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Isolation, Characterization and Serological Study of *Avibacterium paragallinarum field* isolates from Indian Poultry

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Abstract

A total of 65 nasal swab samples from Infectious coryza suspected poultry birds were collected from different geographical locations of India during 2013 to 2015 and subjected to isolation of organism, biochemical and serological characterization of isolates by Page scheme along with confirmation by PCR. Biochemically, the isolates demonstrated abilities to utilize four sugars i.e. Glucose, Mannitol, Sorbitol and Sucrose, whereas two sugars Galactose and Trehalose were not fermented by them. All isolates were able to convert nitrate to nitrite. The isolates were negative for catalase, H2S production and Indole test and showed absence of oxidase activity. Among 17 field isolates of Avibacterium paragallinarum, along with serovar A and serovar C, there is presence of serovar B. Out of 17 isolates, the serovar C was prevalent with 47% and serovar A was 27%, whereas serovar B was found to be only 11% and 2 strains were non-typable. This is the first confirmed report of presence of serovar B in Indian poultry isolates.

Key words*:* *Avibacterium paragallinarum*, Infectious coryza, India, Poultry.

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Introduction

Infectious coryza (IC) is a highly contagious disease of chicken (*Gallus gallus*) affecting primarily upper respiratory tract and is caused by a bacterium, *Avibacterium paragallinarum*. IC affected birds show serous nasal discharge, sneezing, depression and slight facial edema (Chauhan *et al.*, 2007), and results into heavy economic loss due to poor growth performance in growing birds and marked reduction (10-40%) in egg production in layers. Infectious Coryza is a cosmopolitan disease, present everywhere chickens are raised. However, it is considered as an exotic disease in New Zealand, which is the only country that appears to be free from *Avibacterium paragallinarum* (Vargas &Terzolo, 2004).

In India, Infectious Coryza outbreaks have been reported earlier (Rajurkar *et al.*, 2010). It is a continuous threat to both meat chickens and layers. Serological identification of pathogen seems to be an important feature in the development of vaccines. The first serological classification of *Avibacterium paragallinarum* was performed in 1962 by Page who recognized three different serovars termed A, B and C. The Page scheme has been widely used in many parts of the world since then (Poernomo *et al.*, 2000).

In India, Tongaonkar *et al*. (2003) have confirmed the presence of Page serovar A and C. But there is no confirmed report of Page serovar B being recorded till now. However, frequencies of occurrence of the disease, vaccine failure and occurrence of Page serovar B in countries geographically closer to India like China (Zhang *et al.*, 2003), Thailand (Chukiatsiri *et al.*, 2010), Indonesia (Poernomo *et al.*, 2000) have been reported. Unfortunately, information regarding Infectious Coryza outbreaks in India has not been duly published. To understand the magnitude of this detrimental disease with serovar B as a causal factor, it is essential to establish serological involvement of *Avibacterium paragallinarum* in Indian context. The present study is devoted to in this direction.

Materials and Methods

Clinical sample collection

Clinical samples (nasal discharge /swab) of birds of different age groups showing typical symptoms of Infectious Coryza were brought to research laboratory by using Bragg's modified transport medium (Bragg *et al*., 2004). A total of 65 clinical samples were collected from different geographical locations of India during 2013 to 2015 and subjected to biochemical tests along with confirmation of PCR and serological characterization by a Page scheme.

Isolation of organism

The nasal swab samples were streaked on Blood Tryptose Agar (BTA) plates and these agar plates were cross streaked by *Staphylococcus aureus* (feeder culture) to observe satellitic phenomenon (Bragg *et al.*, 2002). The plates were incubated at 37^{o}C for 48 hrs in candle jar. After incubation, the plates were observed

for growth and satellitic phenomenon. The dew drop colonies with satellitic growth were used for further biochemical investigation. TN/SN medium was used to perform biochemical and other tests (Poernomo *et al*., 2000).

Staining, Biochemical and Serological Characterization

I) Gram staining

Gram staining was done to determine the shape and Gram's nature of isolates as described by Chauhan *et al.* (2007).

II) Sugar fermentation test

The sugar fermentation test was performed for six basic carbohydrates i.e. Galactose, Glucose, Mannitol, Sorbitol, Sucrose and Trehalose as per Blackall *et al.* (1997).

III) Other Biochemical tests

Along with sugar fermentation tests, catalase test, indole production, hydrogen sulphide production and nitrate reduction tests were also carried out. Tests were performed to check hemolysis and requirement of factor X and V (Akhtar *et al*., 2001).

IV) PCR test

For final confirmation, species specific PCR (HPG-2 PCR) was carried out as per protocol described by Chen *et al*. (1996). In this test, a pair of primers was used with sequence-

F1 (TGAGGGTAGTCTTGCACGCGAAT) and R1 (CAAGGTATCGATCGTCTCTCTACT).

V) Serotyping of isolates

The serological characterization was performed by using a Page scheme of serotyping in which Haemagglutination (HA) and Haemagglutination Inhibition (HI) tests were carried out as described by Blackall *et al.* (1990).

Results

Isolation Staining, Biochemical and Serological Characterization of organism

All seventeen isolates from suspected samples showed dew drop colonies with satellitic growth as shown in Figure 1. The isolates were further used to carry out Gram staining, sugar fermentation and other tests for biochemical properties. As presented in Table 1 all isolates were found as Gram negative bacilli. The isolates also demonstrated the ability to utilize four sugars i.e. Glucose, Mannitol, Sorbitol and Sucrose which were indicated acolour change from red to yellow whereas two sugars Galactose and Trehalose were not fermented by them. In catalase test, no bubble formation was observed, indicating catalase negative nature of the isolates. No purple colour formation indicated absence of oxidase activity.

Figure 1. Satellitic Growth of *Avibacterium paragallinarum* cross streaked with *Staphylococcus aureus*

Table 1. Biochemical Characterization of *Avibacterium paragallinarum*field isolates

Isolate Code	Gram Nature	Sugar Fermentation Test						Other Biochemical Properties							
		Galactose	Glucose	Mannitol	Sorbitol	Sucrose	Trehalose	Catalase	Indole Production	H2S Production	Nitrate Reduction	Hemolysis on BA	NAD Dependence	HPG2-PCR
1	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
2	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
3	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
4	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
5	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
6	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
7	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
8	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
9	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
10	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
11	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
12	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
13	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
14	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
15	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
16	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+
17	Negative Rods	-	+	+	+	+	-	-	-	-	+	-	+	+

The isolates further showed negative results for H_2S production and Indole test. In Nitrate reduction test, all isolates were able to convert nitrate to nitrite, which is demonstrated by formation of the deep red colour in broth after incubation.

PCR Test

In species specific PCR test (HPG2-PCR), all isolates were able to produce a 0.5kb DNA fragment upon amplification with given combination of N1and R1 primers (Figure 2). No other amplification was observed.

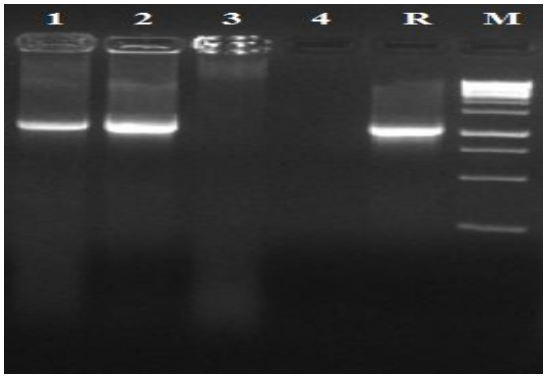

Figure 2. HPG-2 PCR confirmations of field isolates of *Avibacterium paragallinarum*
1 to 3: *Avibacterium paragallinarum* (Field isolates)
4: No Template Control (NTC)
R: Reference strain of *Avibacterium paragallinarum*
M: 100bp DNA Ladder

Serotyping of isolates

The overall serotyping results revealed that all three Page serovars i.e. A, B and C are present in India for Infectious Coryza (Figure 3).Out of 17 isolates, five were serovar A, two were serovar B and eight were serovar C whereas two field isolates were non-typable. The presence of *Avibacterium paragallinarum* was observed in all major poultry rearing states of India including Punjab, Haryana, Maharashtra, Andhra Pradesh, Tamil Nadu, etc.. All three serovars i.e. Serovar A, B and C were found in Punjab and Andhra Pradesh. Similarly two un-typable strains were also recovered from same states (Table 2) .

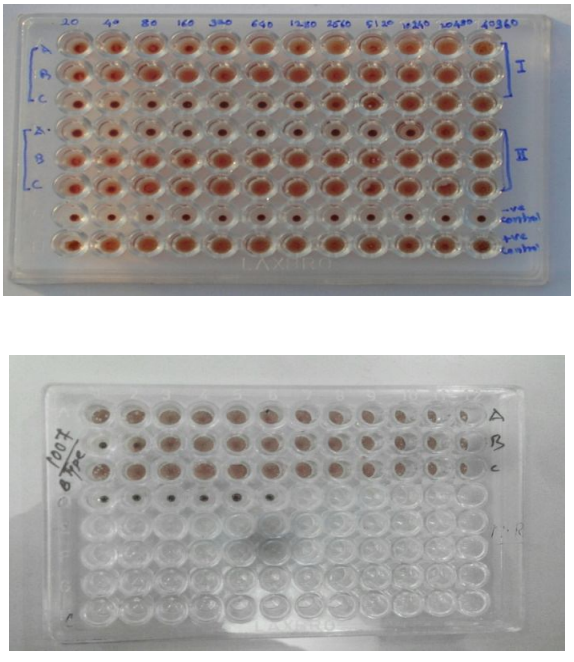

Figure 3. Page Serotyping scheme for *Avibacterium paragallinarum*

Table 2. Serological Distribution of *Avibacterium paragallinarum* field isolates

Field isolates	Bird type	Vaccinated(Yes/ No)	Region	Serotype
Isolate 1	Commercial Layers	Yes	Andhra Pradesh	A
Isolate 2	Commercial Layers	Yes	Andhra Pradesh	A
Isolate 3	Layer Breeders	Yes	Tamil Nadu	A
Isolate 4	Commercial Layers	No	Punjab	A
Isolate 5	Commercial Layers	No	Rajasthan	A
Isolate 6	Layer Breeders	Yes	Punjab	B
Isolate 7	Commercial Layers	Yes	Andhra Pradesh	B
Isolate 8	Commercial Layers	No	Andhra Pradesh	C
Isolate 9	Commercial Layers	Yes	Karnataka	C
Isolate 10	Commercial Layers	Yes	Haryana	C
Isolate 11	Commercial Layers	Yes	Punjab	C
Isolate 12	Commercial Broilers	No	Haryana	C
Isolate 13	Broiler Breeders	Yes	Haryana	C
Isolate 14	Commercial Layers	Yes	Maharashtra	C
Isolate 15	Commercial Layers	Yes	Maharashtra	C
Isolate 16	Commercial Layers	Yes	Andhra Pradesh	NT
Isolate 17	Commercial Layers	Yes	Punjab	NT

Discussion

The results of biochemical tests, HPG2- PCR for all isolates confirmed the presence of *Avibacterium paragallinarum* collected samples. The results of Gram's staining, Carbohydrate fermentation pattern and other biochemical tests matches with results reported by Rajurkar *et al.* (2010); Byarugaba *et al.* (2006) and Bragg *et al.* (2002).The isolates were able to utilize NAD as a growth factor unlike some of the African strains where these were NAD independent (Bragg *et al.*, 1997).

The present study represents the serological data of some of the major states of India where extensive poultry farming is carried out.

It is essential to note that out of 17 field isolates, 13 were collected from vaccinated birds and 4 were from non-vaccinated birds, but all 17 field isolates showed emergence of coryza disease indicating strongly presence of local variant strain(s) of *Avibacterium paragallinarum.* Similar types of results were reported by Zhang *et al.* (2003), Bragg *et al.* (2002), Poernomo *et al.* (2000) and Bragg *et al.* (1997). Along with this improper vaccine handling and vaccination technique may be some other reasons for vaccine failure. But possible local variants may play substantial role in vaccine failure, disease reemergence and increasing magnitude of the disease.

The serological study of *Avibacterium paragallinarum* field isolates shows that 2 out of 17 isolates are serovar B. In India for the first time we have identified presence of this serovar. The only previous characterization of Indian field isolates of *Avibacterium paragallinarum* reported presence of serovar A and C only (Tongaonkar *et al.,* 2003). It is essential to note that some workers have already indicated the presence of serovar B in Asian countries including China (Zhang *et al.,* 2003), Thailand (Chukiatsiri *et al.,* 2010), Indonesia (Poernomo *et al.,* 2000).

In the present investigation, we were able to collect 17 field isolates from seven states of India, out of which serovar C was 47% and serovar A was 27%, whereas serovar B was found to be only 11% and 2 strains were non-typable. This study further explains that there is no region sero- specificity in disease formation of Infectious Coryza.

Conclusion

Overall, this work has provided very important information regarding Indian field isolates of *Avibacterium paragallinarum* causing Infectious Coryza. This set of information also displays the emergence of possible local variants of *Avibacterium paragallinarum* which needs more attention and timely intervention for remedy. Our study indicated that along with serovar A and serovar C, there is presence of serovar B in India. This is the first confirmatory report of serovar B in Indian poultry isolates. This serovar is not only present in India, but possibly also a playing substantial role in vaccine failure and disease (Infectious coryza) reemergence in Indian poultry otherwise.

Conflict of Interest

The authors declare no competing financial interest.

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