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Isolation and molecular characterisation of *Avibacterium paragallinarum* from Japanese quail in Kerala[#]

S. K. Amritha[1*], R. Ambily[2], P. M. Priya[3], K. M. Binu[4], Arun George[5] and S. Surya[2]
Department of Veterinary Microbiology
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University
Kerala, India

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Abstract

Infectious coryza (IC) is an upper respiratory tract disease of poultry, caused by Avibacterium paragallinarum (A. paragallinarum). The disease is of much economic importance due to increased culling rate and marked reduction in egg production. There are no published reports of IC in quail in Kerala. The present study involves isolation and identification of A. paragallinarum from quails with respiratory signs, employing conventional and molecular methods. Swabs from infra-orbital sinus and trachea were collected under sterile precautions from 16 quails brought for disease investigation to Departments of Veterinary Microbiology and Pathology, College of Veterinary and Animal Sciences, Mannuthy, during August 2022 to March 2023. Avibacterium paragallinarum was isolated from one sample in chocolate agar, Haemophilus test medium and blood agar as tiny dewdrop colonies. The isolate was further characterised by biochemical tests and confirmed by PCR targeting HPG-2 gene. Three samples were found positive for both HPG-2 gene and hagA gene. The representative amplicons were sequenced, analysed and compared with sequences in GenBank. Phylogenetic analysis based on hagA gene revealed that the sample clustered together with isolates from different parts of the world.

Keywords: *Avibacterium paragallinarum*, *quail, HPG-2 gene, hagA gene*

Infectious coryza (IC), caused by *Avibacterium paragallinarum* is considered as a

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1. *MVSc Scholar*
2. *Assistant Professor*
3. *Professor and Head*
4. *Associate Professor*
5. *Assistant Professor, Department of Veterinary Clinical Medicine, Ethics and Jurisprudence*

**Corresponding author: amrithaskumar95@gmail.com, Ph. 7306791396*

significant pathogen affecting poultry industry worldwide. While extensively studied in chicken, the impact and dynamics of IC in quail have received comparatively limited attention. Quail, belonging to family *Phasianidae*, plays a pivotal role in both avian ecology and agricultural economies, serving as a vital source of protein. The economic impact of IC is mainly due to increased culling rate, reduced feed intake leading to reduction in body weight and mortality (Blackall *et al*., 2005). The understanding of the pathogenesis, epidemiology and immunology of IC in quail remains in its infancy. This knowledge gap not only hampers effective disease control strategy but also underscore the need for extensive research efforts dedicated to this avian species.

The diagnosis can be made based on a history of rapid disease transmission, clinical signs and pathological alterations brought about by IC. Definitive diagnosis can be made by isolating and identifying the causative organism directly from nasal and infraorbital sinus swabs (Wahyuni *et al*., 2018). Since *A. paragallinarum* infections often occur as coinfections with other bacteria and viruses, morbidity of disease is prolonged and mortality is high and the organism usually gets masked and overgrown by other members of bacterial family, *Pasteurellaceae* (Mutter *et al*., 1985). Highly sensitive and specific molecular detection techniques like HPG-2 gene specific polymerase chain reaction (PCR), enterobacterial repetitive intergenic consensus-PCR and nucleotide sequencing (Anjaneya *et al*., 2014) are useful in diagnosing IC. Furthermore, the molecular characterisation of *A. paragallinarum* using *hag*A gene (encodes haemagglutinin) PCR is critical in elucidating the pathogenesis and evolution of this pathogen. No incidence of infectious coryza in quails has been reported from Kerala. The present study involves diagnosis of IC in quail based on isolation and molecular detection by PCR. It also aims at molecular characterisation of *A. paragallinarum* from quails based on *hag*A gene specific PCR.

Materials and methods

Sample collection

Samples were collected from 16 dead quails brought to Department of Veterinary Microbiology and Veterinary Pathology, CVAS, Mannuthy for disease investigation with a history of respiratory distress and sinusitis. Sixteen tracheal and infraorbital sinus swabs were collected aseptically during post mortem examination along with other tissue samples including trachea, lungs, heart, liver and spleen.

Isolation and identification of Avibacterium paragallinarum

Primary isolation was performed on chocolate (CHOC) agar at 40°C supplemented with seven per cent CO_2 and incubated for 48 h. The suspected colonies were further sub-cultured in blood agar (BA), Haemophilus test medium (HMT), brain heart infusion agar (BHIA), enriched brain heart infusion broth (EBHIB), MacConkey agar (MCA) and eosin methylene blue (EMB) agar. The enriched brain heart infusion broth (EBHIB) was prepared by supplementing one per cent foetal bovine serum (FBS) and 0.0025 per cent nicotinamide adenine dinucleotide (NAD) in brain heart infusion broth (BHIB). All the isolates obtained were stained by Gram's staining and were further subjected to biochemical tests like catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate, nitrate as well as carbohydrate fermentation test using 13 sugar discs, as per standard procedure. The inoculum for the biochemical tests was prepared from pure colonies on CHOC agar. All the procedures were followed as described by Barrow and Feltham (1993).

Polymerase chain reaction for diagnosis and characterisation

Genomic DNA was extracted directly from all the 16 samples for the detection of *A. paragallinarum* using the commercial kit (HiPurA multi-sample DNA extraction kit, HiMedia Laboratories Pvt. Ltd., India) and stored in elution buffer at -20°C, until use. The DNA extracted from the isolate was also tested. For this, colonies were transferred to 1.5 mL of EBHIB and incubated at 40°C for 24 h. (Patil *et al*., 2017). Extraction of DNA was carried out as per manufacturer's instructions. The commercial vaccine (IC inactivated oil adjuvant vaccine, Indovax Pvt. Ltd., Haryana) was used

as the source of positive control. The oil portion was removed using isopropanol as per the method followed by Antony *et al*. (2006) prior to DNA extraction using the kit method.

Initial screening of the samples were carried out using oligonucleotide primers targeting the HPG-2 gene of *A. paragallinarum* genome (Primer N1F: 5 ' T G A G G G T A G T C T T G C A C G C G A A T 3' and Primer F1R: 5' CAAGGTATCGATCGTCTCTCTACT 3') corresponding to an amplicon of approximately 500 bp (Chen *et al.,* 1996). The amplification was carried under the following conditions in a thermal cycler (MJ Mini Bio-Rad thermal cycler, USA): Initial denaturation for 4 min. at 95°C followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 63°C for 1 min. and extension at 72°C for 1 min. with a final extension at 72°C for 10 min.

Amplification of *hag*A gene was done using primer pairs HA1F (TGTAGCTCAAGCAGCTCCACAAG) and HA2R (TCAAGCGATAAGTGCTTTACGACC) to obtain amplicons of approximately 900 bp (Anjaneya *et al.,* 2014) amplicon size. The amplification was carried under the following conditions in a thermal cycler (MJ Mini Bio-Rad thermal cycler, USA): Initial denaturation for 5 min. at 94°C followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 47 for 1 min. and extension at 72°C for 1.5 min. with a final extension at 72°C for 10 min. The amplicon from the isolate of *A. paragallinarum* was sequenced by Sanger's method for confirmation.

Analysis of the nucleotide sequences of *hag*A gene were carried out employing "MEGA 11" programme (Tamura *et al*., 2021) of Lasergene software. The evolutionary history for the constructed phylogenetic tree was inferred by using Neighbour-joining method and the evolutionary distance was determined by Maximum composite likelihood method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). The phylogenetic tree was constructed from the nucleotide sequences retrieved from GenBank. Sixteen sequences of *hag*A gene taken from NCBI database were used to construct the phylogenetic tree. The accession numbers for *hag*A genomic nucleotide sequences obtained from GenBank for analysis were OP376826.1, MK883778.1, KT371936.1, AF491818.1, MN080795.1, KJ621080.1, MK883780.1, MN654930.1, MK883777.1, KJ621070.1, KJ621081.1, AF491820.1, AF491824.1, CP091455.1, MK883779, CP086713.1. The nucleotide sequences and the corresponding amino acid sequences of haemagglutinin protein *hag*A, was analysed by molecular biology and bioinformatics tools such as ExPASy translate, Self-optimised prediction method (SOPMA), Expasy SWISS MODEL, Expasy ProtParam tool, NCBI conserved domain search and Immune Epitope Database (IEDB) and Analysis Resource tool.

Results and discussion

Samples were collected from 16 quails with clinical history of sinusitis, serous oculo-nasal discharge, conjunctivitis and sticky eyelids. On post-mortem examination, no lesions could be detected in the lower respiratory tract. Disease was mostly observed in adult quails of 10-14 weeks of age. The quails are susceptible to this disease in all ages (Thenmozi and Malmarungan, 2013).

Isolation and identification

From the 16 samples collected, *A. paragallinarum* could be isolated from one sample. Small dewdrop colonies were observed on CHOC agar (Fig. 1), seven per cent BA and HTM. On Gram's staining, the isolate was found to be Gram negative pleomorphic short rod. Similar findings were described by Akter *et al*. (2014), Wahyuni *et al*. (2018) and Balouria *et al*. (2019).

Biochemical characterisation was performed on the isolate with phenotypic traits indicative of *A. paragallinarum*. The isolates were found to be negative for catalase and positive for oxidase; Citrate and nitrate reduction reactions were positive. Indole, methyl red and Voges-Proskauer tests were negative and failed to produce acid from galactose and trehalose. Inability to ferment galactose, lactose and trehalose has been reported by Kaur *et al*.

Fig. 1. Small dewdrop colonies on Chocolate agar

(2004) and Rajurkar *et al*. (2009).

It is challenging and time-consuming to isolate and identify *A. paragallinarum*. Additionally, the growth of *A. paragallinarum* will be concealed by other bacteria in co-infections, and their satellitic growth may go unnoticed (Sandoval *et al*., 1994). Reports on emergence of NAD-independent *A. paragallinarum*, not exhibiting satellitic growth, often complicates the conventional method of identification (Wahyuni *et al*., 2018). *A. paragallinarum* have strict nutritional demands when grown *in-vitro*, NAD, oleic albumin complex, chicken serum and thiamine must be used to obtain pure cultures which increase the cost of isolation (Badouei *et al*., 2014). The isolates failed to grow on BHIA, MCA and EMB agar indicating that these media were not supporting the growth due to lack of V factor and the isolate was found to be NAD dependent.

Molecular diagnosis and characterisation

Molecular detection of *A. paragallinarum* has been utilised widely for the diagnosis of IC because of the availability of HPG-2 gene PCR (species-specific) devised by Chen *et al*. (1996), which amplifies 500 bp product. In the current investigation, *A. paragallinarum* was detected from the clinical samples such as tracheal and infraorbital sinus swabs using PCR targeting HPG-2 gene. Additionally, DNA extracted from the colonies of *A. paragallinarum* was also confirmed by PCR. Among the 16 clinical samples subjected to HPG-2 PCR, three were found to be positive (Fig. 2), indicating an incidence rate of 19 per cent. Out of the three HPG-2 PCR positive samples, *A. paragallinarum* could be isolated from one sample in pure culture. *Escherichia coli* and *Pseudomonas* spp. were isolated from the other two samples, suggesting that these fast-growing organisms might have masked the growth of *A. paragallinarum*. Amplification of 500 bp product corresponding to the gene HPG-2 has been reported in many studies (Chen *et al*., 1996; Kaur *et al*., 2004; Anjaneya *et al*., 2014; Muhammad and Sreedevi, 2015 and Patil *et al*., 2016). Thus, PCR is having much relevance as a diagnostic tool for the rapid detection of highly fastidious organisms like *A. paragallinarum*, to diagnose the disease within hours from the clinical samples. Cultural isolation and identification took three to five days for confirmatory diagnosis (Dwivedi *et al*., 2018).

The presence of 900 bp core region of '*hag*A' (haemagglutinin) gene in *A. paragallinarum* isolate was detected using PCR assay. Using HA1 and HA2 primers at an annealing temperature of 47°C, *A. paragallinarum* isolate amplified 900 bp fragment, indicating the presence of virulence determinant, haemagglutinin outer membrane protein, which acts as an adhesion molecule (Fig. 3). These results were in accordance with the result of Anjaneya *et al*. (2014). The *hag*A gene was detected in all the three HPG-2 PCR positive samples. Hobb *et al*. (2002) also hypothesised that every isolate of *A. paragallinarum* had the adhesin-like haemagglutinin OMP, which is a virulence determinant.

The amplicons obtained after PCR targeting *hag*A genes were sequenced by Sanger's dideoxy chain termination method and the sequences revealed 96 to 100 per cent homology with *A. paragallinarum* from India and abroad available in GenBank.

The phylogenic tree is depicted in Figure 4. The results of phylogenetic analysis on the *hag*A gene showed that the isolate of *A. paragallinarum* strain obtained in the present study, designated as Q8, clustered with strains from Korea, India and China and forms paraphyletic group with strains from India and China.

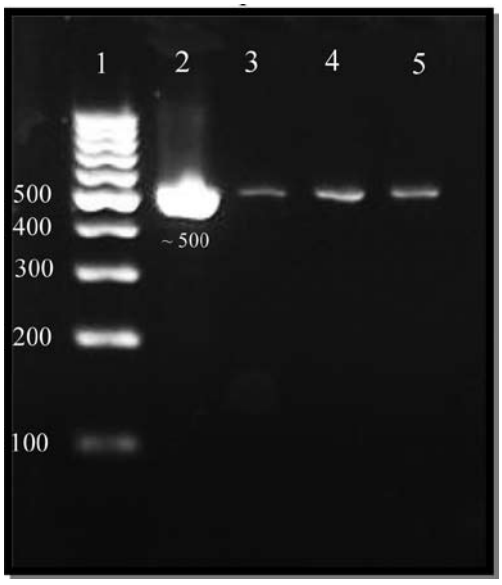

Fig. 2 Agarose gel electrophoresis of PCR amplified products of HPG-2 gene (500 bp)

Lane 1: 100 bp ladder
Lane 2: Positive control
Lane 3-5: IC positive samples

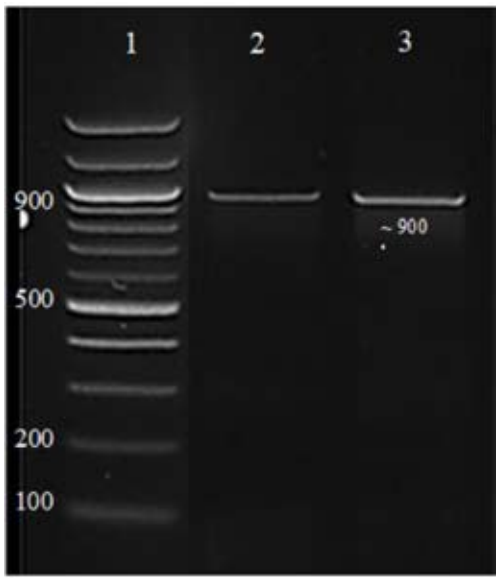

Fig. 3 Agarose gel electrophoresis of PCR amplified products of *hag*A gene (900 bp)

Lane 1: 100 bp ladder
Lane 2: Positive control
Lane 3: Positive sample

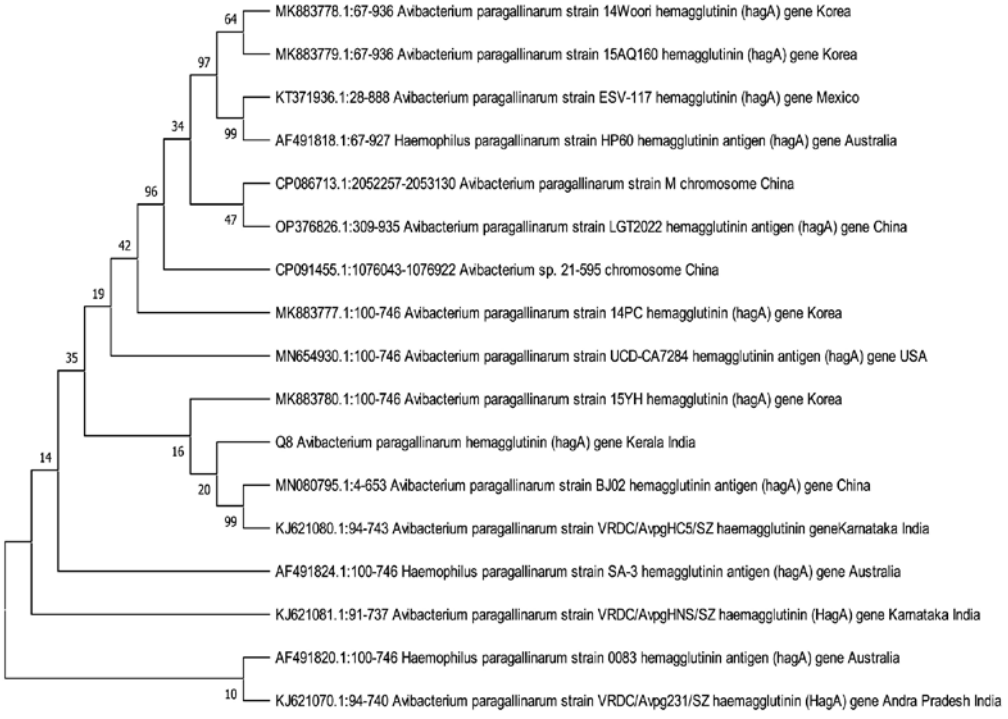

Fig. 4. Phylogenetic tree based on the analysis of 900 bp nucleotides of *hag*A gene. Phylogenetic analysis was done in MEGA XI software, using the Neighbourjoining method and 1000 bootstrap values.

The nucleotide sequence was uploaded in to the ExPASy server for primary structural analysis of *hag*A gene of the sample Q8, confirmed the presence of 277 amino acids. The derived protein sequences of *hag*A gene of Q8 were subjected to NCBI protein BLAST and showed 100 per cent identity with accession number WP264246291.1, 98.56 per cent identity with accession number QEM23765.1 and 98.19 per cent identity with accession number QEM23766.1. Molecular weight and isoelectric point of OMP of Q8 was estimated to be 30125.54 Da and 6.35. Protein structure prediction of OMP of Q8 was done by SOPMA, revealed a predicted secondary structure that contained alpha helices (29.96 per cent), extended strands (14.08 per cent), beta turns (4.33 per cent) and random coils (51.62 per cent). The online software Expasy SWISS MODEL predicted the tertiary structure of the protein based on amino acid sequences as input, which revealed that it contained α helices and β sheets (Fig. 5). The NCBI conserved domain prediction affirmed the presence of a conserved domain, outer membrane protein A domain (residues 1-277). The possible number and composition of epitopes were predicted by linear epitope prediction in IEDB and Analysis Resource tool, which affirmed the presence of seven epitopes with 20 amino acid sequences.

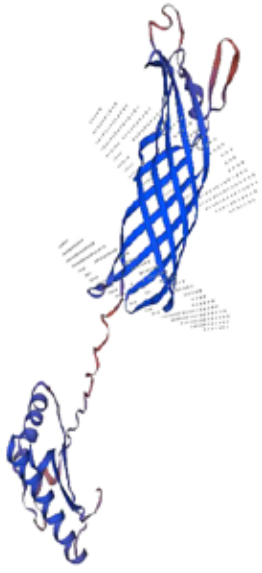

Fig. 5. Predicted tertiary structure haemagglutinin

Conclusion

This research represents the initial isolation and molecular identification of *A. paragallinarum* in quails from Kerala. Three out of sixteen samples found to be positive for IC with HPG-2 gene and *hag*A gene PCR. *Avibacterium paragallinarum* is a slow-growing organism that can take 36-48 h. or longer to manifest noticeable colonies. However, the rapid growth of co-infected bacteria will obscure the growth of *A. paragallinarum*, and the satellitic growth might not be noticed. Isolation revealed the presence of factor V dependent *A. paragallinarum* from one sample. HPG-2 gene specific PCR was found to be a valuable diagnostic tool for rapid and reliable diagnosis of IC. Sequencing of *hag*A gene of the isolate of *A. paragallinarum* revealed the existence of specific haemagglutinin antigen. Phylogenetic analysis revealed that the isolate clustered together with strains from Korea, China and India and nucleotide sequence shared 100 per cent homology with Korean strain.

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Conflict of interest

The authors declare that they have no conflict of interest.

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