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Research Article

Molecular diagnosis of New Duck disease in India by 16SrRNA gene based PCR

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ABSTRACT

Riemerella anatipestifer causes New Duck disease or infectious serositis which is an epizootic infectious disease in poultry resulting in serious economic losses, especially in the duck industry through high mortality, reduced growth rate, poor feed conversion, increased condemnations and high treatment costs. Precise and rapid identification is especially important in case of *R. anatipestifer*, as mistaking it for *Pasteurella multocida* leads to false alarm of fowl cholera, resulting in delayed diagnosis of the disease. This is the first report on molecular biology based confirmation of the occurrence of *R. anatipestifer* infection in ducks of India. In the present study, isolation of *R. anatipestifer* was attempted from suspected field outbreaks of which six isolates were obtained, namely KAL-1, KNB-1, KML-1, KML-2, KML-3, and KTL-1. All the isolates that were culturally and biochemically similar to *Riemerella* spp were subjected to specific 16S rRNA gene based PCR. Sequencing of the amplicons revealed 99% similarity to *R. anatipestifer* strain ATCC 11845 ribosomal RNA partial sequence. The results confirm that *R. anatipestifer* is prevalent among Indian ducks against which proper control and therapeutic strategies need to be developed.

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INTRODUCTION

Riemerella anatipestifer (*R. anatipestifer*) previously known as *Pasteurella anatipestifer* or *Moraxella anatipestifer* causes epizootic infectious disease in poultry resulting in serious economic losses, especially in the duck industry through high mortality, reduced growth rate, poor feed conversion, increased condemnations and high treatment costs. It causes primarily an acute septicaemic disease in younger birds and more chronic and localized lesions in older birds. The disease is known as New Duck disease or anatipestifer syndrome. Mortality varies from 2 to 30%, but it can be as high as 95% influenced by predisposing viral and bacterial infections. Twenty-one serotypes of *R. anatipestifer* have been identified with no significant cross-protection reported.

In India ducks are mostly reared in states like West Bengal and Assam in the eastern part of the country and in southern states like Tamil Nadu and Kerala as a supplementary source of income and animal protein. In Kerala, duck rearing has an important role in generating family income and uplifting of socially backward and deprived sections of society. Ducks are bred and reared on a large scale in Alappuzha, Thrissur, Kottayam and Pathanamthitta districts of Kerala. Severe mortality in ducks of Kerala has been reported since 1990 every year, especially after the onset of monsoon season even in flocks vaccinated against all the prevalent diseases. Reports on the detection of bipolar organisms in young birds immunized against pasteurellosis diverted the attention to *R. anatipestifer*, whose incidence has been previously reported only once in South India (Priya et al., 2008).

The organism is morphologically similar to *Pasteurella* spp, being bipolar in nature. The clinical signs often get confused with *Escherichia coli* infections and salmonellosis leading to diagnostic errors. Hence, the differential diagnosis of New Duck disease is highly imminent for effective treatment and control. This is the first report on molecular biology based confirmation of the occurrence of *R. anatipestifer* infection in duck population of India.

MATERIALS AND METHODS

Bacterial isolates

Samples for the isolation of *R. anatipestifer* were collected mainly from suspected field outbreaks. Samples of heart blood, lung, liver, spleen, ovary and brain, collected from the sacrificed or dead birds, were used for isolation of the organism. Heart blood smears and impression smears from liver and spleen were prepared and stained by Giemsa staining technique to detect the presence of bipolar organisms. The isolates were grown on 10% sheep blood agar plates and incubated at 37ºC for 24 to 48 h in an atmosphere enriched with 5% CO_2. The bacterial isolates were identified based on morphology, cultural characteristics, growth on MacConkey's agar, haemolysis on blood agar and biochemical tests like catalase and oxidase, indole production, gelatin liquefaction and ornithine decarboxylase activity.

Confirmation of *R. anatipestifer* isolates by 16S rRNA gene based PCR

Confirmation of the obtained isolates was done by amplifying partial region (665 bp) of 16S rRNA gene. The supernatant collected after boiling and centrifugation of the

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colonies grown on blood agar was used as the source of DNA for PCR. The primers based on the conserved region of the 16S rRNA gene designed by Tsai et al. (2005) were used for amplification. The oligonucleotides RA20F2 (5´CAGCTTAACTGTAGAA CTGC3´) and RA20R4 (5´ TCGAGATTTGCATCACTTCG3´) were used in a 25µl reaction mixture containing 20 picomoles of each primer, 200 µmol l^{-1} each of dATP, dCTP, dGTP, and dTTP; 1.5 mmol l^{-1} $MgCl_2$; and 1.5 U of Taq DNA polymerase (Genei, Bangalore). The thermal cycling profile consisted of denaturation at 94°C for 30 seconds, annealing at annealing temperature for 30 seconds, and extension at 74°C for 1 min for 35 cycles followed by a final extension at 72°C for 7 min. The annealing temperature ascended from 58 - 68°C. The PCR products were electrophoresed in 2% agarose for 1h.

16S rRNA gene sequence analysis

Using the RA20F2 and RA20R4 primer set, the amplified products were sequenced at the DNA sequencing facility at Sci genome Pvt. Ltd., Cochin, Kerala using an automated DNA sequencer (Applied Biosystems, USA). The sequences were aligned and compared with the sequence data retrieved from GenBank. Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) network provided by the National Centre for Biotechnology Information (NCBI).

RESULTS

Bacterial isolates

Samples were collected from about thirteen suspected field outbreaks, six isolates turned positive for *R. anatipestifer* namely, KAL-1, KNB-1, KML-1, KML-2, KML-3, and KTL-1. The clinical signs of that could be observed included greenish diarrhoea, lethargy, ocular and nasal discharge, tremors of head and neck, swollen limbs and weight loss. Mostly young ducklings of 3-4 weeks of age were affected. In most cases, the gross lesions like congestion of meninges, fibrinous pericarditis, perihepatitis, spleenomegaly and airsacculitis could be detected (Fig 1).

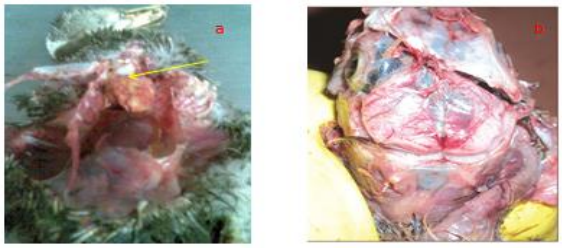

Fig 1
Gross lesions of (a) fibrinous pericarditis and b) congestion of meninges in *R. anatipestifer* affected ducklings

In adult females, the oviducts were filled with caseous yellowish white exudates. Smears prepared from heart blood, liver and spleen upon Giemsa's staining revealed comparatively larger bipolar organisms suggestive of *R. anatipestifer*. All the six isolates produced mucoid, convex, grayish-white and non-haemolytic colonies on 10% sheep blood agar (Fig 2).

All the isolates were non-motile, Gram-negative, coccobacillary, catalase and oxidase positive. None of the six isolates grew on MacConkey's agar. All the isolates that were indole negative, ornithine decarboxylase negative and gelatin liquefaction positive were selected for further analysis by PCR.

Fig 2
Mucoid, convex, greyish-white, non-haemolytic colonies of *R. anatipestifer* grown on sheep blood agar

Confirmation of *R. anatipestifer* isolates by 16S rRNA gene based PCR

Among the range of annealing temperatures tested, the temperature of 54°C gave the optimum amplification. All the six isolates selected were successfully amplified with a 665 bp DNA product (Fig 3).

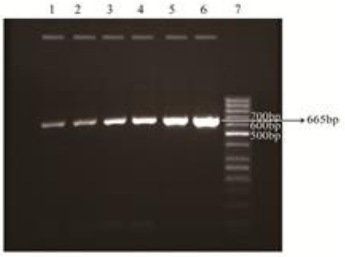

Fig 3
PCR amplified products of 16S rRNA gene confirming the presence of *R. anatipestifer* in all the six isolates. Lane 1: KAL-1, Lane 2: KNB-1, Lane 3: KML-1, Lane 4: KTL-1, Lane 5: KML-2, Lane 6: KML-3, Lane 7: 50bp ladder

16S rRNA gene sequence analysis

The amplicons obtained from all the six isolates were subjected to sequencing. On blast analysis of the sequences generated, about 99% similarity to *R. anatipestifer* strain ATCC 11845 ribosomal RNA partial sequence (Accession no. NR 026025.1) could be noticed with respect to all the isolates.

DISCUSSION

Riemerella anatipestifer is the causative of epizootic infectious polyserositis of domestic ducks. Precise and rapid identification of *R. anatipestifer* is important to avoid diagnostic errors. About twenty-one serotypes of the organism have been identified with no significant cross-protection reported (Loh et al., 1992; Pathanasophon et al., 1995, 2002). The present study is focused on the establishment of the presence of New Duck disease in the duck population of India with the help of 16SrRNA gene based PCR.

The clinical signs of New Duck disease include greenish diarrhoea, lethargy, ocular and nasal discharge, tremors of head and neck and weight loss (Karstard et al., 1970; Kardos et al., 2007). Ducklings less than 5 weeks of age usually die 1 -2 days after the appearance of clinical signs, whereas older birds survived longer. According to Priya et al. (2008) clinical signs included dullness, huddling, poor feed intake, refusal to swim, purulant oculo-nasal discharge, greenish diarrhoea, incoordination and death. The gross lesions noticed like congestion of meninges, fibrinous pericarditis, perihepatitis, spleenomegaly and airsacculitis are in agreement with the previous studies (Dougherty et al. 1955; Zehr and Ostendorf, 1970; Leibovitz, 1972; Kardos et al., 2007). Pillai et al. (1993) showed that the bipolar organisms revealed by Leishman's staining of blood smear were larger than *P. multocida*.

Riemerella anatipestifer is characterized more by the absence than by the presence of specific phenotypic properties (Hinz et

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al. 1998). The cultural and biochemical characteristics shown by the organism under study are suggestive of *Riemerella* spp (Smith et al., 1987; Segers et al., 1993; Shome et al., 2004).

In this study, the 16S rRNA gene based PCR was used to confirm the organism after cultural and biochemical characterization. All the isolates exhibited 99% similarity to the sequence retrieved from GenBank. The 16S rRNA gene-based PCR has been reported to be suitable for the screening of *R. anatipestifer* infections (Tsai et al., 2005). The 16S rRNA gene is considered a stable fragment on the chromosome of all prokaryotes. The phylogenetic relationship among bacteria can be obtained on analysis of their 16S rRNA sequences. The benefit of using 16S rRNA sequence for confirmation of the bacterial isolates is that the gene is highly conserved among all prokaryotes due to the antiquity of the protein synthesizing process. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms. The 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (Clarridge, 2004). The comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels. The number of informative sequence positions makes ribosomal RNA an excellent molecule for comparison of various hierarchial levels (Olsen et al., 1994).

The characterization of *R. anatipestifer* and related bacteria by traditional methods is often inconclusive because of inconsistent reactions and phenotypic diversity. For the same reason, gene sequencing and phylogenetic analysis is essential to allow proper classification. The PCR amplification of a partial region of the rpoB or the 16S rRNA gene with subsequent sequencing is recommended as the fastest way to confirm identification of *R. anatipestifer* (Christensen and Bisgaard, 2010). The results of the present study emphasizes the utility of 16S rRNA based PCR in the prompt diagnosis of New Duck disease which could aid in adopting timely and proper control strategies.

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