



Detection and Molecular Characterization of *Avibacterium paragallinarum* from Poultry

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ABSTRACT

Background: Infectious coryza is an acute, highly contagious respiratory disease of chickens caused by *Avibacterium paragallinarum* causing heavy economical loss to the poultry farmers. The laboratory diagnosis of *Av. paragallinarum* infection is based mainly on conventional methods such as isolation and identification of the organisms. In modern poultry industry, the rapid diagnosis of *Av. paragallinarum* is essential along with detection of variation among the serotypes.

Methods: A total of 128 samples (*viz.*, heart blood swabs, tracheal, eye and infra orbital swabs) suspected for infectious coryza were screened for the presence of *Av. paragallinarum* by isolation and molecular characterization. Information regarding farm/flock strength, age of birds, method of rearing, production performance, clinical symptoms manifested and mortality patterns from various organized poultry farms were recorded. A total of five isolates of *Av. paragallinarum* have been recovered from suspected materials processed for isolation. Two isolates were sequenced and analysed.

Result: All the isolates showed same biochemical characters but exhibited variability in the fermentation of mannose, mannitol and sorbitol. The nucleotide variation of *Av. paragallinarum* present in Indian subcontinent differs in their virulence and pathogenicity. The study was also focused for the molecular characterisation of *Avibacterium* species from different isolates. Though the clinical signs and gross pathological signs were similar, the nucleotide variation between isolates was observed. Sequencing of 'hagA' gene showed that the two isolates were closely related to the strains of China, Australia, Mexico and India which shared 94-100% homology.

Key words: *Avibacterium paragallinarum*, Coryza, hagA gene, Poultry, Sequencing.

INTRODUCTION

Among the primary bacterial respiratory disease of poultry, Infectious Coryza (IC) is an infectious and highly contagious disease affecting the upper respiratory tract of chickens (Blackall and Matsumoto, 2003). The disease occurs worldwide including India. It affects both broiler and layer flocks causing huge economic losses. Infectious coryza is caused by Gram-negative, non-motile, pleomorphic, non-spore forming and capsulated bacteria called as *Avibacterium paragallinarum* (Hinz, 1973).

Infectious coryza is one of the major problems affecting commercial poultry industry, in India. In India, the first incidence of Infectious Coryza in India was reported in 1958 (Adalkha, 1967). The laboratory diagnosis of IC caused by *Av. paragallinarum* infection is based mainly on demonstration and confirmation by conventional methods such as isolation and identification of the organisms.

Characterization of Indian isolates of *Av. paragallinarum* by Page serotyping Scheme has shown that, serovar A and serovar C were prevalent in India but no isolate of serovar B was identified (Tongaonkar *et al.*, 2002). Similarly, Patil *et al.* (2016) found serovar B for the first time in India while serovar C was more prevalent followed by A than B. Later, Patil *et al.* (2017) also found serovar A, B and C by molecular typing. Contrarily, Anne *et al.* (2022) reported higher prevalence of serovar B than A while there was no serovar C in their study.

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Since infectious coryza is one of the major problems detected in different parts of India affecting commercial poultry industry causing significant economic loss that emphasizes the need for molecular diagnosis and

pathological studies of *Av. paragallinarum* serovars circulating in India.

Though few studies on identification of *Av. paragallinarum* is available from India, very scanty literature is available from the state of Tamil Nadu. To the best of our knowledge, little is known about the molecular diagnosis of complicated cases of infectious coryza in the state of Tamil Nadu, India and very scarce systemic pathogenicity studies have been conducted on *Av. paragallinarum*. Keeping in view of the above facts, the present study was conducted for isolation, identification and biochemical characterization of *Av. paragallinarum* from suspected clinical and post-mortem cases of Infectious Coryza. Diagnosis of Infectious Coryza by using gene specific Polymerase chain reaction (PCR) and Pathogenicity studies in chicken were also carried out.

MATERIALS AND METHODS

Sample collection

A total of 128 samples were collected from infectious coryza suspected 73 farms of 85 poultry flocks as well as samples available with Poultry Disease Diagnosis and Surveillance Laboratory, Namakkal, Tamil Nadu. Information regarding farm/flock strength, age, method of rearing, production performance, clinical symptoms manifested and mortality patterns from various organized poultry farms were recorded. The collected samples comprising of heart blood swabs, tracheal, eye and infra orbital swabs. The collected specimens were transported in Ames transport medium and stored in refrigerator till further processing.

Sampling from live birds

Ailing birds with history of respiratory disease and showing signs of infectious coryza viz., swollen infra-orbital sinuses, serous to mucoid oculo-nasal discharge, eye and facial swelling, were selected and gentle pressure was exerted on each infraorbital sinus to flush out mucus through nostrils and sampled using sterile disposable cotton swabs.

Sampling from autopsied birds

Samples were taken from birds either already dead or after scarifying by decapitating the birds showing clinical signs of coryza. The head was swabbed with 70% alcohol and allowed to dry. The mucoid fluid from the infra orbital sinus and nasal or conchal cavities was collected with a nichrome loop.

Primary isolation of NAD-dependent *Av. paragallinarum*

Blood agar (5-7% v/v sheep blood) with haemolytic *Staphylococcus* sp. as feeder culture was used as culture media for preliminary isolation of *Av. paragallinarum* from suspected samples. Chocolate agar with 0.01% (w/v) NADH and Brain Heart Infusion broth were used for further sub culturing and studying growth characteristics as well as purification of genomic DNA. Collected samples were streaked directly onto the chocolate and blood agar plates. The plates were incubated at 37°C for 48 hrs in a traditional candle jar supplying with 5-10% CO₂. The colonies grown

on Blood agar and Chocolate agar at 37°C after 14 hrs of incubation in 5% CO₂ were examined by Gram's staining. The organism was identified as per Cowan and Steel's Manual for Identification of Medical Bacteria (Cowan, 1974). All the isolates were subjected to catalase, oxidase, nitrate reduction, indole production and urea hydrolysis tests.

All the isolates, were inoculated into 5-7 days old embryonated chicken eggs via yolk sac route and inoculated eggs were kept at 37°C for 48 hrs. Embryos dying after 24 hrs of inoculation were considered as infected. After 48 hrs the yolk fluid was harvested, dispensed in screw capped 10 ml glass bottles and stored at -20°C for further use.

Molecular detection

DNA extraction

Genomic DNA was isolated from the overnight grown bacterial culture by using Genomic DNA purification Kit (Promega, USA) and stored at -20°C until use.

Polymerase chain reaction

Initial PCR screening was carried out by using the primer pair targeting the *hpg-2* gene of *Av. paragallinarum* N1 (5'-TGAGGGTAGTCTTGCACGCGAAT-3') and R1 (5'-CAAGGTATCGATCGTCTCTCTACT-3') to amplify specific 500bp fragment (Chen *et al.* 1998). The amplification was carried out using PCR master mix (2X) (Thermo scientific, USA), with an initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 63°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. For sequencing further, the *hagA* gene was amplified using primer pairs HA1 (TGTAGC TCAAGCAGC TCCACAAG) and HA2 (TCAAGC GAT AAG TGC TTT ACG ACC) to amplify 900-bp fragment (Rhonda *et al.* 2002). The amplification was carried out using PCR Master Mix (2X) (Thermo scientific, USA), with an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 46°C for 1 min, 72°C for 1.5 min and a final extension at 72°C for 10 min. PCR products were purified employing ExoSAP-IT® (Affymetrix, USA) and sequenced commercially.

Phylogenetic analysis

The nucleotide sequence data generated were edited and aligned using Sequencing Analyses Software v5.3 (Applied Biosystems, USA) and MEGA 6 software. Reference sequences were downloaded from GenBank database. Phylogenetic analyses and evolutionary associations were inferred in MEGA 6.0 using the Maximum Likelihood algorithm with Kimura-2P correction and 1000 bootstrap replications.

Experimental pathological studies of *Av. paragallinarum*

Pathogenicity of field isolates of *Av. paragallinarum* was studied in experimentally infected 7 weeks old chicken. The birds were examined daily for 7 days for clinical symptoms of Infectious coryza, mortality and progression of diseases. The experimentation was performed after obtaining IAEC and IBSC permission (IAEC approval no. 4/2015, dt.

07.07.2015, VCRI, Namakkal and Lr. No. 1675/DFBS/IBSC/IBSC/2015, dt. 04.09.2015, the Dean, faculty of Basic Sciences, MVC, Chennai).

Preparation of inoculum

The inoculum for the experimental infection of birds was prepared according to Reid and Blackall (1984) and Sobti *et al.* (2001) with slight modifications. Pure bacterial colonies grown on chocolate agar plates were picked and inoculated into Brain Heart infusion broth in test tubes. The test tubes were then incubated for 48 hr under reduced O₂ tension. Cultural suspension of 10¹¹ CFU of each isolate was used as the inoculum for pathogenicity tests.

Infection of birds

A total 90 birds were randomly divided into 6 groups (15 birds each group). Group 1-5 was infected with one isolate each (Isolates 1 to 5) of *Av. paragallinarum*. Group-6 were kept as unchallenged control.

Method of inoculation

An inoculum of 0.2 ml of each isolate with viable organisms was inoculated to susceptible seven-week-old chicks through intranasal and infraorbital sinus route for three days.

Post infection observation and Re-isolation of *Av. paragallinarum*

All the experimentally infected birds were daily observed for clinical symptoms and progression of diseases for 7 days. After the development of clinical signs of infectious coryza, the birds were scarified and decapitated for observation of post-mortem lesions. Tissue samples were collected and the organisms were re-isolated.

RESULTS AND DISCUSSION

Out of 128 samples processed for isolation of *Av. paragallinarum*, five isolates (3 from Infra orbital swabs, 2 from tracheal swabs and 1 from eye swab) of NAD-dependent *Av. paragallinarum* were by isolation and named as PDDSL 1-5. All the isolates yielded characteristic satellite phenomena with barely visible small dewdrop like moist and non-hemolytic colonies on 5% sheep blood agar (Fig 1). On Chocolate agar colonies of all the isolates of *Av. paragallinarum* were barely visible, dew drop like, smooth and moist type (Fig 2). The colonies from solid media revealed distinctive pleomorphic and beaded Gram-negative organisms by Gram's staining. The isolates observed in wet mount smears prepared from brain heart infusion broth revealed non-motile small coccobacillary rods showing pleomorphism (Fig 3).

All the isolates produced similar reactions in biochemical tests as presented in the Table 1 and Fig 4, 5, 6.

Carbohydrate fermentation tests

All the *Av. paragallinarum* isolates tentatively identified by biochemical tests were further characterized by various sugar fermentation tests. All the five field isolates failed to



Fig 1: "Satellite" phenomena with small dew drop like moist and non-hemolytic colonies on 5% sheep blood agar.

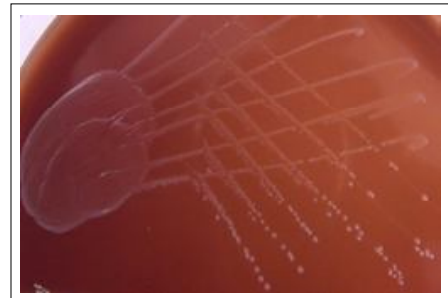


Fig 2: Dew drop like, smooth and moist colonies on chocolate agar.

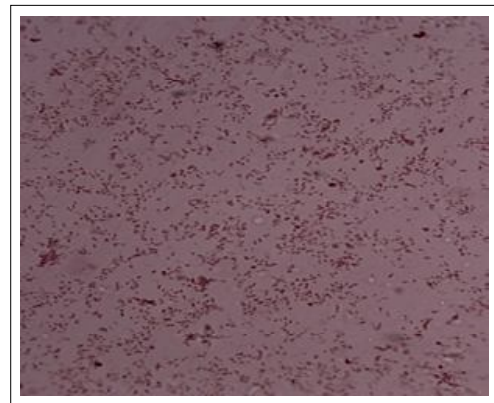


Fig 3: Non-motile small coccobacillary rods showing pleomorphism in Gram's stain.

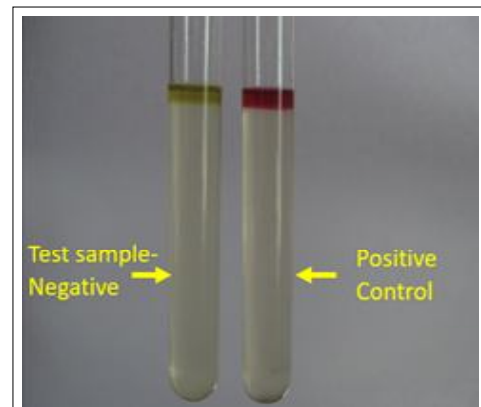


Fig 4: Indole test (Not produced indole).

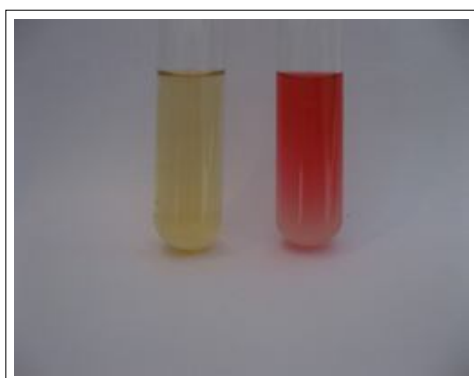


Fig 5: MR -VP test.



Fig 6: Urease test (Not hydrolysed).

ferment Arabinose, Cellobiose, Dulcitol, Galactose, Raffinose and Trehalose, while these isolates showed acid production with Glucose, Sucrose and Fructose without formation of gas. There was variation in acid production from Mannitol, Mannose and Sorbitol. The results of carbohydrate fermentation tests are presented in Table 1.

The overall isolation of *Av. paragallinarum* in this study was 3.9 per cent (5/128). Infra orbital samples showed highest isolation of 9.1 per cent (3/33) followed by tracheal swabs 4.1 per cent (1/24) and eye swab 1.5 per cent (1/67). Among samples collected from affected birds, the isolation was high in birds below 20 weeks of age (5%) followed by layer chicken 20-40 weeks (2.1%).

The results of the *Av. paragallinarum* isolates obtained from various samples are furnished in Table 2 and age wise incidence is furnished in Table 3. Similar findings were reported by Khatun *et al.* (2016), who isolated and characterized *Av. paragallinarum* from commercial broiler chickens in Bangladesh, during field outbreaks using the bacteriological culture of nasal and ocular discharges, tracheal swab, tracheal wash and infraorbital sinus exudates.

Pathology of spontaneous cases of Infectious coryza

Clinical signs

Chickens affected with infectious coryza in most of the natural outbreaks showed serous to mucopurulent oculo-nasal discharges with respiratory rales, conjunctivitis, facial oedema, unilaterally or bilaterally swollen infraorbital and

Table 1: Showing biochemical characteristics of *Av. paragallinarum* isolates.

Biochemical characteristics	<i>Av. paragallinarum</i> isolates				
	1	2	3	4	5
Catalase	-	-	-	-	-
Oxidase	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Indole production	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-
Co2 requirement	+	+	+	+	+
NAD requirement/Symbiotic growth	+	+	+	+	+
Carbohydrate fermentation					
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Mannitol	V	V	V	V	V
Maltose	+	+	+	+	+
Galactose	-	-	-	-	-
Lactose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Trehalose	-	-	-	-	-
Cellobiose	-	-	-	-	-
Arabinose	-	-	-	-	-
Raffinose	-	-	-	-	-
Sucrose	+	+	+	+	+
Sorbitol	V	V	V	V	V
Mannose	V	V	V	V	V

+ = Positive (>90%), - = Negative (>90%), V = Variable reaction.



Fig 7: Facial oedema, swollen infraorbital and nasal sinuses including eyelids.



Fig 8: Catarrhal exudates with necrotic debris was found in upper larynx and eyelids.



Fig 9: Dull birds after 24 hrs infection.

nasal sinuses including eyelids (Fig 7). Foamy lacrimation and purulent exudates stuck to eyes due to fibrino-purulent conjunctivitis. There was drop in egg production and mortality patterns varied with secondary pathogens in environment.

Gross pathology

In the early stages of disease, unilateral or bilateral swelling of periorbital region and conjunctivitis and in advanced cases there were closure of eyes, bilateral infraorbital swelling with mucopurulent oculo-nasal discharge, oedema of wattles and intermandibular region. Catarrhal exudates with necrotic debris was found in upper larynx and nasal cavities (Fig 8). Abdominal air sacs were thickened with yellowish cheesy exudates. Ovaries were congested, softened and ruptured yolk leading to egg yolk peritonitis.

Experimental pathological studies

Clinical signs

The five group of experimentally infected birds were examined in detail at regular intervals of time for clinical signs and pathological changes and the results were compared with those obtained from spontaneously infected cases. All the infected birds exhibited oedematous swelling of left side of infraorbital sinus with secretion of watery nasal discharge after 24 hrs of infection (Fig 9) and swelling of face and infraorbital sinuses of other side after 48 hrs of inoculation (Fig 10). Some birds showed bilateral swelling of face and infraorbital sinuses, conjunctivitis, serous to mucoid nasal discharge with foul smelling, foamy lacrimation and induration of face after 3 days of infection. Respiratory rales, clear watery diarrhoea and swelling of comb and wattles were noticed after 4 days of infection. After 5 days of infection disappearance of nasal discharge and lacrimation and cessation of diarrhoea were observed but the birds still suffered from swelling of face and wattle after 7 days. Two isolates (PDDSL-1 and 3) also caused mortality (Fig 11) with clinical symptoms. The birds were sacrificed after 3rd day of inoculation for observation of post-mortem lesions and collection of nasal and sinus exudates for re-isolation of organisms. Other birds were kept for observation up to 10 days of post infection. Unchallenged group of birds did not reveal any clinical signs of infectious coryza and also did not show any gross and histopathological lesions of infectious coryza. Anjaneya *et al.* (2013) and Xu *et al.* (2019) observed less severe ocular discharges and mild swelling of infraorbital sinus in chicken after experimental infection when compared to other reports. Further they also reported that this could be due to the bacterial strain,

Table 2: *Av. paragallinarum* isolates obtained from various poultry samples.

Kind of sample	Number of samples	No. of Isolates obtained	Sample wise Isolation percentage	Isolate number
Infra orbital swabs	33	03	9.1	PDDSL-1, 2 and 3
Tracheal swabs	24	01	4.1	PDDSL-4
Eye swabs	67	01	1.5	PDDSL-5
Heart blood swabs	4	-	-	-
Total	128	05	3.9	



Fig 10: Facial swelling after 48 hrs of inoculation.



Fig 11: Death after 7 days of post inoculation.

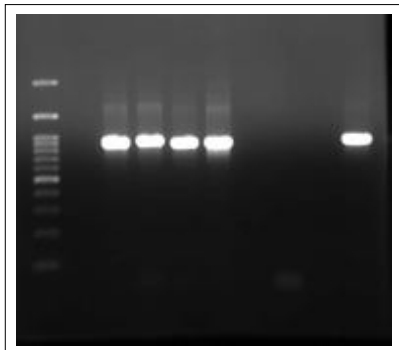


Fig 12: Isolates showing 900 bp amplification with 'hagA' gene.

pathogenicity and host susceptibility. Anjaneya *et al.* (2013) demonstrated the infection of *Av. paragallinarum* in chickens after multiple infusion through direct intra-sinus inoculation of birds over a period of three days and development of clinical signs.

Gross pathology

The nasal cavities were filled with greyish white thick exudates and mucus membrane of nasal cavities showed congestion and oedematous swelling after 2 days of infection. Infraorbital sinuses cavities were filled with greyish white watery exudate and oedema of subcutaneous tissue of periorbital region, mucus membrane showed hydropic swelling after 3 days of infection. Larger amount of yellowish cheesy exudates was noticed after 5 days of infection. Trachea revealed mild congestion of mucus membrane.

Molecular detection of *Avibacterium paragallinarum*

Isolation and identification of *Av. paragallinarum* is difficult and the organism is so fragile the rate of isolation will always be lesser hence, several incidences molecular diagnostic assay are performed (Muhammad and Sreedevi, 2015). Similar to this study Anne *et al.* (2022) reported isolation of 5 *Av. paragallinarum* and also reported the usefulness of PCR over isolation. PCR performed on all the five isolates of *Av. paragallinarum* revealed an amplified product of approximately 500 bp amplicon with *hpg-2* PCR and 900 bp amplification with *hagA* PCR (Fig 12). Similar findings were reported by Muhammad and Sreedevi (2015), using the PCR technique, detected *Av. paragallinarum* from outbreaks of infectious coryza in Andhra Pradesh of India.

Sequencing and sequence analysis of 'hagA' gene

Sequencing and BLAST analysis of 'hagA' of two isolates (PDDSL 1 and 2) of *Av. paragallinarum* indicated that the isolates were closest relationship with *Av. paragallinarum* strains of China, Australia, Mexico and India which shared 94-100% homology (Fig 13).

Zhang *et al.* (2016) showed that the strains circulating in China before 2014 and after 2015 had different ancestors and that new strains found in China were derived from earlier Indian strains, with some genetic differences.

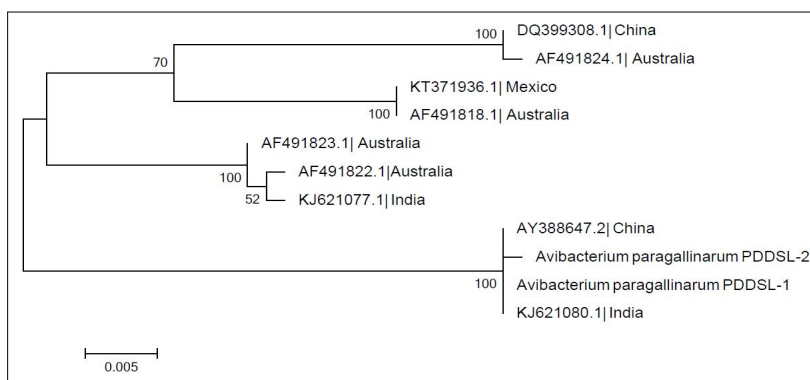


Fig 13: BLAST analysis of 'hagA' gene of *Av. Paragallinarum*.

Table 3: Age wise incidence of *Av. paragallinarum* isolates in poultry birds.

Age of the birds	Number of samples collected	Number of isolates obtained	Percentage
Below 20 weeks	80	4	5
20-40 weeks	48	1	2.1

CONCLUSION

A total number of five NAD-dependent isolates of *Av. paragallinarum* could be recovered from 128 samples collected from different poultry farms of Tamil Nadu. All the field isolates were found to be pathogenic when experimentally inoculated by I/O sinus route in young chickens with expression of typical clinical signs and gross and histopathological lesions. PCR technique was found to be an effective diagnostic tool for rapid and reliable diagnosis of ICand for differentiating *Av. paragallinarum* with other non-pathogenic avian haemophili. The sequencing of 'hagA' gene of two isolate of *Av. paragallinarum* along with other 2 Indian strains (KJ621080.1 and KJ621077.1) revealed that it is closely related to China and Australian strains.

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Conflict of interest: None.

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