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Research Article

MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF *AVIBACTERIUM PARAGALLINARUM* IN AN INFECTIOUS CORYZA OUTBREAK AT A POULTRY FARM IN MEGHALAYA, NORTHEASTERN INDIA

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ABSTRACT: Infectious coryza is a bacterial disease of poultry that results in severe economic losses to the poultry industry. This study investigated an outbreak on an organized farm in Meghalaya, affecting pullets of the BV380 variety specifically bred to lay brown eggs. The pullets exhibited clinical signs of edematous eyes with purulent discharge from the eyes and nostrils, swelling of infraorbital sinuses, and caseous cheesy exudates. Swabs taken from the infra-orbital sinus and caseous exudate underwent PCR-based molecular confirmation to diagnose *Avibacterium paragallinarum*. Phylogenetic analysis revealed close clustering with Chinese counterparts. The outbreak was controlled by oral medication with sulphamethoxazole and trimethoprim, following which the morbid birds recovered uneventfully.

Keywords: Infectious coryza, *Avibacterium paragallinarum*, Meghalaya, Outbreak.

INTRODUCTION

Infectious coryza also called avian coryza or fowl coryza is an acute respiratory disease of chicken caused by a Gram-negative bacterium, *Avibacterium paragallinarum*, formerly known as *Haemophilus paragallinarum*. *A. paragallinarum* is a pleomorphic, non-motile, non-spore forming bacillus or coccobacillus. It is encapsulated and microaerophilic, belonging to the genus *Avibacterium* and the family *Pasteurellaceae*. *A. paragallinarum* is catalase and oxidase negative, utilizes sucrose and mannitol sugars when grown in artificial media producing acids [1]. It also requires V factor (Nicotinamide Adenine Dinucleotide- NAD) for its growth, exhibiting the satellitism phenomenon when cultured along the streaks of *Staphylococcus aureus* in blood agar due to the release of V factor by *S. aureus* and its utilization by *A. paragallinarum* [2, 3, 4]. Based on the Page and Kume scheme, the organism is classified into three serotypes (A, B, and C), which are identified using the plate agglutination test (Page method) and the haemagglutination test (Kume method). Under Kume's method, three serogroups (I, II, III) comprising seven serovars were identified, designated as HA1 - HA-7. Page's serovars A, C, and B correspond to Kume's serogroup I, II, and III, respectively, with serovars A and C are reported to be pathogenic [5,6,7]. Hyaluronic acid capsule, cell wall lipopolysaccharide, haemagglutinin, iron acquisition proteins, and outer membrane proteins (OMPs) are important virulence factors involved in disease pathogenesis facilitating the adhesion and colonization of the pathogen in nasal mucosa [8].

Infectious coryza is reported worldwide, including in India, Thailand, Indonesia, Australia, Pakistan, Malaysia, and Taiwan. Birds with chronic infections and clinically ill carrier birds act as infection reservoirs. Birds that recover from infection are said to be immune for up to a year. The disease is transmitted by direct contact, aerosol route, and through contaminated feed and water [9]. It is reported that feed and water contaminated with *A. paragallinarum* are the probable cause of outbreaks in chicken flocks. All age groups of birds are susceptible to higher susceptibility in pullets than broilers, leading to a drop in egg production (10-40%) and economic loss [10]. In the current study, an outbreak of infectious coryza caused by *A. paragallinarum* was identified in the poultry unit of the College of Agriculture, CAU(I), Kyrdemkulai,

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Meghalaya. The outbreak was confirmed using polymerase chain reaction (PCR), followed by sequencing and phylogenetic analysis of the isolate.

MATERIALS AND METHODS

Sample collection and DNA extraction

In June 2023, one pullet in the poultry unit at the College of Agriculture, CAU(I), Kyrdemkulai, Meghalaya, showed clinical signs of edematous eyes with purulent discharge from the eyes and nostrils, swelling of infraorbital sinuses and caseous cheesy exudate observed in the eyelids when pressed (Fig. 1). Similar clinical signs were observed in six more birds appearing dull, depressed and anorectic, out of which two died, suggesting a possible bacterial disease outbreak *i.e.*, infectious coryza. Swabs from the infra-orbital sinus and caseous exudate were collected from five infected chickens (BV380, a layered variety specifically reared to lay brown eggs). The samples were collected using sterile swabs and sterile sample containers and were transported to the laboratory for further examination. Swabs containing discharges from nasal and infraorbital sinuses were placed in 1.5 mL microcentrifuge tubes containing a lysis buffer to facilitate cell lysis and release of DNA. The genomic DNA was isolated from the collected samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol [11].

Molecular confirmation

The detection and confirmation of *A. paragallinarum* infection in poultry birds were performed by polymerase chain reaction (PCR). The primers used in the study targeting a 510 bp species-specific region (pyrG gene) were F-5'-TGAGGGTAGTCTTGCACG CGAAT-3', R-5'CAAGGTATCGATCGTCT CTCTACT-3' [12]. PCR was carried out in a 20 µL reaction mixture containing 16 µL EmeraldAmp GT PCR Master Mix (Takara Bio, Japan), 1 µL forward primer (10 pm/µl), 1 µL reverse primer (10 pm/µL), and 2 µL DNA template. PCR was performed in a thermal cycler (Eppendorf, Germany) with an initial denaturation step at 94°C for 4 min, 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. The final extension was carried out at 72°C for 10 mins. Around 15 µL of amplified PCR product was electrophoresed on a 1.5% agarose (Himedia, Mumbai, India) gel containing 0.5 µg/mL ethidium bromide (Thermo Fisher Scientific, Waltham, MA, USA). The marker used was a 100 bp DNA ladder (Takara Bio, Japan). Electrophoresis was carried out at 100 volts using 0.5X TBE electrophoresis buffer. The gels were visualized and photographed under a gel documentation system (Vilber Loumat, France).

Sequencing and phylogenetic analysis

The amplicon was commercially sequenced using Sanger sequencing. The sequences obtained underwent a quality check and were assembled and identified by matching with the NCBI-BLAST nonredundant database. A phylogenetic tree was constructed using the Maximum likelihood (ML) algorithm with 1000 bootstrap replicates [13] in MEGA11 [14]. The suitable model was chosen based on Bayesian Information Criterion (BIC) score.

RESULTS AND DISCUSSION

Infectious coryza is an economically important disease in the poultry sector as it affects birds of different age groups. Poor hygienic environments and nutrition are some of the predisposing factors for infectious coryza [9]. Thitisak *et al.* [15] and Poernomo *et al.* [4] reported that poultry birds reared in backyard or semi-intensive systems are most susceptible to the disease, with higher mortality rates compared to Ranikhet disease. Killed vaccines containing serovars A and C have been developed to control the disease. However, despite vaccination efforts, outbreaks of infectious coryza have been reported, indicating the emergence of new serotypes and poor cross-protection among the serovars [16, 17]. Vaccines containing avirulent *A. paragallinarum* mimic natural infection and have been reported to provide better cross-protection among serovars compared to killed vaccines [18].

The present investigation was conducted to detect and confirm the causative agent of infectious coryza in the flock, which is *A. paragallinarum*. The clinical signs reported in the current study, such as swelling of the face, infraorbital sinus, and purulent nasal discharge, which are significant for infectious coryza, were similar to previous reports [19]. Molecular screening using a PCR assay targeting a species-specific region of *A. paragallinarum* showed an amplicon size of 510 bp when analyzed under agarose gel electrophoresis (Fig. 2), confirming the infection. No amplification was observed in the negative control. The samples were collected within 1-2 days of the disease outbreak and showed 100% positive results by the PCR assay. The amplicon sequences matched with *A. paragallinarum* hits in the NCBI database using the NCBI BLAST tool. The assembled sequence is available in the NCBI

Fig. 1. Photographs depicting swollen eyes in infected birds and the caseous material extracted from the eyes.

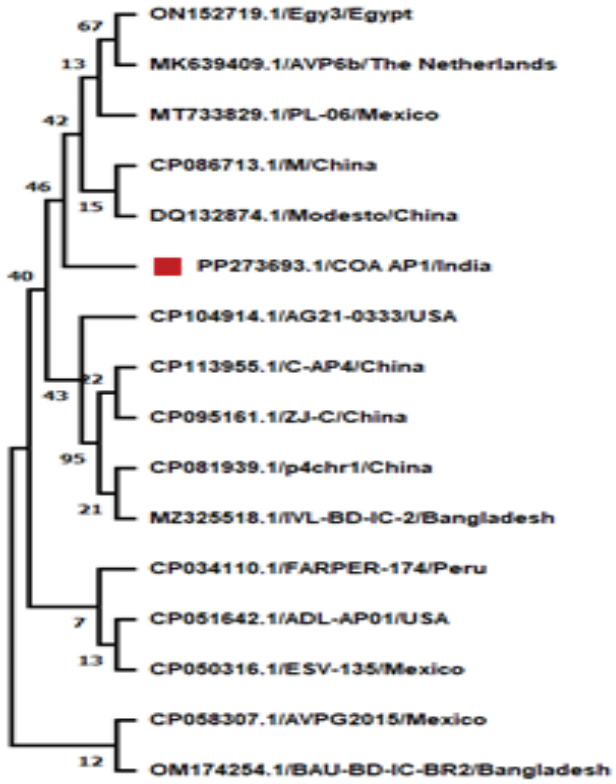

Fig. 3. Maximum likelihood tree constructed using Tamura 3 model with 1000 bootstrap replicates [Sequences obtained in this study is indicated with a red square].

database with accession number PP273693.1. The Tamura 3-parameter model [20] was identified as the best model to compute the ML tree for the obtained sequence, with a BIC score of 1750.018. The phylogenetic tree with 1000 bootstrap replicates revealed a close clustering of the sequence identified in this study with that from China (Fig. 3).

Conventional bacteriological methods, serological tests, and molecular methods were employed for the diagnosis and characterization of *A. paragallinarum*

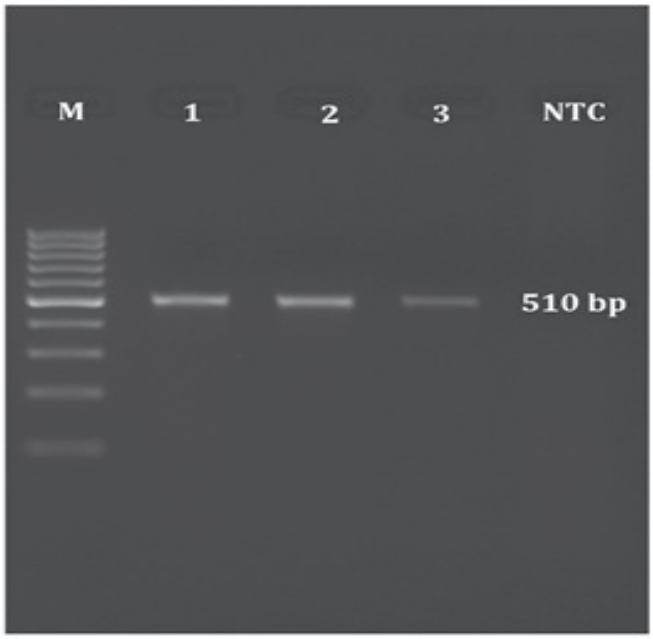

Fig. 2. Amplified PCR product of pyrG gene in agarose gel electrophoresis [Lane M - 100 bp ladder; Lanes 1-3: Samples; Lane NTC - Negative Template Control].

[21, 22]. Conventional diagnosis of *A. paragallinarum* is time-consuming, taking about 24-48 hours, and requires the NAD/V factor for growth and biochemical characterization, making it a tedious task for routine isolation and identification [9]. Molecular diagnostic methods like PCR are rapid, allowing immediate attention to check the spread and administer appropriate treatment [22].

The infected birds were treated using sulphamethoxazole and trimethoprim dispersible powder at a rate of 10 g/8 liters of drinking water for 5-7 days. The morbid birds started recovering, and the treatment successfully restricted the spread of infection to other birds in the flock. In previous studies, *A. paragallinarum* is reported to be sensitive to antibiotics such as ampicillin, amoxicillin, gentamicin, spectinomycin, tylosin, erythromycin, enrofloxacin, sulphamethoxazole and trimethoprim [23, 24, 25]. However, resistance to antibiotics like sulphonamides, streptomycin, erythromycin, and tetracycline has also been reported to varying degrees [25, 26]. In the present outbreak, sulphamethoxazole-trimethoprim was effective in treating the infected birds.

CONCLUSION

In conclusion, PCR proved to be a rapid diagnostic tool for detecting *A. paragallinarum*, the causative agent of infectious coryza. The present study indicated that infectious coryza can be tackled with the help of

sulphamethoxazole and trimethoprim. The occurrence of the disease is linked to aerosol transmission of infection from infected areas, especially as many backyard poultry-rearing practices are observed in nearby villages. Phylogenetic analysis revealed that the strain investigated in the current study was related to strains circulating in neighboring countries. Proper vaccination and biosecurity measures are crucial to prevent the transmission of infectious coryza.

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