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Isolation, Antibiogram and PCR Detection of *Avibacterium paragallinarum* from Poultry Flocks of India

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Infectious coryza (IC), caused by *Avibacterium paragallinarum,* is one of the major problem affecting the commercial poultry industry worldwide including India. Very few reports regarding the prevalence of *Av. paragallinarum* in particular with the application of rapid, sensitive and confirmatory detection by the molecular tools are available from India. In this study a total of 105 field samples collected as nasal and sinus swabs from suspected cases of IC from different geographical locations viz. Bareilly (U.P), Pune (Maharashtra), Bangalore (Karnataka), Indore and Jabalpur (Madhya Pradesh) of India were analyzed. Cultural isolation revealed the presence of four different, NAD-dependent types of *Av. paragallinarum* positive isolates showing typical 'satellite' growth. Biochemically, all the isolates of *Av. paragallinarum* showed negative catalase activity, reduced nitrate to nitrite and unable to produce indole, hydrolysed the urea and fermented glucose and fructose without formation of gas and variability in acid production was observed with mannitol, mannose, and sorbitol. Antibiogram indicated that all field isolates were sensitive to Amoxycillin / Clavulanic acid, Enroflaxacin, Ciprofloxacin, Pefloxacin, Co-Trimoxazole and Chloramphenicol antibiotics. Furthermore, isolates were relatively less sensitive to Neomycin, Cephalaxin, Streptomycin and Furozolidin and resistant to Colistin, Ampicillin, Tetracycline, Oxytetracycline and Doxycycline antibiotics. PCR assay was standardized for direct detection of the causative bacterium on nasal and sinus swabs, culturally isolated colonies of *Av. paragallinarum* and reference strains by using combination of N1/R1 primers (HPG-2 gene based PCR) for evaluating the detection applicability of molecular diagnostic methodology. In conclusion, *Av. paragallinarum* was culturally isolated and biochemically characterized from field cases suspected of infectious coryza in poultry and presence of the causative bacterium was also confirmed by the molecular tool of PCR. Comparatively, PCR was found to be more effective and a sensitive diagnostic tool for rapid and reliable diagnosis of IC. Further, large scale epidemiological studies along with application of diagnostic molecular tools are suggested to know the real magnitude of this important pathogen affecting poultry flocks of the country.

Key words: *Avibacterium paragallinarum*, infectious coryza, poultry, isolation, antibiogram, PCR.

Among the prevailing diseases, respiratory diseases are frequently reported throughout the world causing economic loss to the poultry farmers in terms of mortality and loss of production[1]. The aetiology of respiratory disease is complex, often involving more than one pathogen at the same time arises mainly due to virus, bacteria, fungi and other environmental stress factors[2,3]. Several of the bacterial diseases are now controlled by suitable antibiotic therapy, improved management practices and vaccines. However, despite the best efforts, a few bacterial diseases

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continue to plague the poultry industry worldwide year after year causing severe economic losses. Among the primary bacterial respiratory disease of poultry, infectious coryza is infectious and highly contagious disease affecting the upper respiratory tract of chicken[3]. Infectious coryza is caused by Gram-negative, nonmotile, pleomorphic, non-spore forming and capsulated bacteria called as *Avibacterium paragallinarum* which was previously called as *Homophiles paragallinarum.* The growth requirements of *Av. paragallinarum* is reduced or oxidized form of NAD(H) which is necessary for *in vitro* growth of most of isolates, hence isolation requires co-cultivation on nurse or feeder culture such as *Staphylococcus* spp. The economic impact of the disease is attributed to the resultant increase in unthrifty chickens and significant drop in egg production (10 to 40%) in layer flocks and retardation of growth leading to increased number of culls particularly on multi-aged farms. Presumably infectious coryza have been complicated by the presence of other pathogens such as *Mycoplasma gallisepticum*, *M. synoviae*, *E. coli*, *Pasteurella multocida*, and viral pathogens like Infectious Bronchitis Virus(IBV), Infectious Laryngotracheitis virus (ILT) and Fowl Pox Virus (FPV). Severe and prolonged disease may develop in complicated cases with the clinical picture of a chronic respiratory disease, swollen head-like syndrome, air sacculitis, tarsal arthritis and septicaemia. In India, outbreaks of infectious coryza are often complicated with fowl cholera and can result in high mortalities (50%) in layer flock[5,6]. Since among the infectious diseases of poultry, IC is one of the major problem affecting commercial poultry industry in different parts of India causing significant economic loss and an infected flock may potentially shed the organism as carrier for the rest of the life for other birds. Keeping in view the above facts, the present study reports isolation and identification using cultural, biochemical characterization and molecular detection of *Avibacterium paragallinarum* from materials collected from suspected clinical and post-mortem cases of infectious coryza from different regions of India.

MATERIALS AND METHODS

Field Clinical Samples Collection

A total number of 105 clinical samples were collected from the respiratory tract as swabs from infra-orbital sinuses, exudate from nasal cavities, trachea, lungs and air sacs from live and / or dead birds showing symptoms and lesions like swollen infra-orbital sinuses, serous to mucoid occulo-nasal discharge, eye and facial swelling, suggestive of IC. The samples were collected from various government and commercial private poultry farms from different geographical regions of the country viz. 24 samples from live birds from 03 poultry farms in and around Bareilly district, Uttar Pradesh, brought to the Avian Disease Section, Division of Pathology, for disease diagnosis purpose; 12 samples from 03 poultry farms of Bangalore and Mysore region, Karnataka; 15 samples from Indore and Jabalpur region, Madhya Pradesh; 09 samples from Pune, Maharashtra; 45 samples from dead birds of Central Avian Research Institute (CARI) farm necropised in post-mortem facility of Indian Veterinary Research Institute, Izatnagar, Bareilly. All the swab samples were collected in 30% glycerol phosphate buffer saline (G-PBS) for further studies.

Isolation of pure culture

All the clinical samples collected were processed for cultural isolation of *Av. paragallinarum* following standard protocols. Bacteriological media used for isolation of *Av. paragallinarum* were Blood Agar base, Haemophilus Enrichment Agar base (HA) and Nicotinamide adenine diphosphate (NADH) obtained commercially as dehydrated media from HiMedia Laboratories, Mumbai (India). These media were prepared as per the manufacturer's instructions. Blood agar (5-7% v/v sheep blood) with haemolytic *Staphylococcus* spp. feeder culture was used as primary culture media for preliminary isolation of *Av. paragallinarum* from suspected samples. Other media viz., Haemophilus agar, Haemophilus broth, chocolate agar with 0.01% (w/v) NADH and Brain Heart Infusion broth were used for further sub-culturing to get pure colonies of *Av. paragallinarum*. All the isolates, following isolation in pure cultures, were inoculated into 5-7 days old embryonated chicken eggs via yolk sac

route of inoculation and kept at 37°C for 48 hr. Embryos dying after 24 hr of inoculation were considered as infected. After 48 hr the yolk fluid was harvested, lyophilized and dispensed in screw capped glass bottles (10 ml) and stored at –20° C for further use. Confirmed reference isolates of *Av. paragallinarum* were kindly supplied by Ventry Biologicals, Pune, Maharashtra. The organism was identified as per Cowan and Steel's Manual for Identification of Medical Bacteria[7].

Biochemical Identification Tests

Biochemical properties of *Av. paragallinarum* isolates were evaluated *in vitro* by different biochemical analysis. Oxidase test was used to identify bacteria containing the respiratory enzyme cytochrome oxidase using commercial bacteriological differentiation discs (DD018) HiMedia Laboratories, Mumbai (India) to detect the enzyme oxidase produced by the microorganism. A deep blue purple color within 5-10 seconds was considered positive and no color at all was considered negative. For the catalase test to be performed, a loopfull of the culture of organisms was placed on a sterile glass slide and 3% hydrogen peroxide was pour over it. Catalase activity was estimated on the basis of visual observation of the evolution of oxygen. Urease activity of the bacterial isolates was determined using Christensen's urea agar supplemented with 0.01% NADH, and 1% filtered and heat inactivated chicken serum and 40% urea solution. The results were read as positive if an intense pink colour develops within 24-48 hr of inoculation. Nitrate test was performed as described previously[7], with the addition of 0.01% NADH, and 1% sterile non hemolysed heat inactivated chicken serum. A high dose of the inoculum was used and incubated in a water bath at 37°C for 45 minutes. After this 0.33% sulphanilic acid in 5 M acetic acid was added to the medium followed by 0.5% a-Naphthylamine in 5 M acetic acid. The result was considered into account as positive when the colour of the medium changed to red colour. Indole test was used to identify bacteria capable of producing indole using the enzyme tryptonase. Indole activity was tested by the method of Quin *et al*.[8] where 0.1 per cent L-tryptophan in 0.05M PBS at pH 6.8 was used as substrate. A big loopfull of the suspected *Av. paragallinarum* culture was incubated in this substrate for 4 hrs. The test was shaken with 0.5 ml of Kovac's reagent and red color ring was taken as positive. Carbohydrate fermentation test was used to determine the ability of organism to ferment simple carbohydrates using phenol red as indicator. Commercial carbohydrate discs were inserted on to HA agar and incubated anaerobically at 37°C for 48 hrs. Acid reactions were determined by visual examination and yellow color was considered as positive for acid production.

Antibiogram Assay

Modified Kirby-Bauer disk diffusion method was used to test the antibiotic susceptibility of all the field isolates of *Av. paragallinarum* [8]. Antibiotic discs were obtained from HiMedia Laboratories, Mumbai (India). The sensitivity of the isolates to antibiotics was tested on Haemophilus agar supplemented with 0.01% NADH and 1% horse serum. To prepare the inoculum, single colonies grown on Haemophilus agar was picked with sterile cotton swab, inoculated into HA broth and incubated aerobically at 37°C for 24 hrs. The inoculum was spread onto HA plates with help of sterile cotton by inserting disc (4 disc per plate). Seeded HA plates were incubated for 24 hrs under increased CO_2 at 37°C. Antibiotics to which organisms are sensitive, formed clear zone around it, and to which organisms are resistance, they do not form any zone of inhibition around it. Those that are intermediate, forms a little zone of inhibition. Diameter of the zone of inhibition was measured, compared with the standard zone diameter given in the protocol chart.

Molecular detection

Total genomic DNA was isolated from both clinical samples and *Av. paragallinarum* positive cultures by using Genomic DNA purification kit (Promega, USA) and was stored at -20^{0}C for further use. Polymerase chain reaction was applied as direct PCR on nasal and sinus swabs and as colony PCR for colonies which were confirmed as *Av. paragallinarum* upon biochemical reactions. PCR based detection of DNA extracted from *Av. paragallinarum* isolates was performed according to the protocol reported by Chen *et al*.[10]. The confirmation using oligonucleotide primers used in this study were the combination of N1 (52 - TGAGGGTAGTCTTGCACGCGAAT-32) / R1(52 - CAAGGTATCGATCGTCTCTCTACT-32) primers which amplified a 0.5-kb fragment termed HP-2

PCR[11]. Samples collected in 30% G-PBS and stored at -20°C remained positive for PCR even after 60 days of storage but, the traditional culture method failed to detect the isolates after 48 hrs.

RESULTS AND DISCUSSION

Cultural isolation of *Av. paragallinarum* isolates

Out of 105 samples processed for isolation of *Av. paragallinarum*, four isolates of NAD-dependent *Av. paragallinarum* were recovered by using traditional cultural methods of isolation. Impression smears of nasal sinuses and infra-orbital sinus exudates revealed the presence of characteristic Gram-negative, tiny short rods and few cocco-bacillary form of organisms by Gram's staining (Fig. 1c). For primary isolation of NAD-dependent *Av. paragallinarum* directly from clinical samples, Blood agar (5% sheep blood v/v) cross streaked with *Staphylococcus spp.* feeder culture was used under reduced O_2 tension (5-7% CO_2) incubated at 37°C for 24-48 hr. Selected single young colony appearing adjacent to feeder organism was streaked on chocolate agar with 0.01% NAD or fresh blood agar plates with and without feeder organism. Colonies showing "Satellite" phenomena ('True' NAD dependent organisms) were finally subjected to further confirmation by inoculating on to Haemophilus agar. All the suspected *Av. paragallinarum* isolates produced characteristic "satellite" phenomena with small dew drop like moist and non-haemolytic colonies on 5% sheep blood agar. (Fig.1a). On Haemophilus agar, colonies of all the isolates of *Av. paragallinarum* were barely visible, dew drop like, smooth and moist type (Fig.1b). Similar method of isolation of organisms from clinical samples has been adapted by various workers[12,13,14,15,16].

Table 1. Biochemical characteristics of *Av. paragallinarum* isolates

Biochemical characteristics	*Av. paragallinarum* isolates			
	1	2	3	4
Catalase	-	-	-	-
Oxidases	+	+	+	+
Nitrate reduction	+	+	+	+
Indole production	-	-	-	-
Urea Hydrolysis	-	-	-	-
CO_2 requirement	+	+	+	+
NAD requirement/ Symbiotic growth	+	+	+	+

+ = positive (>90%), - = negative (>90%), V = variable reaction

Biochemical Identification of *Av. paragallinarum* isolates

All the isolates were subjected to catalase, oxidase, nitrate reduction, indole production and urea hydrolysis tests (Fig. 1d-f). Four field isolates and two reference strains obtained from Ventri Biological, Pune, gave similar reactions on the biochemical tests. The isolates were catalase negative, oxidase positive, reduced nitrate to nitrite and none produced indole and

Table 2. Carbohydrate fermentation patterns of *Av. paragallinarum* isolates

Name of the sugar	*Av. paragallinarum* isolates			
	1	2	3	4
Glucose	+	+	+	+
Fructose	+	+	+	+
Mannitol	V	V	V	V
Maltose	+	+	+	+
Galactose	-	-	-	-
Lactose	-	-	-	-
Dulicitol	-	-	-	-
Trehalose	-	-	-	-
Cellobiose	-	-	-	-
Arabinose	-	-	-	-
Raffinose	-	-	-	-
Sucrose	+	+	+	+
Sorbitol	V	V	V	V
Mannose	V	V	V	V

+ = positive (>90%), - = negative (>90%), V = variable reaction

Table 3. Antibiogram of *Av. paragallinarum* field isolates

Category	Diameter of zone of inhibition	Antibiotics
High level of sensitivity (3+)	14 mm	Amoxycillin / Clavulanic acid, Enroflaxacin, Ciprofloxacin, Pefloxacin, Co-Trimoxazole and Chloramphenicol
Less or intermediate sensitivity (2+)	8-13 mm	Neomycin,Ceplaxin, Streptomycin and Furozoli
Resistant	No zone of inhibition	Colistin, Ampicillin, Tetracycline, Oxytetracycline and Doxycycline

hydrolysed urea as presented in Table 1. The absence of catalase activity is a unique feature of *Av. paragallinarum*[17]. Kaur *et al*.[19] suggested that absence of catalase activity is the main biochemical character of *Av. paragallinarum.*

All the *Av. paragallinarum* isolates tentatively identified by biochemical tests were further characterized by various sugar fermentation tests (Fig. 1g,h). Using phenol red as indicator, production of yellow colour in the media was considered as positive sugar fermentation test. All the four field isolates isolated in the present study were able to ferment Glucose and Fructose; however, failed to ferment Arabinose, Cellobiose, Dulicitol, Galactose, Raffinose and Trehalose. There was variation in acid production from Mannitol, Mannose and Sorbital. The results of carbohydrate fermentation tests are presented in Table 2. Similar findings have been reported by Kume *et al*.[20], Rimler [21], Blackall and Reid [22], Piechulla *et al*.[23], and Terzolo *et al*.[24] However these results are not in agreement with those of Page[25] who observed a positive reaction for the sugar galactose.

Antibiogram assay

Antibiogram study of all the four isolates of *Av. paragallinarum was* performed on Haemophilus agar supplemented with 0.01% NADH and 1% horse serum by the modified Kirby-Bauer disc diffusion technique. Antibiogram analysis revealed the high level of sensitivity (3+) for Amoxycillin / Clavulanic acid, Enroflaxacin,

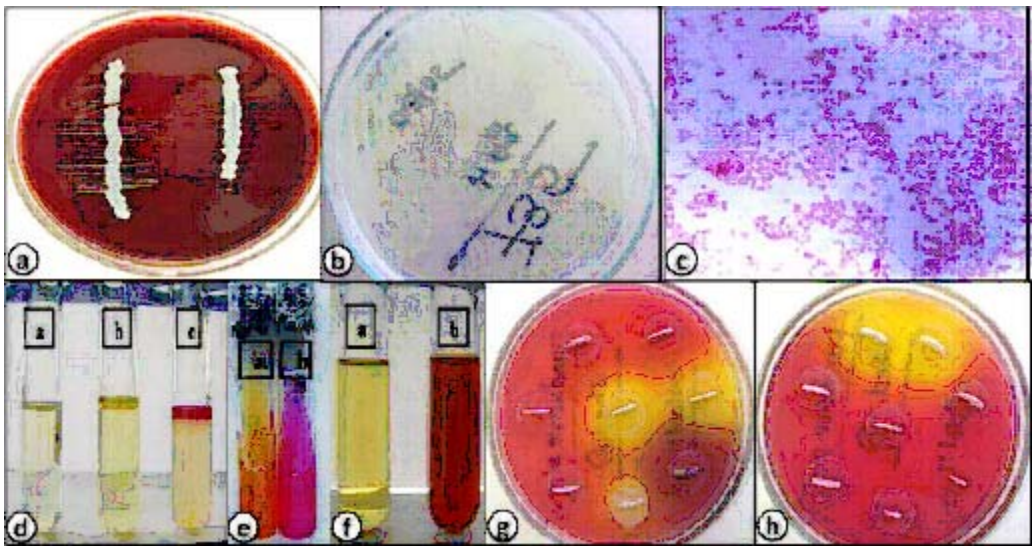

Fig. 1. Cultural, biochemical identification and antibiotic sensitivity of *Av. paragallinarum* isolates: (a) Satellitie growth of *Av. paragallinarum* isolate adjacent to *Staphylococcus* spp. feeder culture sheep blood agar as shiny dew drop colonies, (b) Dew drop like moist colonies of *Av. paragallinarum* isolate on haemophilus agar, (c) Gram negative coccobacilli as beaded and pleomorphic organisms on Gram's staining, (d) Indole test, (e) Urease test, (f) Nitrate test and (g, h) Sugar fermentation tests.

Ciprofloxacin, Pefloxacin, Co-Trimoxazole and Chloramphenicol less or intermediate sensitivity (2+) for Neomycin, Ceplaxin, Streptomycin and Furozolidin and resistance for Colistin, Ampicillin, Tetracycline, Oxytetracycline and Doxycycline as depicted in Table 3. These results were in agreement with the findings of Prabhakar *et al*.[26] and Gayatri *et al*.[27] indicated 100% sensitivity to Enroflaxacin, Ciprofloxacin, Ampicillin. Yamamoto [28] suggested that Haemophilus species were highly sensitive to erythromycin and oxytetracycline but varying degrees of resistance. Similarly, Rimler [21] performed antimicrobial sensitivity tests on *Av.paragallinarum* isolates and found that all of them were sensitive to chloromycetin, erythromycin, furoxone, gentamicin, nalidixic acid, neomycin, novobiocin, spectinomycin and tetracycline. Reece and Coloe [29] reported that one could confidently predict that treatment with streptomycin for treatment of coryza outbreaks could fail to control such outbreaks because of many clear resistant strains.

Molecular detection of *Av. paragallinarum*

Recently, the diagnostic options available for IC have been expanded by the availability of a species-specific PCR for the identification of *Av. paragallinarum*. Chen *et al*. [10] first described the diagnosis of IC by using polymerase chain reaction

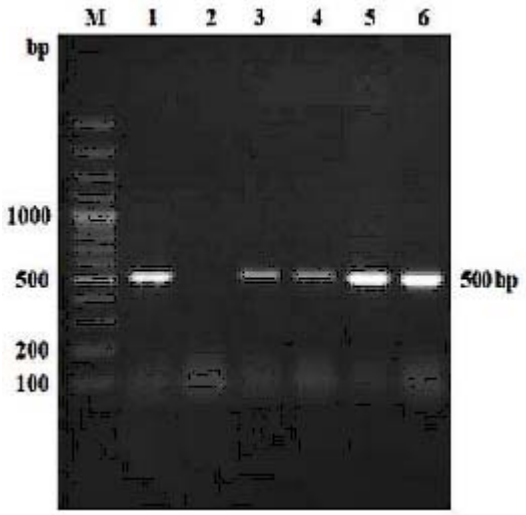

Fig. 2. Colony PCR confirmation for *Av. paragallinarum* using HPG-2 gene specific primers. Lane M : 100 bp plus DNA ladder; Lane 1 : Reference strain of *Av. pargallinarum*; Lane 2 : Negative control ; Lane 3 to 6 : *Av. paragallinarum* isolates

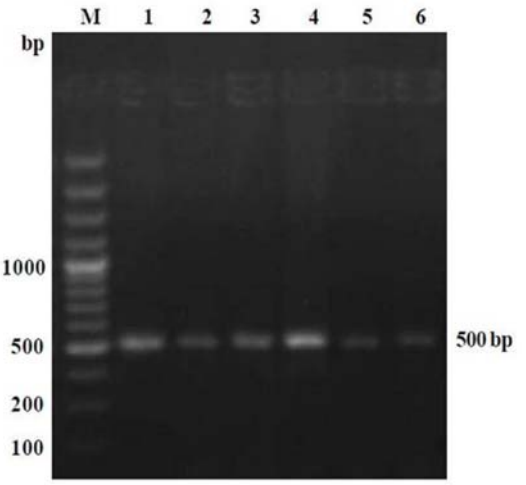

Fig. 3. Direct PCR on Nasal sinus swabs using HPG-2 gene specific primers. Lane M : 100 bp plus DNA ladder; Lane 1 to 6 : *Av. paragallinarum isolates.*

(PCR) assay. In the present study, molecular diagnostic methodology based on gene specific PCR assay was used to detect *Av. paragallinarum* directly on nasal and sinus swabs collected in 30% G-PBS. In addition, colony PCR was carried out to determine its efficacy in differentiating from other non-pathogenic avian haemophili. All the four field isolates of *Av. paragallinarum* as well as reference isolates of *Av. paragallinarum* used in present study produced the expected size of 500 bp amplicons of HPG-2 gene (Fig. 2,3). The diagnosis of *Av. paragallinarum* infection has been greatly strengthened by application of HP-2 PCR even after 60 days of storage at - 20°C as the traditional culture methods failed to detect the isolates even after 48 hr of storage. The results of PCR of all the four isolates of *Av. paragallinarum* in the present study were in accordance with the findings of Chen *et al*. [11] suggesting better sensitivity of PCR than traditional culture method of isolation and identification of *Av. paragallinarum*.

CONCLUSION

In conclusion, the present study demonstrates four culture positive NAD-dependent isolates of *Av. paragallinarum* as isolated and biochemically characterized from different geographical regions. Antibiogram analysis of the isolates indicated high sensitivity (3+) for Amoxycillin / Clavulanic acid, Enroflaxacin, Ciprofloxacin, Pefloxacin, Co-Trimoxazole and Chloramphenicol less or intermediate sensitivity (2+) for Neomycin, Ceplaxin, Streptomycin and Furozolidin and resistance towards Colistin, Ampicillin, Tetracycline, Oxytetracycline and Doxycycline antibiotics. PCR technique was found to be highly effective diagnostic tool for rapid and reliable diagnosis of IC and for differentiating *Av. paragallinarum* from other non pathogenic avian haemophili bacteria. Further extensive epidemiological investigations in this direction especially using molecular tools will help in determining the different isolates and the extent of production losses by this important pathogen of poultry.

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