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Research Article

Isolation and molecular serotyping of *Avibacterium paragallinarum* from desi birds

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Abstract

In this present study, a total of 43 clinical samples were collected from the desi birds of the southern Tamil Nadu region showing swelling in the infraorbital sinus and suspected for coryza infection. Samples were inoculated into the Chocolate agar and kept at 37°C for 48 hrs, of which 05 isolates showed the typical dewdrop colonies in Chocolate agar culture, were further confirmed by the biochemical test and HPG-2 PCR. Direct HPG-2 PCR was also performed in the 43 clinical samples, of which 17 samples were identified as *Avibacterium paragallinarum* by producing a specific amplicon of 500bp. HPG-2 PCR positive samples were further subjected for serotype identification by serotype-specific PCR targeting the *HMTp210* region and 03 samples were identified as serovar A by producing an amplicon of 0.8 kbp, and 14 samples were identified as serovar B by producing an amplicon of 1.1 kbp. But, none of the samples amplified the product for serovar C. This study concludes that the more prevalent circulating serovar in the southern Tamil Nadu region is serovar B, followed by serovar A. To the best of the author's knowledge, this is the second report stating the presence of serovar B in India.

Key words: HPG-2 PCR, Isolation, Serotype specific PCR

Highlights

- Collection of suspected samples from affected birds
- Isolation from Chocolate agar
- Molecular identification by species-specific PCR
- Identification of serotype by serotype-specific PCR

INTRODUCTION

Avian Infectious coryza is a highly contagious disease affecting the upper respiratory tract of poultry caused by *Avibacterium paragallinaum* (*Haemophilus paragallinarum*). The disease is manifested by nasal discharge, swelling of the infraorbital sinuses with conjunctivitis, decreased feed and water consumption, diarrhoea, retarded growth and loss in egg production (10-40%) (Eaves *et al*., 1989; Calnek *et al*., 1991). Coryza is considered as a severe disease, especially in developing countries like India, because it is mostly complicated with other upper respiratory tract infections (Prabhakar *et al*., 1998; Sobti *et al*., 2001) like fowl cholera, fowl pox, chronic respiratory disease (CRD), and *Pseudomonas aeruginosa* infection (Giurov, 1984). *A. paragallinarum* is a Gram negative, polar staining, non-motile bacterium that appears as short rods or coccobacilli and is dependent on NAD (factor V) for their *in vitro* growth. The organism undergoes degeneration within 48-60 hrs, showing fragments and indefinite forms (Schalm and Beach, 1936; Yamamoto, 1991). According to the Page scheme, *A. paragallinarum* is classified into three serovars, A, B and C (Page, 1962). These serovars have been obtained from chickens worldwide, although serovar distribution differs from country to country (Blackall and Soriano-Vargas, 2013). In India, Tongaonkar *et al.* (2003) have confirmed the presence of Page serovar A and C, and recently Patil *et al*. (2016) confirmed the presence of serovar B.

As conventional techniques are considered

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as gold standard method, but *Avibaceterium paragallinarum* isolation and storage of cultures could be quite difficult because of the fragility and easy degenerating properties of the organism. Unfortunately, the outbreaks are more common in south Tamil Nadu, but there are no documented reports on the current status, isolation and identification of prevalent serotypes circulating in the southern Tamil Nadu (India) region. Hence, the objective of the present study is to attempt for isolating *A. paragallinarum* from suspected clinical samples and characterize them by HPG-2 PCR and serotype-specific PCR to make efficient steps to control the disease.

MATERIALS AND METHODS

Sample collection: Samples were collected from Kanyakumari, Tirunelveli and Thoothukudi districts of Tamil Nadu, India. In this investigation, 43 samples were collected from the desi birds suspected of coryza infection from the private poultry farms (Fig. 1). The samples include aspirates or cheesy material from the infraorbital sinus and mucous swabs from the nostrils.

Isolation and identification: A loop full of sinus exudates or the cheesy material from the infraorbital sinus was streaked directly in the Chocolate agar which supplies the V factor necessary for the growth of *A. paragallinarum* and incubated at 37°C for 48 hrs in a candle jar. A single dewdrop colony was obtained on a Chocolate agar after 48 hrs incubation. Pure colonies obtained from Chocolate agar were stained by Gram's staining method (Quinn *et al*., 1994)

HPG-2 PCR: Genomic DNA was extracted from the positive culture. Direct PCR of the clinical samples was also done using Dneasy blood and tissue kit (Qiagen, Germany). The ampliûcation primers for single tube PCR were based on the previously described sequence (Chen *et al.*, 1996). The target DNA used for ampliûcation was a 500 bp fragment of HPG-2, used to identify species-specific *A. paragallinarum.* The reaction was subjected to initial denaturation at 95°C for 5 mins followed by 25 cycles of ampliûcation consisting of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 10 min. The primers used were N1 5′ TGA GGG TAG TCT TGC ACG CGA AT 3′ and R1 5′ CAA GGT ATC GAT CGT CTC TCT ACT 3′.

PCR amplification was carried out in 25 µL reaction mixtures containing 1 µL of DNA template, 10 pM of 1 µL each of forward and reverse primer, 12.5 µL of 2X green dye PCR master mix (Takara) and 9.5 µL of nuclease-freewater. The ûnal PCR ampliûed product was separated by 1.5% agarose gel electrophoresis and gel documented.

Serotype specific mnultiplex PCR: Single tube multiplex PCR which is serotype-specific PCR targeting the *HMTp210* region to identify the serotypes A, B or C. The samples which are positive by HPG-2 PCR were further subjected to serotype-specific PCR. The primer sequence and performance were reported previously by Sakamoto *et al*. (2012). PCR was done by using primer sets, presented in Table 1. Each sample was run using all three sets of the primers that

Table 1. Primer sequence used in serotype-specific PCR

Primer set	Primer	Fragment length	Primer sequence
1.	ABC Forward	0.8 kbp	5′ -GGCTCACAGCTTTATGCAACGAA-3′
	A reverse		5′ -CGCGGGATTGTTGATTTTGTT-3′
2.	ABC Forward	1.1 kbp	5′ -GGCTCACAGCTTTATGCAACGAA-3′
	B reverse		5′ -GGTGAATTTCACCACACCAC-3′
3.	ABC Forward	1.6 kbp	5′ -GGCTCACAGCTTTATGCAACGAA-3′
	C reverse		5′ -TAATTTTCTTATTCCCAGCATCAATACCAT-3′

is ABC/A, ABC/B, and ABC/C. The reaction cycles were as follows: 98°C for 1 min; 30 cycles of 98°C for 10 sec, 56°C for 10 sec and 72°C for 2 min; and final step at 72°C for 7 min. PCR amplification was carried out in 25 µL reaction mixtures containing 1 µL of DNA template, 10 pM of 1 µL each of forward and reverse primer, 12.5 µL of 2X green dye PCR master mix (Takara) and 5.5 µL of nuclease-free water. The final PCR amplified product was separated by 1.5% agarose gel electrophoresis and gel documented.

RESULTS

Isolation and molecular identification by HPG-2 PCR: Out of 43 clinical samples processed, only 05 isolates showed typical small, translucent, tiny dewdrop colonies on Chocolate agar after 48 hrs of incubation (Fig. 2) and on Gram's staining revealed as short rods or coccobacilli forms. Five culture positive isolates were further confirmed by HPG-2 PCR. On the other hand, all the clinical samples (n=43), i.e., cheesy material and the exudates from the infraorbital sinus, were subjected to direct HPG-2PCR, which amplified in 17 samples indicative of *A. paragallinarum* (Fig. 3).

Serotype specific Multiplex PCR: The positive samples (n=17) were further subjected to serotype-specific PCR, in that 03 samples amplified product size of 0.8 kbp indicative of serovar A and 14 samples amplified product size of 1.1 kbp indicative of serovar B (Fig. 4). None of the samples amplified the product for serovar C.

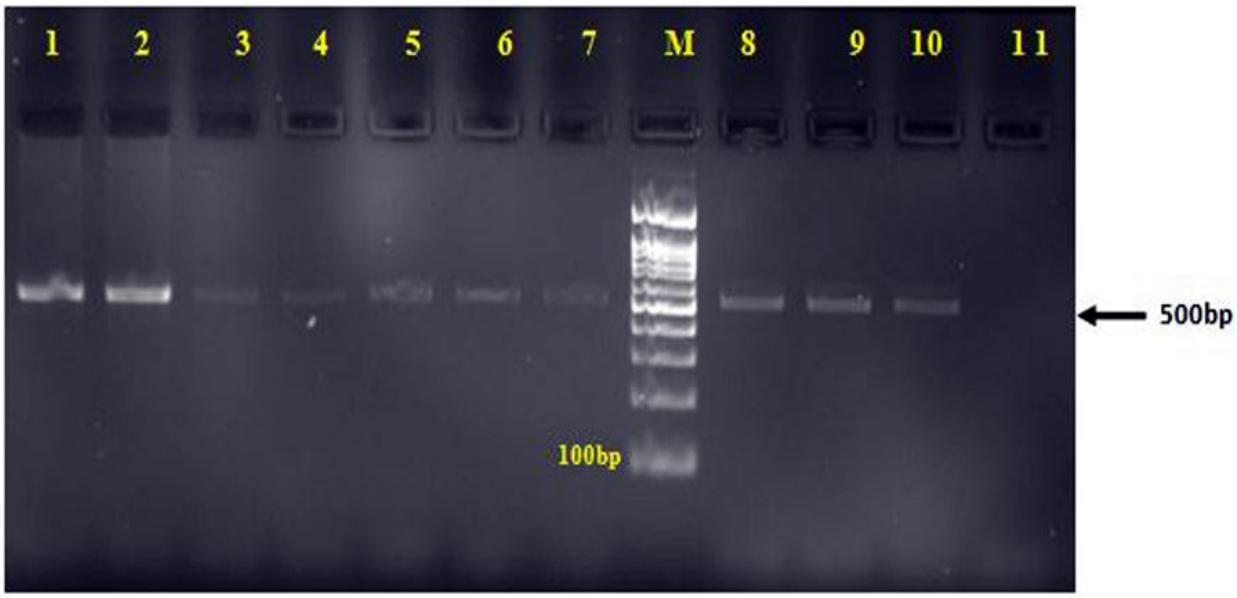
DISCUSSION

Poultry farming in large scale commercial farms and backyard farms is one of the rapidly growing industries in India, especially in Tamil Nadu. The occurrence of diseases in poultry causes economic losses to farmers due to high mortality, loss of egg production and retarded growth rate. Among the diseases in poultry, respiratory diseases contribute maximum, and the infection is complicated due to mixed nature of the infection with *Mycoplasma gallisepticum*, *M. synoviae*, *Pasteurella* spp., *Salmonella* spp. and infectious bronchitis virus. So, a detailed diagnostic procedure may be required to accurately diagnose the disease to identify the exact cause of illness.

In the present study, 43 clinical samples were collected from different poultry farms in various districts of southern Tamil Nadu; from that 05 could be isolated from the clinical samples. It could be because the organism is very fragile and will not retain its viability in refrigeration (Quinn *et al*., 2011). If the organism has a slower growth rate which takes 48 hrs, the other contaminating (Badouei *et al*., 2014) organisms of the upper respiratory tract will predominate, making the isolation difficult (Chen *et al.*, 1998). Growth of organism on Chocolate agar containing NAD produces small, translucent dewdrop like colonies and on Gram's staining revealed pink, gram-negative rods and coccobacilli forms. The growth characteristic pattern obtained in this study was similar to the findings of Keslerk (1997), Quinn *et al*. (2011), Priya *et al*. (2012) and Akter *et al*. (2014).

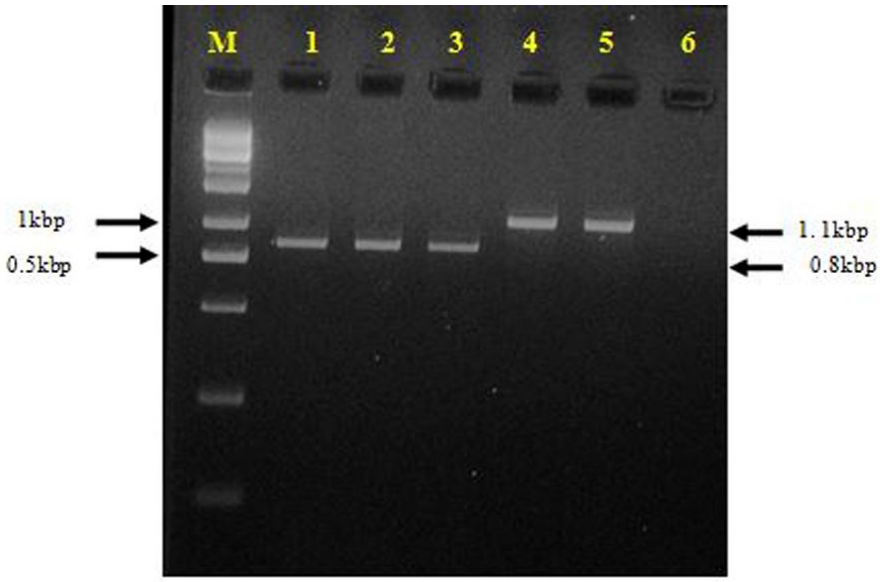
HPG-2 PCR tests appear to be useful diagnostic tools for detecting infectious coryza and its specific species. The HPG-2 PCR test seems to be a potential alternative to culture and is strengthened by the application of HPG-2 PCR even after 60 days of storage at -20°C as the traditional culture methods failed to detect the isolates even after 48 hr of storage (Chen *et al.,* 1998). The PCR results of the isolates are in accordance with the findings of Chen *et al*. (1998), Anjaneya *et al*. (2014), and Patil *et al*. (2017). In this study, 17 samples were positive by direct PCR performed from the clinical samples; whereas only 05 isolates were obtained by culture. Badouei *et al.* (2014) also reported that Direct PCR from clinical samples is a more sensitive approach than culture PCR.

In the present study, from 17 positive samples of *Avibacterium paragallinarum*, only 03 samples belong to serovar A and 14 samples belong to serovar B. In India, a previous report stated that the prevalent serotype is A and C only (Tongaonkar *et al*., 2003). But recently,



[M- 100bp DNA ladder, Lane 1 – Positive control, Lane 2 to 10 – Positive samples, Lane 11 – Negative control]

Fig. 3. Species-specific HPG-2 PCR



[M – 1kbladder, Lane 1 to 3 – Serotype A, Lane 4, 5 – Serotype B, Lane 6 – Negative control]

Fig. 4. Serotype specific PCR

in India, studies reported that serovar B is prevalent as 11% (Patil *et al*., 2016; Patil *et al*., 2017). None of the samples revealed serovar C. This study concludes that the more prevalent serovar in the southern Tamil Nadu region is serovar B and serovar A.

In such cases, *A. paragallinarum* was confirmed for its identity using species-specific HPG-2 direct PCR from clinical samples to make quick preventive measures.This is the second confirmatory report of serovar B in Indian and also found that serovar A and B are commonly circulating strains in the southern Tamil Nadu region. Further, vaccines available against the

prevalent serovars are not much protective due to the emergence of local variant strains making the disease more risky and causing frequent outbreaks (Deshmukh *et al*., 2015; Patil *et al.,* 2016). The serovar (i.e. B), not only present in India but also prevalent in nearby countries like China, and Thailand, could play a role in outbreaks. The re-emergence of disease makes the vaccination challenge.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: All the authors contributed equally to sample collection in different areas from affected birds and collected research articles and information for manuscript preparation.

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