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Isolation and molecular detection of *Pasteurella multocida* and *Gallibacterium anatis* in Poultry from Five Provinces in Iraq

Shaymaa Jameel Owaid A. H. Ulaiwi

[1*2] College of Veterinary Medicine, University of Baghdad, Iraq

E- mail: amjed.h@covm.uobaghdad.edu.iq

Abstract

The current study aimed at isolation and identification with molecular detection of *Pasteurella multocida* and *Gallibacterium anatis* from five provinces in Iraq. Samples were collected from 68-layer farms in different provinces (Baghdad, Diyala, Babylon, Salahuddin, and Wasit). Out of 12 swabs of suspected chicken cases, bacteriological diagnosis detected 5 isolates as *Pasteurella multocida* by using blood agar and biochemical tests, and out of 21 samples, only 6 isolates were *Gallibacterium* anatis using the same blood agar and biochemical tests. Additionally, the positive samples detected by RT- PCR technique was used to confirm the isolates. To conclude, *Pasteurella multocida* and *Gallibacterium* anatis were isolated bacteria from respiratory infections in layer poultry farms in Iraq.

Key words: fowl cholera, PCR, poultry respiratory disease.

I. INTRODUCTION

Pasteurella multocida is a Gram-negative, non-motile, and facultative anaerobic bacterium that belongs to the family *Pasteurellaceae*, in poultry, *Pasteurella multocida* is primarily associated with fowl cholera, a highly contagious and economically devastating disease affecting chickens, turkeys, ducks, and other avian species(1,2).Transmission of *Pasteurella multocida* in poultry typically occurs through direct contact between infected and susceptible birds, or indirectly via contaminated feed, water, equipment, or the environment(3,4). The bacterium can enter the host through the respiratory tract, oral cavity, or skin wounds and subsequently colonizes and multiplies in various tissues, leading to the development of fowl cholera (5, 6, and 7).

Gallibacterium anatis, an opportunistic microbe, is frequently found in the respiratory and genital tracts of domestic animals raised in close proximity, including chickens (8, 9, and 10) .This Gram-negative coccobacillus of family *Pasteurellaceae*, is known to be a cause of mortality in chickens and other domestic animals, and in rare instances, it can also infect humans. In healthy chickens, *G. anatis* is a common inhabitant of the upper respiratory and genitourinary tracts, and it has been shown to cause spontaneous infections in experimental chicken models (11, 12, and 13). In naturally affected poultry *G. anatis* affects the urogenital, gastrointestinal, and respiratory systems in chickens, this bacterium has been involved in septicemia, inflammation of ovaries, follicle degeneration, and enteric, peritoneal, and respiratory tract infections (14, 15, and 16). The aim of this study was to investigate the *P. multocida* and *G. anatis* in layer poultry flocks that suffered from respiratory signs and drop in egg production with PCR accurate diagnostic technique.

II. MATERIALS AND METHODS

Collection of Samples

The liver samples were collected from (68) poultry farms that were previously infected. The infected birds were from five provinces: Baghdad, Diyala, Babylon, Salahuddin, and Wasit. The collection spanned eight months, from November 2022 to July 2023. It is noteworthy that the suspected layers exhibited

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respiratory signs, which were documented along with case history and post-mortem lesions. It is also mentioned that the flock of birds had not received a vaccine against Cholera disease. Each liver sample, approximately 20-30 mg from each organ, was treated with 1ml of normal saline (0.9%) for cleaning (17), The aseptically collected samples were then transferred to Uruk laboratory in Baghdad using a cool box. The liver tissue was obtained by using a sterile spatula to sterilize the surface and a sterile scarble to cut the liver and take inside tissue. The liver tissue was directly inoculated into nutrient broth for enrichment (18).

Isolation and Identification of *Pasteurella multocida*: -

For the isolation of *P. multocida*, liver samples that incubated in broth then cultured on blood agar (prepared according manufacture) and incubated in an aerobic condition at 37c° for 24-28 hrs after that study morphology of growth culture, gram stain and biochemical test (catalase, oxidase, Indole, VP, MR, grow in MacConkey agar) (19).

Biochemical test

Biochemical tests were performed from five diagnosed samples of *P. multocida*, catalase, oxidase, indol synthesis, methyl red, and voges proskaur were used for biochemical identification (20).

Diagnosis of *Gallibacterium anatis*: -

Liver and trachea samples were collected from (68) poultry farms from five provinces (Baghdad, Diyala, Babylon, Salahuddin and Wasit) throughout eight months.G.anatis were detected from the clinical signs and post mortem lesions which similar to that in fowl cholera disease and confirmed the infection by PCR test. All samples were collected from the suspected birds as 20-30 mg of each organ was taken,1ml of normal saline (0.9%) were added to each sample for cleaning (21,22), and the samples were transferred aseptically in cool box to laboratory in nutrient broth to enrichment (8).

Isolation and identification

Liver and trachea samples from nutrient broth were inoculated to 5% sheep blood (Oxoid, USA) and MacConkey agar (NEOGEN/England). The plates were incubated aerobically at 37°c for 18-24 hours, isolation and identification of *Gallibacterium anatis* was done based on colony morphology which were smooth, shiny and greyish, staining properties were performed to identify the Gram negative rods (23,24), Then pure culture inoculated in nutrient broth.

Molecular detection of *Pasteurella multocida:* -

DNA Extraction

Bacterial DNA was extracted by using Kylt RNA/DNA Purification kit, according to the manufacturer instructions.

PCR assay

Real-Time PCR assay (Smart Cycler (R.T-PCR from Cepheid company/USA) was used for the detection of *P. multocida* based on the amplification of the 16S rRNA gene using specific primers as described in Table 1.

Table (1): Primers used for the detection of *P. multocida*

Forward primer	PMA2f, 5'ATAACTGTGGGAAACTGCAGCTAA-3'
Reverse primer	PMA2r, 5'-GGTCCCACCCTTT(A/C)CTCCTC-3'
MGB probe	PMA2, 5'-6FAM-CCGCGTA(A/T) TCTCT-MGBNFQ-3'.

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*MGB: Minor Groove Binding

Real time –PCR program

PCR typically consists of three successive steps firstly by denaturation, annealing, and finally extention of repeated cycles for getting PCR product, amd the same program was used for *P.multocida* and *G.anatis* as shown in Table 2.

Table (2): Real time –PCR program

Step No.	Description	Temperature	Time	Channel detection
1	Activation of polymerase	95 c°	10 min	
2	Denaturation	95 c°	15 sec	
3	Annealing	60 c°	45 sec	42 cycles → FAM,HEX and cCy5
4	Extension	72 c°	45 sec	

Molecular detection of *Gallibacterium anatis*

DNA Extraction

Bacterial DNA was extracted by using Kylt RNA/DNA Purification kit, according to the manufacturer instructions.

PCR assay

Real- Time PCR procedure (Smart Cycler (R.T-PCR from Cepheid company/USA) was used for the detection of *G. anatis* DNA and this done by using specific primers as mention in Table 3. The gene "gyrB" was considered as biomarker for diagnostic tests (25). This gene was amplified by using the following specific primers:

Table (3): Primers used for the detection of *G. anatis*

Forward	5'-CGATTGTGTCCGTTAAAGTGC-3'
Reverse	5'-TGCAAACGCTCACCAACTG-3'
TaqMan probes	FAM-CCACTACACTTTTCACTTCGG AAGAAACCAG-BHQ

*BHQ: Black Hool Quinture.

III. RESULTS AND DISSCUTION

Isolating and identifying

The Gram-negative coccobacilli observed in all blood agar cultures displayed small, smooth-edged, convex, and glossy features. The diameters of the samples ranged from 0.5 to 2.5 mm, and no signs of hemolysis were observed. Additional biochemical analysis confirmed the bacteria's ability to produce indole through the breakdown of tryptophan, a trait that is also found in *P. multocida*. Based on various biochemical, morphological, and growth characteristics, it can be concluded *that P. multocida* is the bacterial species in issue. Figure 1.

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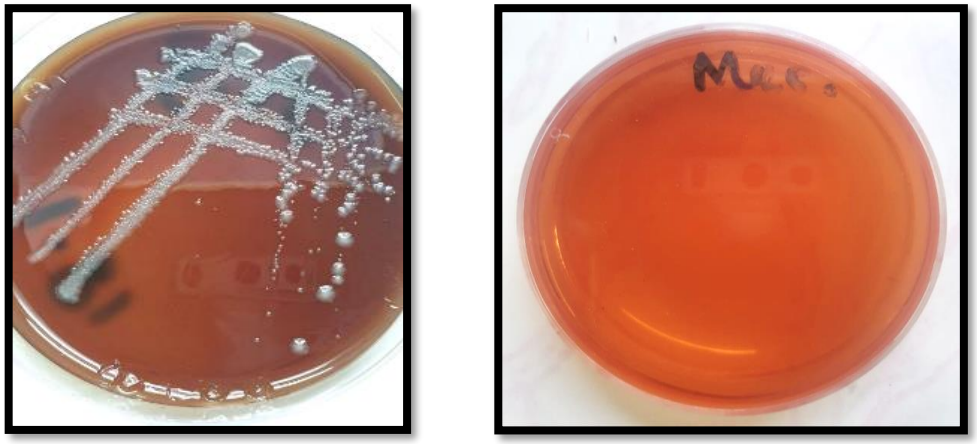
Figure (1): Isolation of *Pasteurella multocida* on blood agar with no growth on MacConkey agar

The procedure for isolating and identifying *Pasteurella multocida* involved cultivating bacterial samples on blood agar, where the colonies exhibited specific characteristics. The colonies had a shiny surface, smooth texture, and well-defined, unbroken edge, with a convex shape indicating a consistent, dome-like structure. These results were agreement with results obtained by (30) who found that, isolating and identifying *P. multocida* involved cultivating bacterial samples on blood agar and there were no occurrences of hemolysis, a common feature in highly virulent bacterial strains. Also those results were agreement with another study



(31,32, and 33).

Molecular detection.

The molecular analysis revealed positive identification for *P. multocida* in 5 out of 12 samples and *G. anatis* in 6 out of 21 samples. Detailed molecular findings pertaining to the isolated respiratory bacteria are elucidated in Table 4. Furthermore, Table 5 delineates the outcomes specific to *P. multocida*, while Table 6 elucidates the results concerning *G. anatis*.

Table (4): Molecular detection of the isolates:

Bacteria	**+ve sample**	**- ve sample**	**Total sample**
P.multocida	5	7	12
G.anatis	6	15	21

As a result, in Table 5, using real-time PCR, twelve sample were tested for *P. multocida*. Conducted a manual real-time PCR with a FAM value of 9.6, and it was found that, out of the 12 sample, five tested positives for *P. multocida*.

Table (5): Result of *P. multocida*

Number of the Well	ID of the tube	Ct, Fam
A2	Sample_2	32.0
A4	Sample_4	32.6

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A6	Sample_6	32.9
A7	Sample_7	32.5
A12	Sample_12	32.6

These results were agreement with results obtained by (34) who found that, two *P. multocida* strains have been successfully isolated from poultry tissue samples, and their presence has been definitively confirmed using PCR. Another study of, (35) investigated the tests for identifying and typing *P. multocida*, it was found two polymerase chain reaction (PCR) tests have shown promising results for *P. multocida*. Althogh These results were consistent with the researches conducted by (36-40)

Table (6): Result of *G. anatis*

Number of the Well	ID of the tube	Ct, Fam
A1	Sample_1	26.8
A3	Sample_3	21.1
A5	Sample_5	26.6
A9	Sample_9	27.2
A10	Sample_10	27.0
A12	Sample_12	27.4

Molecular detection of *Gallibacterium anatis* by Real-time PCR was detected and the results found that, 6 samples tested positive, with Cycle threshold (Ct) values ranging from 21.1 to 27.4. The detection rate of *G. anatis* in these samples was 27.3% as shown in Table 6. These results were significant as they provide insights into the prevalence of *G. anatis* in the sampled population, contributing to understanding of the distribution and potential effect of this pathogenic bacterium in poultry. According to results of (41) who investigated that, the prevalence of *G. anatis* broiler flocks using qPCR, finding a similar positive rate (27.3%), it explored the association between *G. anatis* detection and flock health parameters, which our might find relevant for interpreting the results in this study.

As well as a study of (42) who described the development and validation of a sensitive and specific qPCR assay for *G. anatis* detection. While us haven't mentioned the specific assay used, comparing to positive rate with data from similar studies using validated assays can strengthen the findings in this study. Furthermore, a study of (43) who investigated the compared qPCR with traditional culture methods for *G. anatis* detection, it was Emphasized the benefits of qPCR in sensitivity and speed, which complements the importance of our Real-time PCR analysis.

IV. CONCLUSION

Respiratory infections exert a substantial impact on poultry performance. Our investigation successfully isolated and identified *Pasteurella multocida* and *Gallibacterium anatis* within the laying population. The findings presented herein offer vital insights into the prevalence and diversity of bacterial pathogens influencing global poultry health, encompassing our region. Also molecular detection with different ways of several Bacterial *Spp.* was more accurate.

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