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Dr. Debasish Behera
Assistant Professor, Department of Veterinary Pathology, College of Veterinary Sciences and animal Husbandry, R. K. Nagar, West, Tripura, India

Dr. Sukanta Datta
JRF, SERB-DST Project, Department of Veterinary Pathology, College of Veterinary Sciences and animal Husbandry, R. K. Nagar, West, Tripura, India

Dr. Kanika Kalai
Assistant Professor, Department of Veterinary Pathology, College of Veterinary Sciences and animal Husbandry, R. K. Nagar, West, Tripura, India

Dr. Aparajita Das
Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Sciences and animal Husbandry, R. K. Nagar, West, Tripura, India

Corresponding Author:
Dr. Debasish Behera
Assistant Professor, Department of Veterinary Pathology, College of Veterinary Sciences and animal Husbandry, R. K. Nagar, West, Tripura, India

Isolation and molecular detection of *Riemerella anatipestifer* from Ducks in Tripura

Dr. Debasish Behera, Dr. Sukanta Datta, Dr. Kanika Kalai and Dr. Aparajita Das

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Abstract
The present study was carried out to isolate and identify the *Riemerella anatipestifer* in ducks in the Tripura state of North East India and this organism is responsible for new duck disease or duck septicaemia. *Riemerella anatipestifer* is a gram-negative, non-motile, non-spore forming, rod-shaped bacterium and leading to high morbidity and mortality among ducks. The clinical manifestation in this study revealed, fibrinous serositis, arthritis and often leads to rapid fatality due to septicaemia. A total of 453 samples were collected from five districts of Tripura such as West, Sepahijala, Gomati, Khowai and Unokoti. Pharyngeal swab, tracheal scrapings, heart blood, lung, liver, spleen, ovary, and brain as samples from live and dead birds were part of this study. Samples were inoculated in Brain Heart Infusion (BHI) broth, 5% sheep blood agar and Trypticase soy agar plates and isolates were identified based on their morphology, cultural characteristics, growth on MacConkey's agar, and haemolysis on blood agar. Based on microbiological characteristics, 167 samples out of 453 were suspected positive for *Riemerella anatipestifer*. Further the confirmation of the isolated samples was obtained by analysing the amplification of 16s rRNA gene with amplicon size was 665 bp by PCR and a total of 144 samples were confirmed as *Riemerella anatipestifer* in this study. The positive isolates were preserved for further research work such as molecular epidemiology, pathology, antibiogram.

Keywords: 16SrRNA, isolation, molecular detection, *Riemerella anatipestifer*

Introduction
Riemerella anatipestifer causes septicaemia disease in ducks resulting in serious economic losses through high mortality, reduced growth rate, poor feed conversion and increased condemnations of carcasses. *Riemerella anatipestifer* is a gram negative, microaerophilic, non-motile, bipolar bacterium under the family Flavobacteriaceae (Gong *et al*., 2020) [3]. The disease was first reported and described by Riemer in 1904. *R. anatipestifer* affects both domestic and wild birds globally, with ducks being particularly susceptible, followed by geese, turkeys, and chickens. In India ducks are mostly reared in states like West Bengal, Assam and Tripura out of these Tripura state is one of the upcoming vibrant state with respective to the duck production in the North East India and having second highest duck population after Assam. Mortality varies from 2 to 30%, but it can be as high as 95% by presence of predisposing viral and bacterial infections (Tsai *et al*., 2005 and Yu *et al*., 2008) [17, 18]. Till date, more than 21 serotypes of *R. anatipestifer* have been identified worldwide with no effective cross-protection between them (Chang *et al*., 2019) [1]. Different serotypes show wide variations in virulence factors (Huang *et al*., 2002) [4] and involvement of multiple serotypes has been reported in the same farm flock (Pala and Radhakrishnan *et al*., 2014) [8]. The transmission of the disease occurs primarily through respiratory tract and skin abrasions (Sandhu, 2003) [12]. The disease manifests as an acute form in ducks less than 8 weeks of age, with ducklings up to 3-4 weeks of age being the most vulnerable but the disease remains as chronic form in older birds. Major clinical signs are such as fibrinous serositis, caseous salpingitis, and arthritis in acute form and results in rapid fatality due to septicaemia. The chronic carrier state may be virtually asymptomatic without distinct lesions. Post Mortem lesions revealed fibrinous pericarditis, perihepatitis, and air-sacculitis (Ryll *et al*., 2001) [11]. In India, the disease has been reported in ducks from Assam and Kerala (Shome *et al*. 2004 and Priya *et al*., 2008) [15, 10]. Molecular techniques play a vital role in analysing microorganisms from various sources and in different environment. These techniques also provide a means to study the genetic diversity of individual organisms, offering definitive information on their characterization, identification, and biodiversity.

Taking in to consideration of the above facts, this study was conducted to unravel the tryst of this duck septicaemia by isolation and molecular detection by employing species specific gene 16s RNA and studying other microbiological characteristics for future prospect in terms of diagnosis.

Materials and Methods

The present study was carried out in the Department of Veterinary Pathology, College of Veterinary Sciences and Animal Husbandry, R. K. Nagar, West, Tripura. A total of 453 samples were collected from the villages or places from five districts such as West, Sepahijala, Gomati, Khowai and Unokoti. The villages and places were selected on the basis of information of outbreak like morbidity and mortality among ducks. The swab samples were collected from both diseased and healthy ducks of mixed age groups. Pharyngeal swabs, tracheal piece, heart blood, lung, liver, spleen, ovary, and brain were collected from sacrificed or deceased birds for this study. The samples were inoculated in Brain Heart Infusion (BHI) broth and incubated in microaerophilic environment at 37 ºC overnight. After incubation, the inoculum was streaked into 5% sheep blood agar and Trypticase soy agar plates. These plates were then incubated at 37 ºC for 24 to 48 hours in a candle glass jar rich with carbon dioxide to create optimal growth conditions. Further, in the present study in order to detect the presence of bipolar organisms, heart blood smears, impression smears from the liver and spleen were stained by Giemsa stain.

The bacterial isolates were identified based on their morphology, cultural characteristics, growth on MacConkey's agar, and haemolysis on blood agar. Cultural characteristics were recorded based upon colony appearance, colour, and texture on agar media. Growth on MacConkey's agar helped to differentiate between lactose fermenters and non-fermenters, while haemolysis on blood agar indicated the ability of the bacteria to lyse red blood cells.

Identification of *R. anatipestifer* using 16SrRNA gene-based PCR

Confirmations of the microbiologically and culturally suspected isolates were subjected for amplifying partial region of 16SrRNA gene. Suspected colonies grown in blood agar and trypticase soy agar were selected for DNA isolation. For that the supernatant collected after boiling and centrifugation was used as the source of DNA for PCR amplification. The primers based on the conserved region of the 16SrRNA gene were used for amplification. The oligonucleotides forward (5´CAGCTTAACTGTAGAACTGC3´) and reverse (5´TCGAGATTTGCATCACTTCG3´) were used. Twenty picomoles of each primer, Emerald GT master mix, nuclease free water (HiMedia) and sample DNA were used for the preparation of 25 µl reaction mixture for simplex PCR reactions. The thermal cycling profile standardized as initial denaturation at 95 °C temperature for 4 minutes, followed by 35 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C temperature for 1 minute, extension at 74 °C temperature for 1 minute and a final extension at 72 °C for 7 minutes. Thermal cycling was performed using T100 Thermal Cycler (Biorad). The PCR products were electrophoresed in 1.5% agarose for 1hour.

Results

The clinical signs and gross lesions of duck septicaemia disease were quite similar with the diseases like duck pasteurellosis and *E. coli* infection. Therefore, the best method of diagnosis was adopted in this study that, the isolation of the bacteria was done in appropriate media and preferred samples for more probability of getting desired bacteria and that was found to be lower portion of trachea. Apart from that, the positive isolates were also isolated from the heart blood, lung, liver, spleen, ovary and brain. Based on microbiological and cultural characteristic out of 453 total samples, 167 were found to be positive for *Riemerella anatipestifer*. Cultural characteristics such as small dew drop whitish to milky colony were recorded on BHI agar media (Fig.1). A portion of colony was taken for Gram stain and it revealed Gram negative organism with a coccobacillus of variable morphology from short rods to filamentous in appearance (Fig.-2). Further studies revealed that, no growth on MacConkey's agar and no haemolysis on blood agar was found to be characteristics towards isolation of positive isolates of *Riemerella anatipestifer* (Fig.3). The culturally positive isolates of *Riemerella anatipestifer* was confirmed based upon the detection of 16SrRNA gene. Out of the total 167 culturally positive isolates, only144 numbers of isolates were found to be confirmed as *Riemerella anatipestifer* by analysing the amplification of 16SrRNA gene with amplicon size of 665 bp (Fig.4).

Fig 1: Small dew drop whitish to milky colony on BHI agar

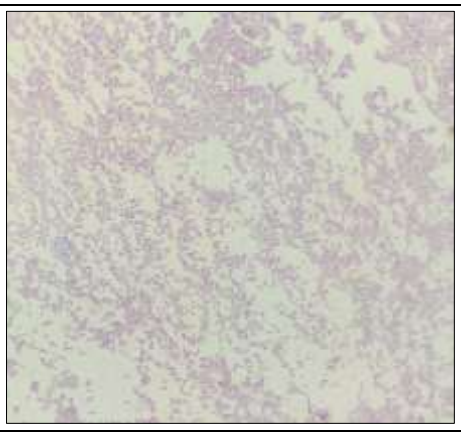

Fig 2: Gram negative *Riemerella anatipestifer* with variable morphology

Fig 3: *Riemerella anatipestifer* growth on blood agar without haemolysis

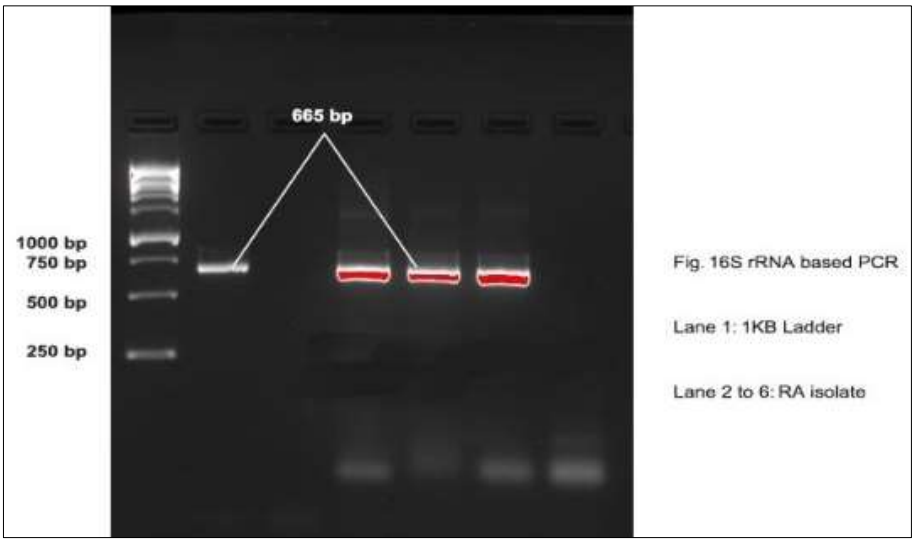

Fig 4: Riemerella anatipestifer PCR amplification of 16SrRNA gene with amplicon size of 665 bp, Lane-1- 1KB ladder; L-2: Positive control; L-3: Negative control, and L-4 to L-6: Field RA isolates

Discussions

Riemerella anatipestifer is the causative of new duck disease or duck septicaemia and characterised by polyserositis and fibrinous pericarditis of domestic ducks. Earlier research studies established that, twenty-one serotypes of the organism have been identified with no significant cross-protection (Loh *et al*., 1992 and Pathanasophon *et al*., 1995) [6, 9]. The present study was aimed to establishment the presence of duck septicaemia in the duck population of Tripura by detecting 16SrRNA gene in PCR. The clinical manifestation in the present study recorded as diarrhoea, swelling of face with oculo-nasal discharge, nervous signs of head and neck movement and parallel findings were also recorded earlier (Kardos *et al*., 2007) [5]. In this study, the mortality and morbidity were observed in ducklings of less than 5 weeks of age and death usually occurred within short time period after manifestation of clinical signs such as greenish-white diarrhoea, off fed, torticollis, tremor of head and neck, incoordination of movement and similar clinical findings were recorded by Priya *et al*. (2008) [10]. The current study suggested that, by using commonly available media there is always over growth of other saprophytic organisms made the isolation difficult. The biochemical profile also does not reveal any special biochemical property based on which correct identification could have been done. So, the molecular technique becomes an important tool by detecting 16SrRNA PCR assay to identify the bacterium and the amplicon size obtained was 665bp. In this study, the 16S rRNA gene based PCR was used to confirm the organism after microbiological and cultural and characterization. The 16S rRNA gene-based PCR was reported to be suitable for the screening of *R. anatipestifer* infections and the primers and thermal profile used for this technique as described by Tsai *et al.* (2005) [17] and same result also has been recorded by Shancy *et al.* (2018) [14]. In the present study PCR helped fast and proper identification of *R. anatipestifer* infection in ducks and differentiated easily from *Pasteurella multocida* and similar results were opined by Soman *et al*. (2014) [16]. The effectiveness of 16S rRNA based PCR in the prompt diagnosis of New Duck disease which could aid in adopting timely and proper control strategies as suggested by Pala *et al.* (2013) [7] and PCR amplification of 16S rRNA gene with subsequent sequencing was recommended as the fastest way to confirm identification of *R. anatipestifer* (Christensen and Bisgaard, 2010) [2].

Conclusion

In Tripura, duck rearing has established itself as one of the important family income generating sources and it helps in uplifting of socially backward and deprived sections of society. The meat of ducks is being considered as a symbol of delicacy and hence demand is always high among all sections of people in this state. Ducks are bred and reared on a large scale and it has proved itself as best farming for the sustainable livelihood. Therefore, isolation and molecular diagnosis of duck septicaemia disease among ducks is a wakeup call for the farmers. Further studies on pathogenesis, pathology, virulence of the infectious agent and antibiogram will be evinced to be a best possible way for diagnosis and prevention of this health menace of duck population of Tripura.

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Authors' contributions: DB: performed all aspects of the experiments, drafted and revised the manuscript and overall monitoring the project. SD: conceived and designed all aspects of the molecular works and authored the initial draft of the manuscript, KK: sample collections, tour travels and designing the concept for manuscript. AD: analyzed and interpreted the data and assisted in the data interpretation and all the authors read and approved the final manuscript.

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Availability of data and materials: All data generated or analyzed during this study are included in this manuscript.

Declarations

Ethics approval and consent: Not applicable

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests

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