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Molecular detection and phylogenetic tree analysis of *Salmonella Pullorum* isolated from broiler chicken in Al-Diwanyia province, Iraq

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Abstract

The current molecular-based analytical study was initiated to detect the infection occurrence of *Salmonella Pullorum* in broiler chicken plus to understand some of the evolutional features of the study isolates and compare them with global isolates. Here, 25 cloaca fecal samples were collected from 25 broiler chickens of a breeding hall in Al-Diwaniyah City, Iraq. The samples were subjected, first, to polymerase chain reaction (PCR)-based exploring for the presence of this bacterium. Then, some of the positive samples from the PCR were sent out for partial sequencing. Both PCR and sequencing employed *16S rRNA* gene as a target. The sequencing results were studied for evolution using phylogenetic analyses that were ended up with building a phylogenetic tree to compare our isolates with some global isolates of this bacterium. The PCR method showed the occurrence of *S. Pullorum* in 8 (32%) out 25 samples. The sequencing, for four-PCR-positive samples, confirmed the presence of this bacterium in the positive samples. When studied the phylogeny, the confirmed isolates (No.1 (MH752058), No.3 (MH752060), and No.4 (MH752061)) showed 99% similarity to the NCBI isolate, MH421997.1, an isolate from Colombia. Moreover, the current study isolate No.2, MH752059, revealed 99% matching to the NCBI isolate, MH446374.1, another isolate from Colombia. The current molecular and phylogenetic study reveals the actual present of *S. Pullorum* in the broiler chickens of the studied city plus shows evolutional characteristics and links to some global isolates.

Keywords: *16S rRNA* gene, broilers, PCR, *Salmonella Pullorum*, sequencing.

INTRODUCTION

The *Salmonella enterica subsp. enterica serovar Pullorum*, a gram negative bacterium, induces the pullorum disease in chickens (1). The disease is characterized by the presence of certain clinical features such as anorexia, diarrhea, and high mortality in chicks and declines in egg production and reproduction-based features such as fertility and hatchability and high mortalities in adult birds. For deeper look at the disease, some characteristics generated via macroscopic and microscopic-based detections are manifested by the presence of splenitis, hepatitis, myocarditis, omphalitis, pneumonia, peritonitis, and synovitis in chicks. However, signs of oophoritis, orchitis, salpingitis, and perihepatitis are noticed in adult birds (2,3). The disease is transported to other birds vertically via transovarian infection to the eggs and subsequently chicks. Selective and non-selective media are used to isolate the bacterium and could further be differentiated via the use of ornithine-rapid decarboxylation which the bacterium is positive for this test (2,4). Serological tests such as tube agglutination and stained-antigen for whole blood are used to identify the presence of infections by *S. Pullorum* (2,5–7). As rule of thumb, Eradication of the disease could be performed using serological tests and getting rid of the infected birds. Prevention and treatment using vaccines and antibiotics respectively could be used in general. The disease is still a big problem in the development-impaired countries; however, the disease is completely removed from the developed countries in the world such as the United States of America (2,8–10). The current molecular-based analytical study was initiated to detect the infection occurrence of *S. Pullorum* in broiler chicken plus to understand some of the evolutional features of the study isolates and compare them with global isolates.

MATERIALS AND METHODS

Sampling

In the current work, 25 cloaca fecal samples were aseptically collected from 25 broiler chickens of a breeding hall in Al-Diwaniyah City, Iraq.

Extraction of genomic DNA

The DNA of the samples was extracted employing Stool DNA Kit (Bioneer, Korea). The kit was used according to the accompanied instruction of the company. The resulted DNA was quantified and qualified using a NanoDrop and was stored in -20^{0}C until the next tests were performed.

PCR method

The technique used in this study utilized the *16S rRNA* gene as a main target to detect *Salmonella Pullorum* using specific primers, GenBank: FR686362.1, that were designed using the NCBI-based website and Primer 3 Plus online and purchased from (Bioneer, Korea). These primers are F: GTTGGCTTGAGACCGGAAGA and R: GCCATGAGGACTTGACGTCA that amplify a 562bp region of the gene. The reaction mixes were done using AccuPower kit (Bioneer, Korea). The instructions with the kit were followed to complete the process. The PCR conditioned used in the thermocycler (Mygene Bioneer, Korea) were 95 ^{0}C lasted for 5min to begin with the first denaturation, 30 cycles of (95°C lasted for 30s to do the principle denaturation, 58°C lasted for 30s to perform the annealing, 72°C for 1min to do the extension), and 1 cycle at 72°C lasted 5min to perform the final extension. The products of the PCR test were run on a agarose gel at 1% contained the dye of ethidium bromide and electrophoresed to be later visualized under a UV imager.

Sequencing

For purifying PCR products from the PCR gel, EZ EZ-10 Kit (Biobasic, Canada) was used to perform this process. Four of these products were mailed to Macrogen Co. in Korea to complete the process of sequencing of *18S rRNA* primer (AB DNA-based sequencing system). NCBI-based websites and Mega v6 were used to process the results of the sequencing and generate the phylogenetic tree using the Neighbor Distance Phylogenetic tree analysis (Mega version 6).

RESULTS

The PCR

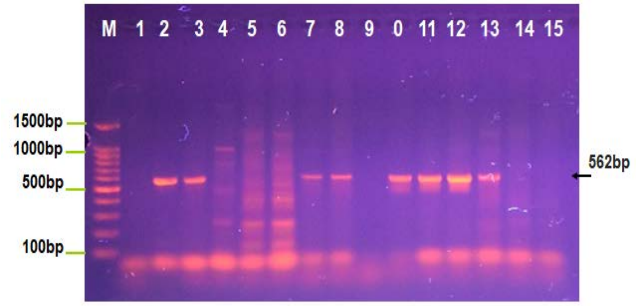

Figure 1: The agarose gel image of the PCR products of *S. Pullorum* targeted 16S rRNA gene. M indicates the ladder at 1500 to 100bp. At 562bp product size, the positive samples are 2 and 3, 7 and 8, and 10-13.

The PCR method showed the occurrence of *S. Pullorum* in 8 (32%) out 25 samples, figure 1. These products were at the size of 562bp of the 16S rRNA gene.

The Partial sequencing

The sequencing, for four-PCR-positive samples, confirmed the presence of this bacterium in the positive samples. When studied the phylogeny, the confirmed isolates (No.1 (MH752058), No.3 (MH752060), and No.4 (MH752061)) showed 99% similarity to the NCBI isolate, MH421997.1, an isolate from Colombia. Moreover, the current study isolate No.2, MH752059, revealed 99% matching to the NCBI isolate, MH446374.1, another isolate from Colombia. These results are shown in the phylogenetic tree, figure 2.

DISCUSSION

The *Salmonella enterica subsp. enterica serovar Pullorum*, a gram negative bacterium, induces the pullorum disease in chickens. The disease is characterized by the presence of certain clinical features such as anorexia, diarrhea, and high mortality in chicks and declines in egg production and reproduction-based features such as fertility and hatchability and high mortalities in adult birds (2). For diagnosis, selective and non-selective media and serological tests such as tube agglutination and stained-antigen for whole blood are used to identify the presence of infections by *S. Pullorum* (2,5,6). However, definite diagnosis might need developed tools such as PCR methods. Here, the PCR method used provided information about the presence of this bacterium in the fecal samples of the chickens. These results agree with (11) who developed a PCR method to detect the presence of these bacteria in addition to the classical tools used in laboratories.

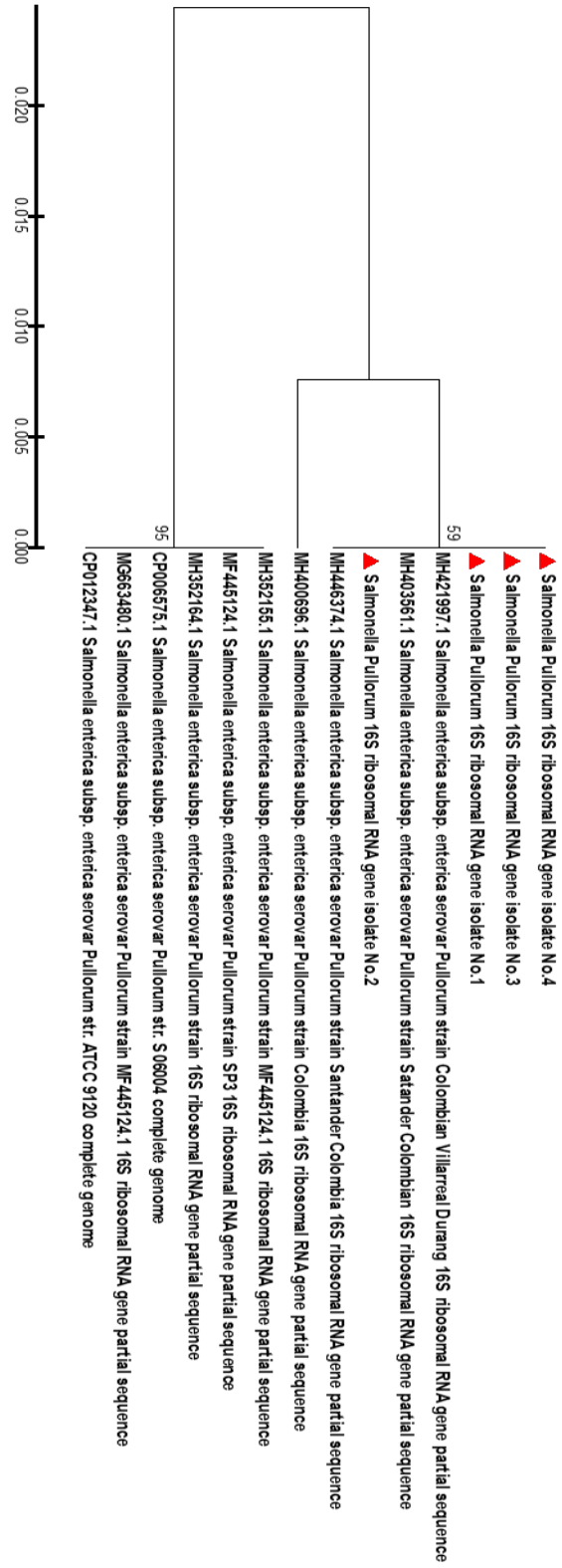

Figure 2: Phylogenetic tree using partial sequencing of 16S rRNA gene of *S. Pullorum* isolates. The confirmed isolates (No.1 (MH752058), No.3 (MH752060), and No.4 (MH752061)) showed 99% similarity to the NCBI isolate, MH421997.1, an isolate from Colombia. Moreover, the current study isolate No.2, MH752059, revealed 99% matching to the NCBI isolate, MH446374.1, another isolate from Colombia.

Moreover, (12) who used a rapid and cost-effective PCR method that employed the O and H antigen alleles as targets and indicated successful use of this method rather depending on the use of serological tests. Furthermore, (13) reported a PCR method that could be utilized in differentiation between serovars of *Salmonella* strains. For sequencing and phylogeny, (14,15) found successful rates to differentiate between *Salmonella* isolates were detected, and this agrees with the current results that revealed 4 isolates that showed similarities at 99% with global isolates. This indicates that our isolates and the NCBI isolates might have descended from the same *Salmonella* lineage. The current molecular and phylogenetic study reveals the actual present of *S. Pullorum* in the broiler chickens of the studied city plus shows evolutional characteristics and links to some global isolates.

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