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Co-existence of fowl cholera and lymphoid leucosis in Desi chickens

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Abstract
Sudden death of birds were reported in a poultry farm located at Chengalpattu district in Tamil Nadu, India. Flock strength of the farm was 372 Aseel cross birds in which 79% mortality and 87% morbidity was reported over a week period of time. Disease investigation was started with post moterm examination of dead birds revealed hepatomegaly with necrotic foci along with swollen foot. Bacterial isolation and PCR confirmed the *Pasturella multocida* infection in this flock. The flock was also found to be positive for Avian lymphoid leucosis by PCR. Further, PCR was carried out to identify the subgroups of ALV (A, B, C, D and E) which has revealed that the samples were positive for ALV-E which is endogenous subgroup and negative for other exogenous subgroups. This study reports the co-infection of Fowl cholera and Avian lymphoid leucosis subgroup E in chickens.

Keywords: Co-infection, fowl cholera, lymphoid leucosis, molecular diagnosis

1. Introduction
Fowl cholera is a contagious disease of domestic fowls and many other wild birds, caused by a bacterium, *Pasteurella multocida* which is of major economic importance worldwide. High morbidity and mortality can occur in the acute form of infection (Glisson *et al*., 2008)[7] creating a greater economic loss on a farm but chronic and asymptomatic infections were also reported. In acute fowl cholera, finding a huge number of dead birds without previous signs is usually the first indicator of the disease. Chronic form is characterized by localized infection of the joints, swollen wattles and eyelids with respiratory gurgles (rales). Birds that recover continue to be disease carriers, carry the infection for the rest of their lives and can serve as a reservoir for future outbreaks (Sathish *et al*., 2015) [15]. Avian lymphoid leucosis (ALL) is a lymphoproliferative disease of poultry species (Sathish *et al*., 2015) [15] associated with a reduction in the quality and quantity of egg production creating a greater economic impact. The natural host for the Avian lymphoid leucosis virus (ALV) is chickens. It was also reported in ostriches (Garcia-Fernandez *et al*., 2000) [6], green peafowls (Khordadmehr *et al*., 2017) [8], and budgerigar (Nouri *et al*., 2011) [10]. ALV belongs to the alpha retrovirus genus of the family Retroviridae and are divided into subgroups based on their viral envelope antigens. Subgroups A, B, C, D, and J are classified as exogenous viruses and E is endogenous. Though there are no specific clinical signs observed in the affected birds, nodular tumours can be found in the internal organs upon post moterm examination. In this communication, we report co-infection of Fowl cholera with Avian lymphoid leukosis virus in Aseel cross chickens.

2. Materials and Methods
2.1 Study area and Farm description: A poultry farm located in Chengalpattu district in Tamil Nadu, India with a flock strength of 372 Aseel cross birds (200 numbers of 2-8 weeks old chicks and 172 numbers of 13-17 weeks of growers) was reported with 79% mortality and 87% of morbidity over a week period of time in November 2022. Affected birds were dull, depressed and off feed. Litter material in the affected shed was wet and caked up. Necropsy examination of dead birds revealed hepatomegaly with necrotic foci (Fig. 1) and swollen joints and foot (Fig. 2).

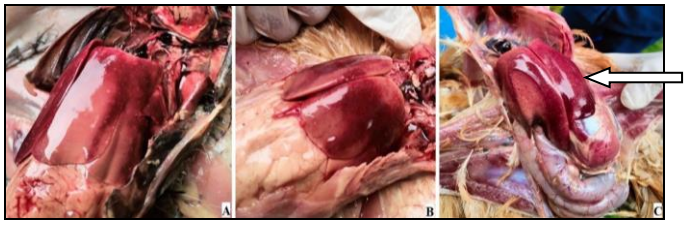


Fig 1: Liver of affected birds showing the multiple necrotic foci with hepatomegaly

Fig 2: Swollen foot of the affected bird

2.2 Collection of samples

Samples such as liver, lung, heart, long bones, heart blood swabs and impression smears of liver and spleen were also collected to rule out Fowl cholera. Further, organs like liver, spleen, lung, kidney, brain, proventriculus, sciatic nerve and bursa were collected to rule out the viral diseases such as Avian lymphoid leukosis and Marek's disease (MD).

2.3 Bacterial etiology

2.3.1 Culture, isolation and staining of bacteria

The liver, lung and spleen impression smears were stained using Leishman stain. The suspected samples were inoculated in Blood agar, McConkey agar and Brain Heart Infusion agar for screening of the bacterial organism. *Pasteurella multocida* (*P. multocida*.) organisms were cultured according to the standard method described by Cowan *et al*. (1965) [5]. Based on the colony characteristics, subsequent selective subculture was done to obtain pure culture of *P. multocida*. The isolated pure culture was subjected to Gram staining and biochemical tests (Catalase, Oxidase, Indole, Urease, Citrate and Nitrate reduction) for further confirmation.

2.3.2 Antibiotic sensitivity test

The Kirby /Bauer disc diffusion method was followed for antibiotic sensitivity of the sample using a total of 6 different antibiotic discs (Oxytetracycline, Sulphadimidine, Chloramphenicol, Gentamicin, Ciprofloxacin and Cefotaxime) commonly used in field condition (Bauer *et al*., 1966) [2].

2.3.3 PCR for Fowl cholera

DNA was isolated from the culture using boiling method mentioned by Queipo-Ortun *et al*. (2008) [12] with slight modification. A loopful of bacteria was picked up from the culture plate by swiping and mixed in 200 µL of deionized water. The mixture was then heated in boiling water for 10 min followed by dipping into ice for 10 min and centrifugation was done at 13,000 rpm for 10 min. The supernatant was collected and used in PCR. PCR was performed targeting the KMT1 gene using primers KMT1T7 and KMT1SP6 (Table 1) (Townsend *et al*., 2001)[20].

Table 1: Primers used to rule out Fowl cholera

S. No	Name of the disease	Gene target	Primer sequence	Product size
1	*Pasteurella multocida*	KMT1T7	F –ATCCGCATTTACCAGTGG,	*460bp*
		KMT1SP6	R-GCTGTAAACGAACTCGCCAC	

PCR amplification was carried out under the following conditions: 3 min at 94℃ for initial denaturation, followed by 37 cycles of 30 s at 94 ℃, 30 s at 50 ℃, and 45 s at 72 ℃, for denaturation, primer annealing, and extension respectively. The final extension was carried out at 72 ℃ for 10 min. Products of the amplification reaction were separated electrophoretically using 1.5% agarose gel. Samples that returned an amplicon of 460 bp using KMT1T7 and KMT1SP6 primer for all serogroup (A, B, D, E, F) were considered positive for Fowl cholera.

2.4 Viral Etiology

2.4.1 PCR for Avian lymphoid leucosis and Marek's disease

The DNA was extracted from 10% tissue homogenate of liver, spleen, lung, kidney, brain, proventriculus, sciatic nerve and bursa by Phenol chloroform method of extraction. PCR was carried out from the extracted DNA to rule out ALV and MD.

2.4.1.1 PCR for Avian lymphoid leucosis virus

The H5, AD1 and H7 oligonucleotide primers used in the study (Table 2) were derived from the published sequences (Smith *et al*., 1998) [18]. Primer H5 was created to target the 3' region of the pol gene and was found to be conserved in multiple ALV subgroups. A highly conserved section of the variant viruses gp85 sequence served as the basis for the creation of primer H7. Meanwhile AD1 sequence was derived from a consensus sequence of RAV-1 sequence. Primers H5 and AD1 were used for the detection of subgroups A-E, whereas, H5 and H7 primers were used for the detection of subgroup J of ALV. Samples that returned an amplicon of 295-326 bp using H5 and AD1 primer for subgroup A-E and 545 bp using H5 and H7 primer for subgroup J were considered positive for Avian lymphoid leucosis virus. PCR amplification was carried out under the following conditions: 3 min at 94 ℃ for initial denaturation, followed by 37 cycles of 30 s at 94 ℃, 30 s at 50 ℃, and 45 s at 72 ℃ for denaturation, primer annealing, and extension respectively. The final extension was carried out at 72 ℃ for 10 min. Products of the amplification reaction were separated electrophoretic ally using 1.5% agarose gel (Smith *et al*., 1998) [18].

Further ALV subgroup specific PCR was also carried out individually for ALV-A, B/D, C and E as per the protocol described by Silva *et al*. (2007) [17]. The details of primers used are given in Table 2.

Table 2: Primers used in this study to screen Avian lymphoid leucosis

Primer	Sequence(5'-3')	Product size	Reference
A to E of ALV	H5: GGATGAGGTGACTAAGAAAG AD1: GGGAGGTGGCTGACTGTGT	295-326 bp	Smith *et al*., 1998 [18]
J of ALV	H5: GGATGAGGTGACTAAGAAAG H7:CGAACCAAAGGTAACACACG	545 bp	Smith *et al*., 1998 [18]
ALV-A	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'CCCATTTGCCTCCTCTCCTTGTA3'	1.3kbp	Silva *et al*., 2007 [17]
ALV-B and D	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'AGCCGGACTATCGTATGGGGTAA3'	1.1 Kbp	Silva *et al*., 2007 [17]
ALV –C	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'CCCATATACCTCCTTTTCCTCTG3'	1.5kbp	Silva *et al*., 2007 [17]
ALV –E	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'GGCCCCACCCGTAGACACCACTT3'	1.25kbp	Silva *et al*., 2007 [17]

2.4.1.2 PCR for Mareks Disease

PCR was also carried out to rule out the presence of MD in this flock using MDV1 specific meq gene based primers as per Chang *et al*. (2002) [4].

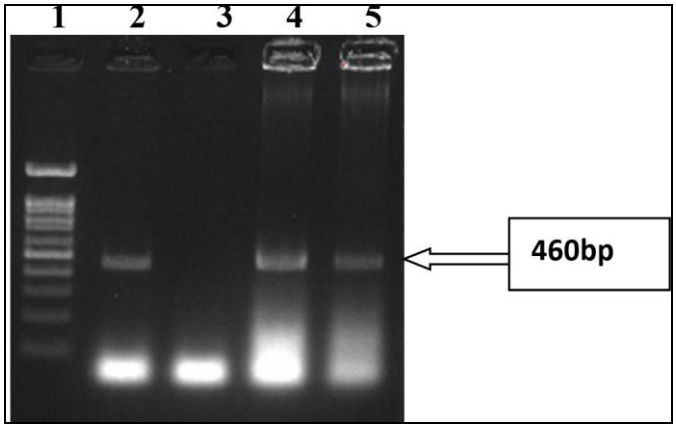
3. Results and Discussion

The infectious bacterial disease known as "Fowl cholera" affects both domesticated and wild bird species. It is also referred to as "avian cholera," "avian pasteurellosis," and "avian hemorrhagic septicemia." When the disease strikes older birds, it usually manifests as a fulminating disease with severe bacteraemia and major morbidity and mortality rates. Mortality rates of 5%–20% can occur in the early stages of disease. Mortality may even reach much higher rates upto 100% (Pilatti *et al*., 2016) [11]. The present study also reports 79% mortality and 87% morbidity over a week period of time. Further post moterm findings of dead birds revealed hepatomalagy with necrotic foci along with swollen foot which were suggestive of Fowl cholera.

Laboratory confirmation by staining the impression smears also revealed bipolar organisms. The isolated pure culture was non motile and subjected to gram staining for morphological identification of the bacteria which were found to be gram negative coccobacilli. On bacterial isolation in BHI agar, whitish, opaque, circular, translucent colonies could be observed. Whitish, opaque, circular, translucent, non hemolytic, mucoid colonies could be observed in blood agar. There was no growth observed in MacConkey agar. The isolated bacteria were found to be positive for catalase and oxidase enzymes and produced indole. They were urease and citrate negative and positive for nitrate reduction. These results were in accordance with the characters described by Quinn *et al*. (1994) [13], Balakrishnan and Parimal Roy. (2012) [1].

Chemotherapy is widely used in the treatment of Fowl cholera. Variety of chemotherapeutic agents is often effective. Because of the variation in the responsiveness of *Pasteurella multocida* to this agents *in vitro* sensitivity testing is recommended. Antibiotic sensitivity pattern of isolated *Pasteurella multocida* in the present study showed resistance to oxytetracycline and sulphonamides. Whereas sensitive to gentamicin and chloramphenicol. Ciprofloxacin and Cefotaxim were found to exhibit intermediate sensitivity. Balakrishnan and Parimal Roy. (2012) [1] recorded 100 percentage sensitivity to gentamicin. Sensitive to gentamicin also recorded by Bhattacharya. (2005) [3] and Shivachandra *et al*. (2004) [16]. Hence, the present study recommends to perform ABST prior to treatment in cases of Fowl cholera. In situations, where performing ABST is not feasible then gentamicin could be given preferential consideration over other antibiotics.

Further, PCR using KMT1T7 F - ATCCGCATTTACCAGTGG, KMT1SP6 R- GCTGTAAACGAACTCGCCAC primers confirmed *Pasteurella multocida* infection in this flock with an amplicon of 460bp (Fig 3). This is in agreement with the findings of Townsend *et al*. (1998) [20]. Therefore the outbreak of Fowl cholera in the flock was confirmed.



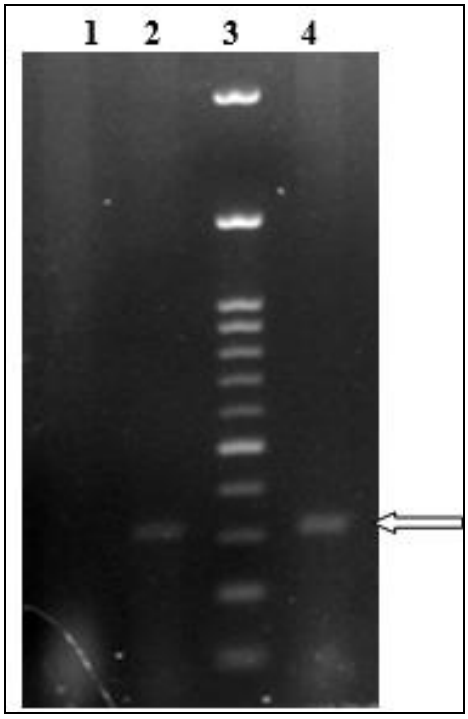
Lane 1- DNA ladder 1kb, Lane 2 - Positive control, Lane 3 - Negative control, Lane 4 and 5 - Positive samples of affected birds

Fig 3: PCR results for Fowl cholera showing 460bp amplicon

Poor nutrition, environmental temperature, poor litter material, concurrent viral infections may induce immunosuppression and serves to be a predisposing factor for causing Fowl cholera (Rhoades *et al*., 1991) [14]. Here the wet and caked-up litter observed in the affected shed could served to be a major factor in transmitting the bacterial disease as reported by Turner *et al*. (2008) [21].

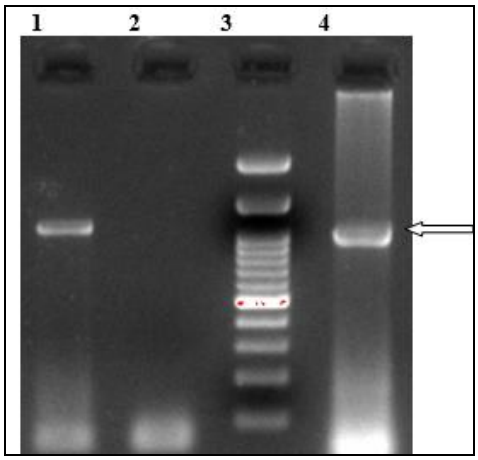
Hepatomegaly without tumour is also seen in Avian lymphoid leucosis in chickens as per the findings of Soujanya *et al*., 2019 [19]. Hence, this flock was also screened for ALV along with MD to rule out other possible viral etiologies. The results of screening for MD using meq gene did not show the expected amplicon, this confirms that the flock was negative for MD.

Screening for ALV J subgroup using primer H5 and H7 primer did not show any specific amplification, whereas A-E subgroup-specific primer H5 and AD1 had shown a positive amplicon of 295-326 bp (Fig 4). The obtained results confirmed that the given flock had co-infection of ALV with Fowl cholera. Further, PCR was carried out to know the subgroup of ALV. The results showed that the flock was positive for Subgroup E of ALV by producing an amplicon of 1.25 kbp (Fig 5). Commonly, birds around 4 months of the group will be affected by ALV (Nair *et al*., 2022) [9].



Lane 1-Negative control, Lane 2-Positive control, Lane 3- DNA ladder 1kb,
Lane 4 - Positive sample of affected bird

Fig 4: PCR results for avian lymphoid leucosis using H5 and AD1 primer showing an amplicon of 295-326 bp



Lane 1- Positive control, Lane 2- Negative control, Lane 3- DNA ladder 1kb,
Lane 4 - Positive sample of affected bird

Fig 5: PCR results for Avian lymphoid leucosis using subgroup E primer showing an amplicon of 1.25 Kbp

In contrast to the existing literature, the chicks (2-8 weeks) and growers (13-17 weeks) of this flock were positive for ALV by PCR in the present study. However, it was identified as subgroup E of ALV which is purely an endogenous origin and the proviral DNA could have been vertically transmitted from parent stock than mere infection. The results depicts that the PCR has identified the integrated genome of ALV-E which are generally non-oncogenic and alter the response of the birds to exogenous ALV infection by inducing immunity or enhancing tolerance.

4. Conclusion

Disease investigation was carried out in November 2022 whch is considered to be moonsoon season in Chengalpattu district with high humidity. The increased humidity could have attributed for the caked up litter in this flock which could be a major predisposing factor for Fowl cholera outbreak in this flock.

5. Acknowledgements

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