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A report of natural concurrent infection with *Avibacterium paragallinarum* and *Mycoplasma gallisepticum* in chicken

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Abstract
Infectious diseases form one of the major impediments to the growth of poultry industry in India. Among the infectious diseases, Infectious coryza (IC) and mycoplasmosis play a significant role causing high economic losses. A concurrent infection of *Avibacterium paragallinarum* and *Mycoplasma gallisepticum* in chicken is described in this paper. The bird was presented with a history of whitish diarrhoea, respiratory distress and weight loss. A multiplex polymerase chain reaction (m PCR) was used for the detection of important poultry pathogens and positive results were obtained for IC and mycoplasmosis. This study emphasis the usefulness of m PCR in the rapid detection of various poultry pathogens and also ascertain the need for investigating the presence of mixed infection in poultry.

Keywords: Avibacterium paragallinarum, *Mycoplasma gallisepticum*, multiplex PCR, poultry

1. Introduction
Indian poultry industry has taken a quantum leap with the conversion of conventional unscientific farming practices to more scientific commercial farming systems [1]. However, one of the major impediments to the growth of this industry is the mortality and morbidity associated with infectious diseases. Among various diseases, significant one is poultry respiratory disease complex, which is the name designated for a group of respiratory diseases producing similar symptoms [2]. One of the major challenges faced by poultry industry is the control of mixed infection with multiple pathogens. The present case report deals with the occurrence of concurrent infection of infectious coryza (IC) along with mycoplasmosis in a flock of chicken.

Infectious coryza is an economically important disease caused by the bacteria *Avibacterium paragallinarum*, which is a member of the family *Pasteurellaceae* and is cosmopolitan in occurrence. The economic losses caused by this disease are mainly due to reduced egg production, increased culling rate and poor growth performance [3]. The disease remains endemic due to the fact that chronically infected birds act as carriers [4]. Though the first isolation of this organism in India was reported in 1958[5], the second isolation was reported only after 11 years [6] which emphasis the difficulty in isolating the agent. The molecular techniques play an important role in detection of this organism [7]. The concurrent infections aggravate the losses due to IC.

Chronic respiratory disease (CRD) is considered as another economically significant disease of poultry, caused by several pathogens. Among them, *Mycoplasma gallisepticum* is the most important one in chickens [8]. The disease in chicken is characterised by coryza, conjunctivitis, respiratory rales and sneezing. The isolation of *Mycoplasma* is very tedious and time consuming due to its highly fastidious nature and molecular techniques helps in early identification of this bacteria [9]. This report deals with the rapid molecular detection of the concurrent infection of IC and mycoplasmosis.

2. Materials and methods
2.1. Samples
A two month old ailing chicken with the history of whitish diarrhoea and respiratory distress was presented to the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy for disease diagnosis. Representative samples collected aseptically following necropsy, from lungs, spleen, liver, intestine and heart blood formed the materials for investigation.

2.2. Isolation of bacterial organism and antibiotic sensitivity test

Representative samples of liver, lung, heart blood, spleen, and intestine were cultured onto 7% ox blood agar and incubated aerobically and anaerobically at 37°C for 48 h. The intestine and liver enriched in selenite broth was cultured on MacConkey's agar. Antibiotic sensitivity test of the isolates obtained were done as per the standard single disc diffusion method [10] employing Mueller Hinton's agar. Antibiotic discs of known concentrations as depicted in paranthesis in micrograms (mcg) or International units/disc were employed for this study. The following discs were used: Colistin (10mcg), Co-trimoxazole (25 mcg), Cefalexin (30mcg), Tetracycline (30mcg), Ceftriaxone-Tazobactam (10 mcg) and Enrofloxacin (10 mcg).

2.3. Extraction of nucleic acids and PCR

The DNA was extracted from the pooled tissue samples using commercial kit (Hi Pur A Multisample DNA purification kit, Himedia, MB554-50PR) following manufacturer's instructions. The extracted DNA was quantified using Nanodrop (Thermofisher, USA).

The viral RNA for detection of Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) was extracted from 250 µl supernatant of the processed tissue samples as per standard protocol of Trizol method of RNA extraction. The subsequent reverse transcription was done for synthesizing cDNA using Verso cDNA synthesis kit (Thermo Scientific, USA) following the manufacturer's instructions. The reaction mix prepared was incubated for an initial incubation at 25ºC for five minutes followed by 42°C for one hour followed by 70°C for five minutes. The resultant cDNA was used as template for m PCR.

The extracted nucleic acids were subjected to an *in-house* developed multiplex PCR targeting Infectious Bronchitis (IB), Mycoplasmosis, Infectious Coryza (IC), Infectious Laryngotracheitis (ILT) and New castle Disease (ND) which are the major pathogens causing avian respiratory diseases. The PCR reactions were performed with 100 ng of DNA sample, in a volume of 25µl using Emerald Amp PCR mastermix (Takara, Japan). DNA extracted from field samples that had been confirmed for IB, Mycoplasmosis, IC and ILT and commercial vaccine for ND were used as positive control. The DNA samples were subjected to 35 cycles of denaturation at 94ºC for 45sec, annealing at 55.3ºC for 60sec and extension at 72ºC for 60sec. The amplified products were subjected to agarose gel electrophoresis using 1% agarose stained by Ethidium bromide and visualised in gel documentation system.

3. Results

3.1. History, Clinical Signs and Gross lesions

As per the owner's report, about thirty birds from a flock of 150 were showing signs of severe respiratory distress, emaciation and whitish diarrhoea with high mortality rate. This clinical signs were observed in chickens of age group two to six months. On necropsy, the carcass was found to be emaciated. No gross lesions could be detected except bilateral congestion of lungs.

3.2. Isolation and Antibiogram

Culture of liver, lung, heart blood, spleen, and intestine on 7% ox blood agar did not yield any significant bacterial growth after 48 hrs of incubation, whereas intestine and liver on MacConkey's agar yielded bright pink lactose fermenting colonies in pure form after incubation at 37 ºC for 24 h. On Gram's staining, the isolate revealed Gram negative medium sized bacilli that were positive for catalase and negative for oxidase tests. Antibiotic susceptibility pattern using six different antibiotics were carried out. All the isolates were sensitive to colistin, co-trimoxazole and cefalexin and resistant to tetracycline, ceftriaxone-tazobactam and enrofloxacin. The result was communicated to the owner for further action.

3.2 Polymerase Chain Reaction

On multiplex PCR, amplified products corresponding to 500 bp and 187 bp were documented for IC and *Mycoplasma gallisepticum* respectively (Figure 1), in sample and positive control. No amplicons were obtained for IB, ND and ILT samples and negative control.

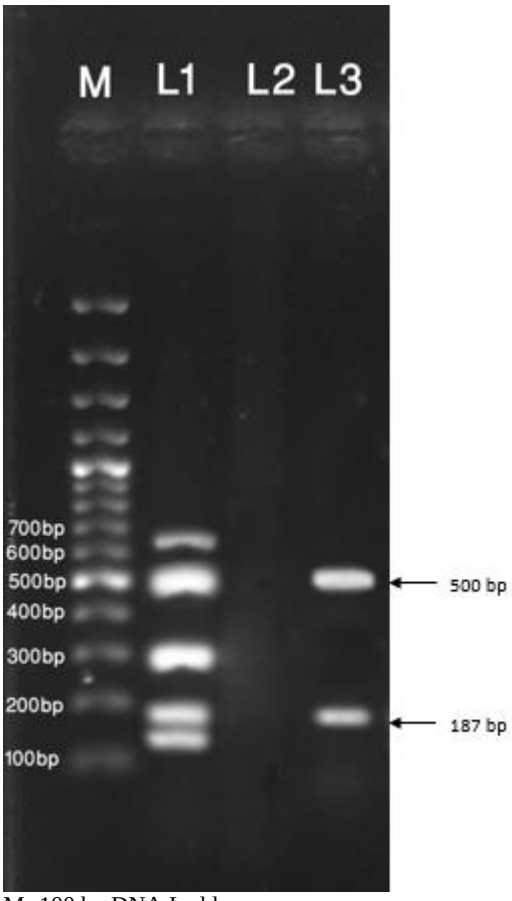
4. Discussion

Avibacterium paragallinarum and *Mycoplasma gallisepticum* are the predominant bacterial pathogens causing respiratory diseases in poultry with a high impact on the economy. *Avibacterium paragallinarum* causes infectious coryza in poultry which is manifested as respiratory distress and frothy oculo-nasal discharge whereas, *Mycoplasma gallisepticum* causes a wide variety of clinical signs ranging from loss of production to respiratory distress [11].

Mixed infections form one of the major challenges in poultry industry. Concurrent infection of mycoplasmosis with various other pathogens are common [2, 12] Reports of synergism of mycoplasma with other other bacteria and viruses shows its importance in poultry production [12]. Similarly, co-infection of *Avibacterium paragallinarum* with other infectious agents has been reported from different parts of the world [13, 14].

Though, the co-infection of different agents causing respiratory syndrome is common in poultry, the diagnosis of mixed infection remains a challenge as it demands the use of multiple methodologies. The diagnosis of the organism is usually complicated by many factors. Fastidious nature of *Mycoplasma* makes the isolation tedious with the requirement of special techniques [11], whereas, the requirement of V factor or NAD and slow growing nature of *A. paragallinarum* complicates its diagnosis by isolation and biochemical characterisation [2]. Reports of NAD in dependant strains [15] and presence of opportunistic pathogens further complicates the diagnosis of IC [16]. Hence, molecular techniques like PCR form an easy, reliable and sensitive technique for the detection of *A. paragallinarum* and *M. gallisepticum* [2].

Fresh samples collected asceptically after sacrificing the ailing birds in acute stages of infection and before the commencement of antibiotic therapy increases the accuracy of PCR detection [17, 18]. In the present investigation, PCR in a multiplex format could accurately detect the multiple pathogens of IC and mycoplasmosis rapidly. During the investigations of diseases affecting poultry, before proceeding, it should be bear in mind the possibility of multiple pathogens involved and multiplex PCR could offer an effective platform for the sensitive, rapid and accurate detection of the pathogens simultaneously thereby, effective strategies can be employed to combat the diseases.



M- 100 bp DNA Ladder
L1- Positive Control
L2- Negative Control
L3- Sample

Fig 1: Multiplex PCR for avian respiratory complex

5. Conclusion

In the present study, concurrent infection with *A. paragallinarum* and *M. Gallise pticum* is described. As the isolation and biochemical characterisation of both the organisms are laborious, time consuming and require special techniques, molecular methods are used for rapid detection of the organisms in this study. The study emphasise the importance of ruling out the occurrence of concurrent infections during poultry disease investigation. The study also suggest m PCR assay as an accurate and easy method for the diagnosis of respiratory disease complex in poultry.

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