preventive Veterinary Medicine*, **12(1-2):** 95-100.þDOI: org/10.1016/0167-5877(92)90072-N.

Brito, J.R.F., Y. Xu, M. Hinton and G.R. Pearson (1995). Pathological findings in the intestinal tract and liver of chicks after exposure to Salmonella serotypes typhimurium or Kedougou. *British Veterinary Journal*, **151(3):** 311-323. DOI: org/10.1016/S0007.

De Oliveira, G.H., Â. Berchieri Júnior, H.J. Montassier and A.C. Fernandes (2004). Assessment of serological response of chickens to *Salmonella gallinarum* and *Salmonella pullorum* by Elisa. *Brazilian Journal of Poultry Science*, **6(2):** 111-115. DOI: 10.1590/S1516-635X2004000200007.

Erbeck, D.H., B.G. McLaughlin and S.N. Singh (1993). Pullorum disease with unusual signs in two backyard chicken flocks. *Avian Dis.*, **37:** 895-897. Available at: https://www.jstor.org/stable/1592049?seq=1#metadata_info_tab_contents.

Foley, S.L. and A.M. Lynne (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *Journal of animal science*, **86(suppl_14):** E173-E187. DOI:.org/10.2527/jas.2007-0447.

Guo, X., J. Chen, L.R. Beuchat and R.E. Brackett (2000). PCR detection of *Salmonella enterica* serotype *montevideo* in and on raw tomatoes using primers derived from hila. *Applied and Environmental Microbiology*, **66(12):** 5248-5252. DOI: 10.1128/AEM.66.12.5248-5252.2000.

Judicial Commission of the International Committee on Systematics of Prokaryotes (JCICSP) (2005). The type species of the genus Salmonella lignieres 1900 is *Salmonella enterica* (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987, with the type strain LT2T and conservation of the epithetenterica in *Salmonella enterica* over all earlier epithets that may be applied to this species. Opinion 80. *Int. J. Syst. Evol. Microbiol.*, **55:** 519-520. DOI 10.1099/ijs.0.63585-0.

Jordan, F.T.W. (1956). The transmission of *Salmonella gallinarum* through the egg. *Poultry Science*, **35(5):** 1019-1025. DOI.org/10.3382/ps.0351019.

Kabir, S.M. (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *International journal of environmental research and public health*, **7(1):** 89-114. DOI:.org/10.3390/ijerph7010089.

McWhorter, A.R. and K.K. Chousalkar (2018).A long-term efficacy trial of a live, attenuated *Salmonella typhimurium* vaccine in layer hens. *Frontiers in microbiology*, **9:** 1380. DOI: org/10.3389/fmicb.2018.01380.

Mohammed, A.H. (2015). Synovitis associated with Salmonellae infections of broiler chickens in Kerbala province. *Journal of Kerbala University*, **13(3):** 183-187. https://www.iasj.net/iasj?func=fulltext&aId=106039.

Okwori, A.E.J., R.J. Ogbe, S.C. Chollom, G.O.A. Agada, A. Ujah, E. Okwori, O.N. Adeyanju and G.O.N. Echeonwu (2013). Isolation of *Salmonella gallinarum* from Poultry Droppings in Jos Metropolis, Plateau State, Nigeria. *IOSR J. Agric. Vet. Sci.*, **5(2):** 14-44.þ http://www.iosrjournals.org/iosr-javs/papers/vol5-issue2/G0524144.pdf?id=8060.

Oliveira, G.H.D., A. Berchieri Junior and A.C. Fernandes (2005). Experimental infection of laying hens with *Salmonella*

enterica serovar *gallinarum*. *Brazilian Journal of Microbiology*, **36(1):** 51-56. DOI:.org/10.1590/S1517-83822005000100011.

Reeves, P. and G. Stevenson (1989). Cloning and nucleotide sequence of the *Salmonella typhimurium* LT2 gnd gene and its homology with the corresponding sequence of Escherichia coli K12. *Mol. Gen. Genet.*, **217:** 182-184. https://link.springer.com/article/10.1007/BF00330960.

Shivaprasad, H.L. (2000). Fowl typhoid and pullorum disease. *Revue Scientifiqueet Technique Office International des Epizooties*, **19(2):** 405-416. https://pdfs.semanticscholar.org/f33d/81b98ebd62ff49e11b34e28ecdd5e735fde3.pdf.

Tindall, B.J., P.A.D. Grimont, G.M. Garrity and J.P. Euzeby (2005). Nomenclature and taxonomy of the genus Salmonella. *International J. of systematic & evolutionary microbiology*, **55(1):** 521-524. DOI: org/10.1099/ijs.0.63580-0.

Turnbull, P.C.B. (1979). Food poisoning with special reference to Salmonella- its epidemiology, pathogenesis and control. *Clin. Gastroenterol.*, **8:** 663-713. https://ci.nii.ac.jp/naid/10024215416.

Wigley, P., A. Berchieri, K.L. Page, A.L. Smith and P.A. Barrow (2001). *Salmonella enterica* serovar *pullorum* persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. *Infection and immunity*, **69(12):** 7873-7879. DOI: 10.1128/IAI.69.12.7873-7879.2001.

Xiong, D., L. Song, S. Geng, J. Tao, S. An, Z. Pan and X. Jiao (2016). One-step PCR detection of *Salmonella pullorum*/*gallinarum* using a novel target: the flagellar biosynthesis gene flhB. *Frontiers in microbiology*, **7:** 1863.DOI: org/10.3389/fmicb.2016.01863.

Xu, L., Z. Liu, Y. Li, C. Yin, Y. Hu, X. Xie and X. Jiao (2018). A rapid method to identify *Salmonella enterica* serovar *gallinarum* biovar *pullorum* using a specific target gene ipaJ. *Avian pathology*, **47(3):** 238-244. DOI: org/10.1080/03079457.2017.1412084.