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Original Article

Isolation and Molecular Identification of *Salmonella pullorum* from Broiler Chicken in Iraqi Fields

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Abstract

Pullorum disease (PD) is one of the most common diseases in the world, with devastating consequences. In the chicken sector, there have been financial losses. It is brought on by *Salmonella enteric subspecies serovar Gallinarum biovar pullorum*; definitive detection requires culture followed by biochemistry analysis and serotyping. This study aimed to verify the presence of bacteria by culture, biochemical characterization, PCR assay, and sequencing. One hundred samples were collected from 12 broiler chicken flocks of different ages for 8districts of Baghdad province, including cloacal swabs (65), visceral organs (15), and dropping (20). Salmonella colonies were identified by selective culture broth and agar with biochemical description for 75% of the total samples, with a higher incidence in visceral organs than dropping and cloacal swabs. ‹The Sequencing and phylogenetic tree analysis of 16S rRNA gene for representative Salmonella isolates. The presence of Salmonella *pullorum* isolates in global genetic strains; was revealed a matching NCBI isolates similarity of 99.02% with (MF445124.1) and 98% with (MH352164.1), respectively. In the current state of molecular and genetic research, phlyogentic research announced the real presence of *Salmonella pullorum* in Baghdadprovince's broiler chicken, also showing the phylogentic characteristics and links to some global isolates. The detection of *Salmonella pullorum* in broiler flocks of the current study extent of health risks to other uninfected birds present in the free range.

Keywords: *Salmonella pullorum*, Biochemical Tests, PCR, Phylogenetic Analysis

1. Introduction

Pullorum disease (PD) isconsidered one of the most dangerous bacterial infections and causes substantial financial losses in the poultryindustry, affects food security in Iraq and is caused by Salmonella enterica serovar Gallinarum biovars Pullorum (1). This bacteria is rod Gram-negative and prefer to be present in the liver, spleen, and reproductive tract (2). Common symptoms of PD include gasping, lack of appetite, lethargy, ruffled feathers, loud chirping, lameness, and diarrhea s in young chicks, and effects on the reproductive systeminclude a significant decline in egg output, fertility, and hatchability in adult birds (3). *Salmonella pullorum* is isolated using both selective and non-selective enrichment methods (4). Biochemical testing, on the other hand, carbohydrate fermentation tests, and PCR to determine the genomic DNA of bacterialserotyping were used to determine infection (5). *Salmonellasp.* colonies show distinct characteristics subject tobiochemical examinations, whichexplain the metabolic process of *Salmonella pullorum* so that they can be used as confirmatory tests for bacterial isolation (4). *Salmonella pullorum* is not like the other*Salmonellasp*. in the fermentation of glucose, ornithine, and lycine and gives negative results with urea, lactose, citrate, indol, and sorbitol (2). Various forms of culture media are employed, such as triple

sugar iodine, Salmonella Shigella agar, McConkey agar, Mortality indole ornithine, Simmons citrate, Methyl Red-Voges Proskauer, and Eosin Methylene Blue (2, 4). PCR tests have been proven as diagnostic confirmatory Salmonella detection tools in broiler and layer chickens in terms of Workload reductions andreducing the time it takes to diagnose (6). In this study, samples (dropping, cloacal swabs, and visceral organs) taken from broiler chickens were examined for the isolation of *Salmonella pullorum* bacteria by culture, biochemical identified, and confirmed by PCR assay for 16S rRNA gene.

2. Materials and Methods

2.1. Sample Collecting

(100) samples were collected randomly from the broiler chicken fields of Baghdad (from 12 broiler chicken flocks of different ages for 8 districts province. Each sample includedncludingcloacal swabs (n=65), visceral organs (n=15), and dropping (n=20) collected in aseptic condition and placed in a container made of sterilized plastic in a refrigerated box until delivered to the laboratory.

2.2. Identification and Isolation of *Salmonella pullorum*

The samples were cultured in a non-selective Peptone water brothfor 24 hours at 37 °C and 10 ml Tetrathionate broth for 24 hours at 41.4 °C (2). The cells were then cultivated on a specific broth (Himedia's Selenite F broth) and incubated at 37 °C for 24 hours. A loopful of each broth was culturedstreaked across the surface of MacConkey agar plates (Oxoid), Xylose Lysine Desoxycholate agar (Himedia), Salmonella and Shigella agar (Himedia), Brilliant green agar (LAB), and chromogenic agar (Conda) for further incubation at 37 °C for 24 hours (2). Gram's stainingwas used to determine the staining features of the isolated bacteria and the purity of the culture (according to the manufacturer of the Gram's stain kit). Biochemical assays on Triple Sugar Iodide (TSI) agar, Motility Indole Ornithine agar, Simmons Citrate agar, MacConkey agar, Salmonella Shigella agar, and Eosin Methylene Blue agarwere used to indicate the species of isolates (7).

2.3. Molecular identification

Extraction of genomic DNA was done bystool DNA Kit was used to extract DNA from the samples (Bioneer, Korea). The kit was used following the directions of the company (8). The DNA was measured and certified with a NanoDrop before being stored at -20ºC until the subsequent tests were run. The 16S rRNA gene was employed as a critical objective of *Salmonella pullorum* and was detectedusing particular primers GenBank: FR686362.1 (Table 1). The PCR conditions were utilized according to table 2. The results of the PCR test were conducted on a 1% agarose gel with ethidium bromide dye and electrophoresed before being viewed with a UV.

Table 1. The *Salmonella pullorum* primer sequence was utilized to detect the bacteria

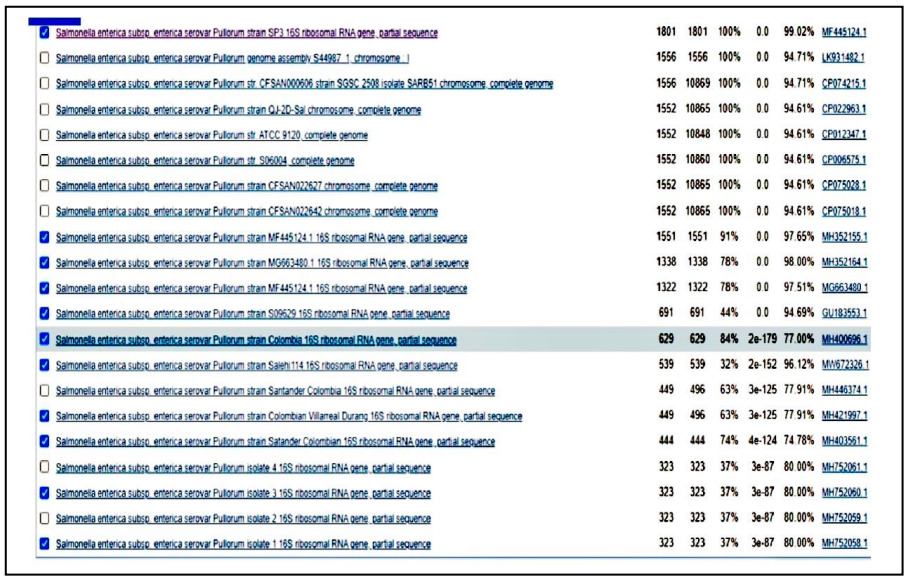
Gene	Primer Sequences (5˚-3˚)		Product Size (bp)	Reference
16SrRNA	F	AGAGTTTGAT CCTGGCTCAG	1500 bp	Ojha, Yean Yean (9)
	R	GGTTACCTTGT TACGACTT		

Table 2. Program of the thermal condition of PCR for amplification of *16SrRNA* gene

Genes	Step	Temperature	Time	Cycle
16SrRNA	Initial denaturation	95.0 C^0	5 min	1
	Denaturation	95.0 C^0	30 sec	35
	Annealing	55.0 C^0	1 min	
	Extension	72.0 C^0	1 min	
	Final Extension	72.0 C^0	10 min	1

2.4. Sequencing

The process of purifying PCR products from the PCR gel was carried out using the EZ EZ-10 Kit (Biobasic, Canada). Two samples of PCR products were sent to Macrogen Co. in Korea to sequence the 16S rRNA gene (AB DNA-based sequencing system). The Neighbor Distance Phylogenetic tree analysis was utilized to analyze the sequencing findings and create the phylogenetic tree utilizing NCBI-based websites and Mega v6 (Mega version 6) (Figure 1).



Salmonella enterica subsp. enterica serovar Pullorum strain SP3 16S ribosomal RNA gene, partial sequence	1801	1801	100%	0.0	99.02%	MF445124.1
Salmonella enterica subsp. enterica serovar Pullorum genome assembly S44987_1, chromosome : I	1556	1556	100%	0.0	94.71%	LK931482.1
Salmonella enterica subsp. enterica serovar Pullorum str. CFSAN000606 strain SGSC 2598 isolate SARB51 chromosome, complete genome	1556	10869	100%	0.0	94.71%	CP074215.1
Salmonella enterica subsp. enterica serovar Pullorum strain QJ-2D-Sal chromosome, complete genome	1552	10865	100%	0.0	94.61%	CP022963.1
Salmonella enterica subsp. enterica serovar Pullorum str. ATCC 9120, complete genome	1552	10848	100%	0.0	94.61%	CP012347.1
Salmonella enterica subsp. enterica serovar Pullorum str. S06004, complete genome	1552	10860	100%	0.0	94.61%	CP006575.1
Salmonella enterica subsp. enterica serovar Pullorum strain CFSAN022627 chromosome, complete genome	1552	10865	100%	0.0	94.61%	CP075028.1
Salmonella enterica subsp. enterica serovar Pullorum strain CFSAN022642 chromosome, complete genome	1552	10865	100%	0.0	94.61%	CP075018.1
Salmonella enterica subsp. enterica serovar Pullorum strain MF445124.1 16S ribosomal RNA gene, partial sequence	1551	1551	91%	0.0	97.65%	MH352155.1
Salmonella enterica subsp. enterica serovar Pullorum strain MG663480.1 16S ribosomal RNA gene, partial sequence	1338	1338	78%	0.0	98.00%	MH352164.1
Salmonella enterica subsp. enterica serovar Pullorum strain MF445124.1 16S ribosomal RNA gene, partial sequence	1322	1322	78%	0.0	97.51%	MG663480.1
Salmonella enterica subsp. enterica serovar Pullorum strain S09629 16S ribosomal RNA gene, partial sequence	691	691	44%	0.0	94.69%	GU183553.1
Salmonella enterica subsp. enterica serovar Pullorum strain Colombia 16S ribosomal RNA gene, partial sequence	629	629	84%	2e-179	77.00%	MH400696.1
Salmonella enterica subsp. enterica serovar Pullorum strain Saleh114 16S ribosomal RNA gene, partial sequence	539	539	32%	2e-152	96.12%	MW672326.1
Salmonella enterica subsp. enterica serovar Pullorum strain Santander Colombia 16S ribosomal RNA gene, partial sequence	449	496	63%	3e-125	77.91%	MH446374.1
Salmonella enterica subsp. enterica serovar Pullorum strain Colombian Villarreal Durang 16S ribosomal RNA gene, partial sequence	449	496	63%	3e-125	77.91%	MH421997.1
Salmonella enterica subsp. enterica serovar Pullorum strain Satander Colombian 16S ribosomal RNA gene, partial sequence	444	444	74%	4e-124	74.78%	MH403561.1
Salmonella enterica subsp. enterica serovar Pullorum isolate 4 16S ribosomal RNA gene, partial sequence	323	323	37%	3e-87	80.00%	MH752061.1
Salmonella enterica subsp. enterica serovar Pullorum isolate 3 16S ribosomal RNA gene, partial sequence	323	323	37%	3e-87	80.00%	MH752060.1
Salmonella enterica subsp. enterica serovar Pullorum isolate 2 16S ribosomal RNA gene, partial sequence	323	323	37%	3e-87	80.00%	MH752059.1
Salmonella enterica subsp. enterica serovar Pullorum isolate 1 16S ribosomal RNA gene, partial sequence	323	323	37%	3e-87	80.00%	MH752058.1

Figure 1. The sequence analysis of the PCR products of *S. pullorum* targeted the 16S rRNA gene in NCBI

3. Results

In the current investigation, 75 of the 100 samples tested positive for *Salmonella pullorum*. At the beginning of the isolation stage, the culture medium of bacteria on peptone water was cloudy and had an unpleasant smell. Due to Salmonella's non-lactose fermentation capabilities, all isolates generated On MacConkey agar media, colorless, smooth, pale, and clear raised colonies; on the XLD and BGA plates, there were tiny black and pink colonies. The recovered bacteria had a tiny rod shape, were Gram-negative, and were solitary or paired in Gram's staining. Indole, ureas, and oxidase were all negative in all Salmonella; however, catalase, lysine iron agar, and simmone's citrate were all positive. Alkaline slant (red), acid butt (yellow), and H2S and gas production all indicated positive results on triple sugar iron agar (TSI) (Figure 2).

Figure 3 shows that *S. pullorum* was found in samples using the PCR technique. The size of these products was 1500bp of the 16S rRNA gene. Sequencing of parts The existence of this bacteria in the positive samples were confirmed by sequencing the PCR-positive samples. The confirmed isolates (OM988162.1) exhibited 99.02 percent similarity to the NCBI isolate (MF445124.1) and 98 percent (MH352164.1), respectively, an isolate from Colombia when the phylogeny was examined. Furthermore, the current research isolates. Figure 4 depicts these findings in the form of a phylogenetic tree.

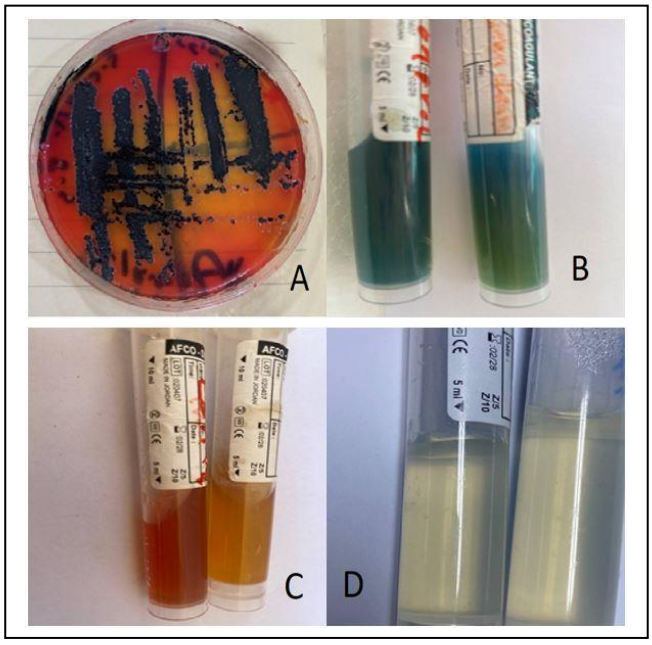


Figure 2. A: Salmonella -Shigella agar. **B:** Simmons citrate agar. **C:** Triple sugar iron agar. **D:** Motility test

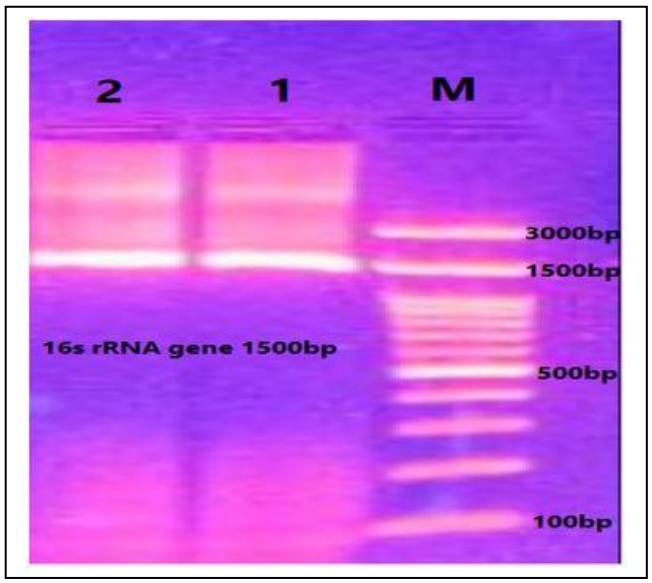


Figure 3. *S. Pullorum* PCR results targeting the 16S rRNA gene on an agarose gel. Ladder (100bp) M, the positive samples 1 and 2

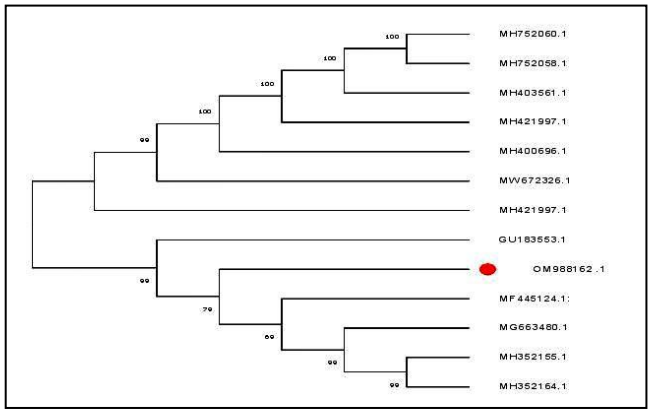


Figure 4. Phylogenetic tree study of *S. pullorum* PCR results targeting the 16S rRNA gene

4. Discussion

Salmonella pullorum is one of the important bacterial pathogens that leads to high mortality in young birds, and in adult birds, it is associated with reduced egg laying, weight loss, and economic losses. The prevalence of *S. pullorum* infection in poultry samples was 75 percent, which is nearly identical to the number reported by AL-Iedani, Khudor (10) in Basrah region, where the prevalence of Salmonella was 80 percent and 92 percent, respectively. A survey of the most common digestive disorders in laying hens and parent stock was conducted in Mosul, and Salmonellosis accounted for 70% of the situations (11). This discrepancy could be attributed to variances in environmental and management factors. Its regional spread because the survey did not focus solely on Salmonella infection but also covered the most common digestive illnesses. The culturing and biochemical results are consistent with the findings of Ayesha, Mahmood (12) and Rumi, Rahman (13). On SS agar media, red and white colonies with black centers were seen in all Salmonella suspicious isolates. The same result was shown by Ashraf, Ahmed (14) and Abd El-Ghany, El-Shafii (15) - the Salmonella spp. Colonies Detailed diagnosis, on the other hand, may necessitate using sophisticated instruments such as PCR methods. The PCR approach was employed in this case to determine the presence of this bacterium in the chickens' feces. These findings are consistent with Xiong, Song (16) that devised a PCR approach to detect the presence of these bacteria in addition to the traditional laboratory methods. Furthermore, Saud and AL-Zuhariy (17), employed a quick and low-cost PCR method with the O and H antigen alleles as targets and reported success rather than relying on serological assays.

Furthermore, Majchrzak, Krzyzanowska (18), described a PCR approach that may be used to distinguish between Salmonella serovars. Sequencing and phylogeny were used to detect successful differentiation rates amongst Salmonella isolates (19, 20); this is consistent with the recent findings, which demonstrated that one sample had 99 percent similarity to worldwide isolates. Current molecular and phylogenetic research analysis showed that *S. pullorum* is present in broiler chickens in the analyzed area, as well as evolutional traits and linkages to several global isolates. As a result, preventative programs to prevent salmonella infections must be considered in the case of broiler chickens.

Authors' Contribution

Study concept and design: R. G. H.
Acquisition of data: M. T. B.

Analysis and interpretation of data: M. T. B.
Drafting of the manuscript: M. T. B.
Critical revision of the manuscript for important intellectual content: R. G. H.
Statistical analysis: R. G. H.
Administrative, technical, and material support: R. G. H.

Ethics

We hereby declare all ethical standards have been accepted by the ethics committee of the University of Baghdad, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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References

1. Brooks BW, Lutze-Wallace CL, Blais B, Gauthier M, Deschenes M. Monoclonal antibodies to lipopolysaccharide O antigens of enterohemorrhagic Escherichia coli strains in serogroups O26, O45, O103, O111, O121, and O145. J Food Prot. 2015;78(7):1252-8.
2. Revolledo L. Vaccines and vaccination against fowl typhoid and pullorum disease: An overview and approaches in developing countries. J Appl Poult Res. 2018;27(3):279-91.
3. Sharif YM, Tayeb BA. Estimation of limit of detection of Salmonella typhimurium in artificially contaminated chicken meat by cultured-based and polymerase chain reaction techniques. Iraqi J Vet Sci. 2021;35(4):621-5.
4. Sabri JB, Al-Sultan II, Altaif K, Peter S, Saadh MJ. Pathogenesis of Salmonella enterica serovar Albany in experimental infected SPF BALB/c Mice. Iraqi J Vet Sci. 2020;34(2):339-44.
5. Xu Y, Hu Y, Guo Y, Zhou Z, Xiong D, Meng C, et al. A new PCR assay based on the new gene-SPUL_2693 for rapid detection of Salmonella enterica subsp. enterica serovar Gallinarum biovars Gallinarum and Pullorum. Poult Sci. 2018;97(11):4000-7.
6. Siala M, Barbana A, Smaoui S, Hachicha S, Marouane C, Kammoun S, et al. Screening and detecting Salmonella in different food matrices in Southern Tunisia using a combined enrichment/real-time PCR method: Correlation with conventional culture method. Front Microbiol. 2017;8:2416.
7. Markey B, Leonard F, Archambault M, Cullinane A, Maguire D. Clinical veterinary microbiology e-book: Elsevier Health Sciences; 2013.
8. Moore E, Arnscheidt A, Krüger A, Strömpl C, Mau M. Simplified protocols for the preparation of genomic DNA from bacterial cultures. MMEM. 1999;1(1):1-15.
9. Ojha SC, Yean Yean C, Ismail A, Banga Singh K-K. A pentaplex PCR assay for the detection and differentiation of Shigella species. BioMed Res Int. 2013;2013.
10. AL-Iedani AA, Khudor MH, Oufi NM. Isolation and identification of Salmonella spp. from poultry farms by using different techniques and evaluation of their antimicrobial susceptibilities. Bas J Vet Res. 2014;1(1):246-59.
11. Al-Niema A, Youkhana S. Survey on the most important diseases of the digestive system in laying hens and parent stock in Mosul. Iraqi J Vet Sci. 2012;26:241-5.
12. Ayesha K, Mahmood M, Iftikhar H, Faisal S, Azhar R, Asif I, et al. Bacteriological and epidemiological investigations of Pullorum disease in selected poultry farms of faisalabad, Pakistan. Glob Vet. 2014;12(4):455-60.
13. Rumi N, Rahman M, Akter M, Fakhruzzaman M, Hossain M. Evaluation of the effectiveness of commercially available disinfectants against salmonellae isolated from internal organs of dead chickens. Bangladesh J Vet Med. 2011;9(1):43-52.
14. Ashraf A, Ahmed MA, Aisha RA, Fatma I, Mohammed E. Detection of common (inv A) gene in salmonellae isolated from poultry using polymerase chain rection technique. Benha Vet Med J. 2013;25:70-7.
15. Abd El-Ghany WA, El-Shafii SS, Hatem M. A survey on Salmonella species isolated from chicken flocks in Egypt. Asian J Anim Vet Adv. 2012;7(6):489-501.
16. Xiong D, Song L, Geng S, Tao J, An S, Pan Z, et al. One-step PCR detection of Salmonella

Pullorum/Gallinarum using a novel target: the flagellar biosynthesis gene flhB. Front Microbiol. 2016;7:1863.

17. Saud BH, AL-Zuhariy MT. Using T cell lymphokines to enhance the immune response against Newcastle disease in vaccinated broiler chickens fed naturally contaminated diet with different mycotoxins. Iraqi J Vet Sci. 2020;34(2):427-33.

18. Majchrzak M, Krzyzanowska A, Kubiak AB, Wojtasik A, Wolkowicz T, Szych J, et al. TRS-based PCR as a potential tool for inter-serovar discrimination of Salmonella Enteritidis, S. Typhimurium, S. Infantis, S. Virchow, S. Hadar, S. Newport and S. Anatum. Mol Biol Rep. 2014;41(11):7121-32.

19. Zou Q-H, Li R-Q, Liu G-R, Liu S-L. Genotyping of Salmonella with lineage-specific genes: correlation with serotyping. Int J Infect Dis. 2016;49:134-40.

20. Feng Y, Johnston RN, Liu G-R, Liu S-L. Genomic comparison between Salmonella Gallinarum and Pullorum: differential pseudogene formation under common host restriction. PLoS One. 2013;8(3):59427.