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Concurrent infections of duck viral enteritis and pasteurellosis among ducks in Kerala[#]

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Abstract

Duck farmers face significant economic losses due to infections caused by duck virus enteritis (DVE) virus and Pasteurella multocida (P. multocida), leading to high morbidity and mortality. These pathogens exhibit similar clinical manifestations. Thus, differentiating these infections based on clinical signs alone is highly challenging. The present study focuses on the detection of DVE coinfection among the pasteurellosis-suspected duck samples collected in Kerala during a 16 months study period. P. multocida was isolated from 19 samples in blood agar as mucoid, tiny dewdrop colonies. The isolates were further characterised by biochemical tests and confirmed by species-specific polymerase chain reaction (PCR). Among the 19 samples, 11 samples that were PCR positive for P. multocida (PM) revealed amplicons specific for DVE on PCR targeting UL2 gene of the DVE virus. The current study revealed the occurrence of concurrent infections of DVE and pasteurellosis in ducks that were immunised with the DVE vaccine. Despite vaccination against pasteurellosis in a few cases, the occurrence of P. multocida infection was observed in the study.

Keywords: *Pasteurella multocida*, *duck viral enteritis, ducks, concurrent infection*

Duck farming is a profitable sector on a global scale due to its contributions in terms of eggs, meat and feathers. Every year, there is a rise in both the occurrence of duck mortality events and the diverse pathogenic infections contributing to such mortality. Infectious diseases like duck viral enteritis (DVE/ duck plague), pasteurellosis (duck cholera), riemerellosis (duck septicaemia) and duck viral hepatitis are encountered as the major cause of economic losses in the duck industry. Hence, timely disease surveillance is necessary to detect the presence of infectious and non-

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infectious pathogens in the duck population and promptly implement countermeasures to enhance duck productivity.

The DVE is caused by *Anatid alphaherpesvirus*-1 or DVE virus of the subfamily *Herpesviridae*, genus *Mardivirus*. It has been observed in domestic ducks and ducklings of various ages, ranging from 7 days old to mature breeders. The first signs in susceptible flocks are often sudden, high, and persistent mortality, accompanied by a reduction in egg production. The immunosuppressive state triggered by duck plague might result in secondary bacterial infections with *P. multocida* (PM). Since *P. multocida* organisms typically reside as commensal in the respiratory tracts of various avian species, it might occur as a primary or opportunistic pathogen of respiratory tract infections. It is more frequent and fatal in ducklings below 8 weeks of age than in adult birds. In Kerala, an outbreak of pasteurellosis in ducks was reported for the first time in a flock that had been vaccinated against DVE (Pillai *et al*., 1993). Since then, duck mortality has been ongoing, either due to pasteurellosis or as a result of coinfection by *Pasteurella* and the DVE virus. Due to the similarity in clinical signs observed during outbreaks of DVE and pasteurellosis in ducks, molecular tools have proven to be highly specific for rapid identification. The present study involves molecular detection of concurrent infections of DVE and pasteurellosis in ducks by PCR. It also aims at the isolation and identification of *P. multocida* from clinical samples by conventional methods.

Materials and methods

Sample collection

Samples were collected from a total of 50 ailing/ dead ducks suspected of pasteurellosis brought to the Departments of Veterinary Pathology and Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy during a study period of 16 months from July 2022 to October 2023 for disease investigation with a history of loss of appetite, greenish diarrhea and sudden death (Table 1). Vaccination history revealed that, in some cases, flocks were vaccinated against DVE and few against pasteurellosis. The tissue samples including heart blood, lung, liver, spleen and brain were collected aseptically. Heart blood smears and impression smears from liver and spleen were prepared for direct microscopical examination.

Table 1. Details of samples collected from ducks

Samples collected	Vaccination history	No. of Samples
Heart blood, lung, liver, spleen and brain	DVE	17
	Pasteurellosis	14
	Unvaccinated	19
	Total	**50**

Isolation and identification of P. multocida

Blood agar (BA) was prepared by supplementing with sterile defibrinated bovine blood at five per cent level and used for primary isolation. All clinical samples were streaked directly onto BA plates and incubated at 37°C for 24 to 48 hours in a candle jar, as it is facultatively anaerobic. Colonies suggestive of *P. multocida* were stained by Gram's method to study the morphological features and staining character and were selected for further identification. A list of first and second stage biochemical tests were performed according to Quinn *et al*. (1994). Morphology, cultural characteristics, haemolysis on BA, reaction for catalase and oxidase and growth on MCA were noted as first stage tests. This was followed by secondary identification tests like indole production, methyl red (MR) test, Voges-Proskauer (VP) reaction, citrate utilisation, urease activity, nitrate reduction, ornithine decarboxylase activity, gelatin liquefaction and oxidation-fermentation (O/F) reaction.

Molecular detection by PCR

DNA extraction

Genomic DNA from bacterial isolates and tissue samples were extracted using the commercial kit (HiPurA multi-sample DNA extraction kit, HiMedia Laboratories Pvt. Ltd., India) and stored in elution buffer at -20 °C, until use. For this, the colonies of the isolates

were dispensed in 1.5 mL of sterile Brain heart infusion broth (BHIB) and incubated overnight at 37 °C in a candle jar. Extraction of DNA was carried out as per the manufacturer's instructions.

***Polymerase chain reaction – Pasteurella multocida* species-specific PCR (PM-PCR)**

The morphologically positive isolates were confirmed by PCR, targeting the *P. multocida* species-specific (*kmt1*) gene using primers (KMTISP6: GCTGTAAACGAACTCGCCAC and KMTIT7: ATCCGCTATTTACCCAGTGG) designed by Townsend *et al*. (1998) corresponding to an amplicon of approximately 460 bp. The amplification was carried out under the following conditions in a thermal cycler (MJ Mini Bio-Rad thermal cycler, USA): Initial denaturation for 4 min. at 95°C followed by 29 cycles of denaturation at 95°C for 45 sec., annealing at 55°C for 45 sec. and extension at 72°C for 45 sec. with a final extension at 72°C for 6 min. *Riemerella anatipestifer* species-specific PCR (RA-PCR) with primers designed by Kardos *et al*. (2007) using tissue samples to rule out the presence of any positive cases of *R. anatipestifer*.

Polymerase chain reaction - Amplification of* UL2 *gene

Molecular detection of DVE using the collected tissue samples were carried out employing oligonucleotide primers (F: ATCGCATGTAGACGTTGGTT and R: AGACAGCGGTGATGGATGG) targeting the unique long region 2 (UL2) gene (172 bp), which is a highly conserved region of the DVE virus (Yao *et al*., 2019). The amplification was carried under the following conditions in a thermal cycler (MJ Mini Bio-Rad thermal cycler, USA): Initial denaturation for 5 min. at 95°C followed by 35 cycles of denaturation at 95°C for 30 sec., annealing at 56.7°C for 30 sec. and extension at 72°C for 40 sec. with a final extension at 72°C for 10 min.

Results and discussion

Sample collection

The birds submitted for disease investigation belonged to different age groups ranging from 45 days to two years. The birds were presented with varying clinical signs such as anorexia, ruffled feathers, greenish diarrhea, listlessness, ocular and nasal discharge and sudden death. On post-mortem examination, gross lesions observed were tissue haemorrhages, pericarditis with diffuse petechial and ecchymotic haemorrhage in the epicardium, perihepatitis with the presence of necrotic foci on the liver, haemorrhage in the intestinal serosa and enlarged spleen. The lesions were consistent with earlier studies by Punnoose *et al*. (2021) and Shawky *et al*. (2000).

Direct microscopic examination

Smears prepared from the heart blood, liver and spleen when stained by Leishman's stain revealed the presence of bipolar organisms suggestive of *P. multocida* (Fig. 1), which was in accordance with the findings of Shome *et al*. (2004) and Amany *et al*. (2017). Pillai *et al*. (1993) opined that bipolar organisms of *R. anatipestifer* were morphologically distinguishable from *P. multocida* in heart blood and liver impression smears by the size variation.

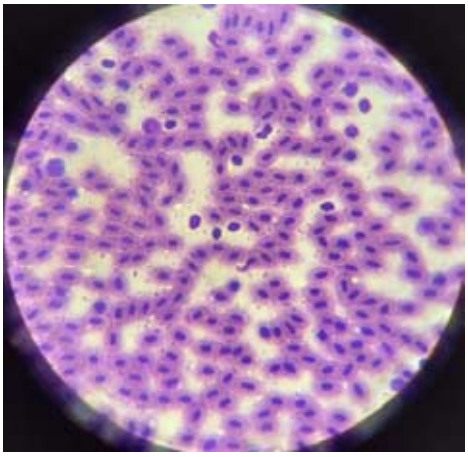

Fig. 1. Bipolar organisms in blood smear **(**Leishman's staining, 1000x)

Isolation and identification

In the present study, out of the 50 ducks screened, samples from 19 birds showed colonies suggestive of *P. multocida* in BA. All the PM isolates produced colonies

Fig. 2. Colonies of *P.multocida* on blood agar

that were convex, mucoid, greyish-white and dew drop-like appearance (Fig. 2). There was a characteristic odour detected upon opening the plates. Similar observations have been made by Mutters *et al*. (1989). Due to the genotypic and phenotypic similarities and their similar clinical manifestations in ducks, *R. anatipestifer* could often be confused with *P. multocida*. However, variations in their biochemical characteristics could be employed to distinguish between them (Surya *et al*., 2016).

All the PM isolates (PM-1 to PM-19) were Gram-negative, cocco-bacillary in morphology, non-motile, did not grow on MCA and catalase and oxidase positive. These results are in agreement with those of Antony (2004). Most of the PM isolates were non-haemolytic on BA except PM-1 to PM-4, which exhibited a narrow zone of greenish discolouration on BA. Manuselis and MacGill (2007) had reported the similar findings. In the second stage of identification, all the 19 PM isolates were positive for indole production, ornithine decarboxylase activity and nitrate reduction tests and negative for urease, H_2S production, MR, VP and citrate utilisation tests (OIE, 2021). Based on the first and second stage biochemical tests, the isolates were identified as *P. multocida*.

Molecular confirmation of the isolates by PM-PCR

To validate the results of the biochemical tests, PM-PCR, developed by Townsend *et al.* (1998) was employed to detect the organism from culture as well as from clinical samples. On amplification, all of the PM isolates generated a product of ~460 bp size (Fig. 3). This was in harmony with the results of Antony (2004). The overall detection rate of *P. multocida* isolates from the pasteurellosis suspected ducks was 38 per cent. Nineteen PM-positive samples were obtained from cases with a vaccination history of DVE (11/17), pasteurellosis (4/14) and unvaccinated (4/19). Despite being vaccinated against pasteurellosis, occurrences of the disease were noticed in four

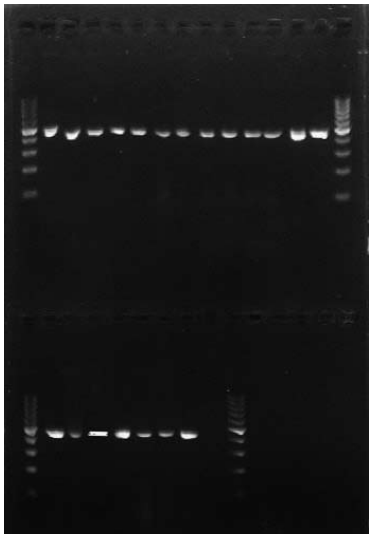

Fig. 3. PM-PCR (~460 bp)
Lane 1, 15, 16, 25: 100 bp ladder
Lane 2-14, 17-22: Positive samples
Lane 23: Positive control
Lane 24: Negative control

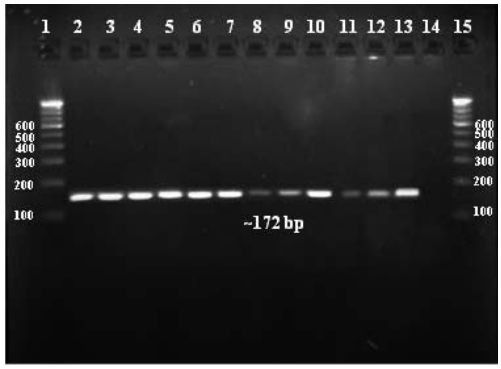

Fig. 4. DVE PCR (~172 bp)
Lane 1, 15: 100 bp ladder
Lane 2-12: Positive samples
Lane 13: Positive control
Lane 14: Negative control

out of 14 samples, raising questions about the efficacy of the vaccination.

All the 19 tissue samples tested negative in the RA-PCR assay. This confirmed the absence of *R. anatipestifer* infection in all the 19 PM-positive cases. Therefore, all the 19 isolates were identified solely as *P. multocida* by both conventional and molecular methods.

Molecular diagnosis of DVE

To detect the presence of coinfection, genomic DNA extracted from the tissue samples, whose isolates tested positive for PM-PCR, exhibited amplicons specific to DVE PCR. Among the 19 clinical samples subjected to DVE PCR, 11 were found to be positive (Fig. 4). This confirmed the presence of concurrent infections of DVE and duck pasteurellosis with an incidence rate of 57.9 per cent. Out of the 17 samples with a history of vaccination against DVE, 11 samples have revealed the presence of coinfection. Since the DVE virus primarily targets the lymphoid organs which results in lymphocyte depletion and subsequent immunosuppression, which could potentially pave the way for secondary bacterial infections by *P. multocida* (Punnoose *et al*., 2021). Similar findings were reported by Soman *et al*. (2014), who documented concurrent infections of DVE and duck pasteurellosis in the same farm employing PCR. According to Megahed *et al*. (2023), secondary infections of *P. multocida* during natural outbreaks of DVE in ducklings might arise from an immunosuppressive state triggered by the disease. The same author also reported a coinfection of DVE in five ducks with *P. multocida*, which was analogous to the present study results. In a study by Neher *et al*. (2019), the presence of DVE virus in tissue samples was detected by Sandwich-ELISA (S-ELISA) and amplification of the UL 44.5 as well as DNA polymerase genes of the DVE virus by PCR, with the highest number of per cent of positivity observed in PCR (78.68 per cent) compared to S-ELISA (56.05 per cent). The findings of the present study illustrate that PCR assays facilitate the rapid and precise identification of concurrent infections in ducks.

Conclusion

The present study documents the presence of DVE coinfection with pasteurellosis among ducks that were immunised with the DVE vaccine. Despite vaccination against pasteurellosis in 14 cases, the occurrence of *P. multocida* infection was observed in four cases. The clinical signs were similar in both disease conditions, so often they go undiagnosed. Based on the current study findings, it could be concluded that the presence of DVE infection resulted in great stress on the duck immune system increasing the susceptibility of birds to infection especially pasteurellosis which resulted in an increased mortality rate. As a preventive measure, immunization with a modified-live vaccine against DVE and an oil-adjuvanted vaccine against pasteurellosis is commonly employed to control these diseases in ducks. Despite vaccination, there are reports of pasteurellosis and DVE outbreaks in vaccinated ducks. These reports question the duration of immunity offered by the current vaccines and it warrants a detailed investigation of the problem.

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Conflict of interest

The authors declare that they have no conflict of interest.

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