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# Genomic diversity of *Riemerella anatipestifer* associated with outbreaks of New Duck disease in India

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ABSTRACT

*Riemerella anatipestifer* causes New Duck disease in poultry, primarily in domestic ducks. The present study confirms the occurrence of genetically diverse isolates of *R. anatipestifer* in the ducks of India. Isolation of *R. anatipestifer* was attempted from 13 suspected outbreaks of duck septicaemia in different regions of Kerala, India and 6 isolates could be obtained, namely KAL–1, KNB-1, KML-1, KML-2, KML-3, and KTL-1. All the isolates were subjected to specific 16S rRNA gene based PCR and sequencing, which revealed 99 % similarity to *R. anatipestifer* strain ATCC 11845 ribosomal RNA partial sequence. Molecular fingerprinting of the isolates by ERIC-PCR produced 10–12 bands per isolate ranging in size from 150 bp to 2000 bp. The phylogenetic analysis of these fingerprints revealed that the variants obtained from commercial flocks are genetically more diverse compared to the variant associated with outbreak in an organized instructional farm.

**Key words:** ERIC-PCR, Genetic variants, Genomic diversity, *Riemerella anatipestifer*, New Duck disease

*Riemerella anatipestifer* causes epizootic infectious disease in poultry, primarily in domestic ducks. The disease is variously known as New Duck disease or duck septicaemia or anatipestifer syndrome or infectious serositis. It has a worldwide distribution, resulting in serious economic losses to duck industry (Helfer and Helmboldt 1977, Bisgaard 1982) especially when the birds are co-infected with other pathogens. Approximately 21 serotypes of *R. anatipestifer* have been identified (Pathanasophon *et al*. 1995) and no cross-protection was reported with inactivated bacterins made from different serotypes of the organism (Layton and Sandhu 1984, Sandhu and Leister 1991). The occurrence of different serotypes in birds at any one time and changes in serotypes from year to year within a single farm was reported (Subramaniam *et al*. 2000). *Riemerella anatipestifer* is phenotypically similar to *Pasteurella* spp, and this similarity may cause diagnostic problems (Kardos *et al*. 2007).

In India ducks are mostly reared in backyard units by individual households in rural areas of states like West Bengal and Asom in the eastern part of the country and Kerala in south India as a supplementary source of income and animal protein. In Kerala, ducks are also reared in large flocks of 5,000 to 50,000 birds each by individual or groups of nomadic farmers with minimal infrastructure. In lean season the flocks are maintained in temporary thatched sheds on unconventional feed resources with minimum bio-security (Jalaludeen *et al*. 2004). Duck farmers of Kerala have been reporting heavy mortality in young flocks for the last few years, with bipolar staining organisms identified in many cases, especially after the onset of annual seasonal rains or monsoons.

*Riemerella anatipestifer* infection in an organized duck farm of Kerala with 12.5 % mortality (Priya *et al*. 2008) and an outbreak in Meghalaya with 16 % mortality (Shome *et al*. 2006) were reported but epidemiological investigations have not been undertaken to unravel the extent of the disease in India. The objective of this study was to explore the genetic diversity of *R. anatipestifer* circulating in domesticated duck population of Kerala, a state in India.

## MATERIALS AND METHODS

*Bacterial isolates:* Isolation of *R. anatipestifer* was attempted from samples of heart blood, lung, liver, spleen, ovary and brain collected from affected ducks of different farms in central and Kuttanad areas of Kerala, India. Heart blood smears and impression smears from liver and spleen were prepared for direct microscopical examination. Blood and tissue impression smears prepared were stained by Giemsa staining technique and examined microscopically to detect the presence of bipolar organisms. All the clinical samples were directly streaked on to sheep blood agar plates and incubated at 37°C for 24 to 48 h under 5 % $CO_2$. The bacterial isolates were identified as per Rimler *et al*. (1998) based on morphology, cultural characteristics, growth on MacConkey's agar, haemolysis on blood agar and

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biochemical tests like catalase and oxidase, indole production, gelatin liquefaction and ornithine decarboxylase activity.

*Confirmation by 16S rRNA gene based PCR:* The isolated organisms were confirmed as *R. anatipestifer* using 16S rRNA gene based PCR. Colonies were collected from blood agar and suspended in 500 µl of distilled water and boiled at 100°C for 15 min. After centrifugation at 17,000 × g for 10 min the supernatant was collected and used as the source of DNA for PCR. Two primers based on the conserved region of the 16S rRNA gene designed by Tsai *et al*. (2005) were used. The sense primer used was RA20F2 (5´CAGCTTAACTGTAGAACTGC3´) and the antisense primer used was RA20R4 (5´ TCGAGATTTGCATCAC TTCG3´). The reactions were performed in a total volume of 25µl containing 2.5 µl 10× assay buffer; 1 µmol $l^{-1}$ 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. The thermal cycling profile consisted of 35 cycles of 94°C for 30 sec, annealing temperature of 54°C for 30 sec, and 74°C for 1 min. The amplicons generated were confirmed for their expected size in 1.5 % (w/v) agarose gel in 1X TAE buffer as per Sambrook *et al*. (1989) using submarine electrophoresis apparatus.

*16S rDNA sequence analysis:* Using the RA20F2 and RA20R4 primer set, the amplified products were sequenced using an automated DNA sequencer. The sequences were aligned and were compared with the sequence retrieved from the Genbank, NCBI database. Sequence similarity search was performed using Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI).

*ERIC-PCR analysis:* Genomic diversity of the confirmed isolates was studied using ERIC-PCR. The oligonucleotides ERIC F (5´ATGTAAGCTCCTGGGGATTCAC3´) and ERIC R (5´AAGTAAGTGACTGGG-GTGAGCG3´) (Kiss *et al*. 2007) were used in a 25µl reaction mixture containing 1 µmol $l^{-1}$ of each primer; 2.5 ml 10X assay buffer; 200 µmol $l^{-1}$ each of dATP, dCTP, dGTP, and dTTP; 3.5 mmol $l^{-1}$ $MgCl_2$; and 2.5 U of Taq DNA polymerase. The reactions were carried out with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 40°C for 3 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 7 min. The amplicons were electrophoresed in 2 % agarose at 80V for 4 h.

The gel images were compared and analyzed using the GelCompar II software. The fingerprints were analyzed using the Pearson correlation and cluster analysis was performed by use of the UPGMA algorithm. The fingerprints of the isolates under study were compared and a dendrogram was constructed from the fingerprints. Two strains were considered to be genetically related if their fingerprints showed 90 % or more similarity.

## RESULTS AND DISCUSSION

The clinical signs of *Riemerella anatipestifer* included greenish diarrhoea, lethargy, ocular and nasal discharge, tremors of head and neck and weight loss. Those samples which revealed the presence of bipolar organisms which were comparatively larger when compared to *Pasteurella* sp. were selected for further isolation and characterization of the organism.

Isolation of *R. anatipestifer* was attempted from 13 suspected outbreaks out of which 6 isolates could be obtained which were named as KAL-1, KNB-1, KML-1, KML-2, KML-3 and KTL-1. Among the samples collected, liver and brain were found to be ideal organs for the isolation compared to lung, heart and ovary. The samples collected were streaked on 5 % sheep blood agar and incubated for 24–48h at 37°C in an atmosphere containing 5% $CO_2$. Following incubation mucoid, convex, grayish-white and non-haemolytic colonies were obtained. The isolates, which were non-motile, Gram-negative and coccobacillary in nature and showed no growth on MacConkey's agar were selected for further identification studies. In the second stage of isolation those isolates which were catalase and oxidase positive, indole negative, ornithine decarboxylase negative and gelatin liquefaction positive, were selected for 16S rDNA sequencing based confirmation.

All the selected isolates were subjected to 16S rRNA based PCR. The annealing temperature was optimized at 54°C. All the 6 field isolates were successfully amplified with a 665bp product (data not shown) as depicted by Pala *et al*. (2013). The GenBank accession numbers obtained for these isolates and place of the outbreak are showed in Table 1. These sequences when subjected to multiple sequence analysis showed about 99 % identity with *R. anatipestifer* type strain ATCC 11845 ribosomal RNA partial sequence (NR 026025.1) retrieved from GenBank. The 16S rRNA gene is typically utilized for the determination of phylogenetic relationship among bacteria. Based on 16SrRNA gene sequence analyses, *R. anatipestifer* belongs to the family *Flavobacteriaceae* in RNA superfamily V (Subramaniam *et al.*1997). The 16S rRNA gene based PCR with subsequent sequencing is the fastest way to diagnose New Duck disease (Christensen and Bisgaard 2010, Pala *et al*. 2013).

Presently the 16S rRNA based PCR and subsequent sequencing of the obtained 6 isolates emphasized that the molecular findings were in good agreement with those obtained from direct microscopy and bacterial cultural

Table 1. GenBank accession numbers of partial 16SrDNA sequences of different isolates of *R. anatipestifer*

| GenBank accession No. | Isolate | Place of the outbreak |
|---|---|---|
| KF577849 | KAL-1 | Ambalapuzha |
| KF577850 | KML-1 | Mangombu |
| KF577851 | KML-2 | Mangombu |
| KF577852 | KTL-1 | Thrissur |
| KF577853 | KML-3 | Mangombu |
| KF577854 | KNB- 1 | Nedumudi |

characteristics. All the isolates exhibited 99 % similarity to the sequence retrieved from GenBank. The results obtained are consistent with previous studies where 99 % sequence similarity was reported between 2 *R. anatipestifer* and 6 *R. anatipestifer*-like strains (Ryll *et al.* 2001). When 4 published sequences were included in the analysis, similarity became 98–99 %. In an earlier study it was observed that the 16S rRNA genes of *R. anatipestifer* are more varied than previously known. However, this heterogeneity would not alter the phylogenetic relationship of the *R. anatipestifer* strains, when compared to other bacteria (Tsai *et al.* 2005).

Genetic diversity among the isolates was studied by means of ERIC-PCR. Detailed analysis of the fingerprints of the 6 isolates of *R. anatipestifer* obtained by ERIC-PCR revealed 10–12 bands per isolate which were of approximately 150 bp to 2000 bp in size (Fig. 1). One band approximately 700 bp was common to all 6 isolates. In some fingerprints, the major bands were less intense and the minor bands were difficult to be visualized, but the presence or absence of DNA fragments was very consistent. A dendrogram was constructed from the data and was analyzed. Cluster analysis of the gel pattern divided all the isolates into 4 variants namely E1, E2, E3, and E4 (Fig. 2). Two isolates were considered to be genetically related if their fingerprint showed at least 90% or more similarity. KML-1 and KML-2 showed 98% similarity and were grouped into E1. KNB-1 and KML-3 showed 94% similarity and formed E2 variant. The third variant E3 contained only 1 isolate which had 84% similarity to E2. E2 and E3 variants showed 74% similarity to the variant E1. KTL-1 fingerprint pattern was different from all other isolates indicating an additional variant, E4. By visual inspection, 3 bands approximately 850 bp, 1100 bp and 1300 bp were absent in the isolate KTL-1 compared with the other isolates. This was also recognized in the computer-assisted analysis, in which this isolate was clearly discriminated from all other variants.

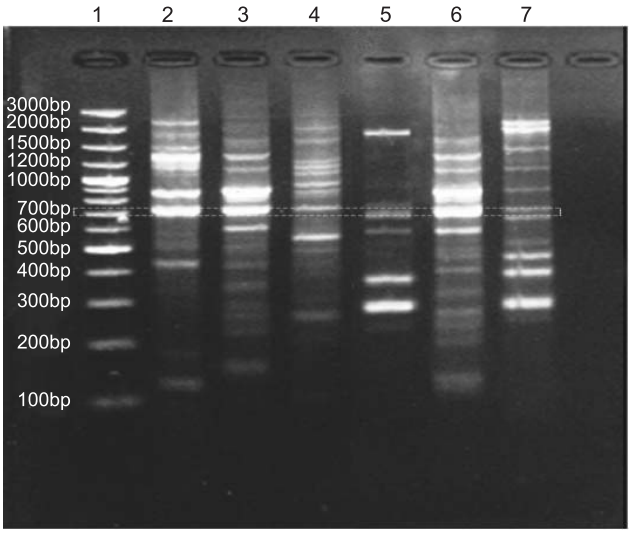


Fig. 1. ERIC-PCR amplification products of all the 6 isolates of *R. anatipestifer*. Lane 1: 100bp plus ladder, Lane 2: KAL-1, Lane 3: KNB-1, Lane 4: KML-1, Lane 5: KTL-1, Lane 6: KML-3, Lane 7: KML-2.

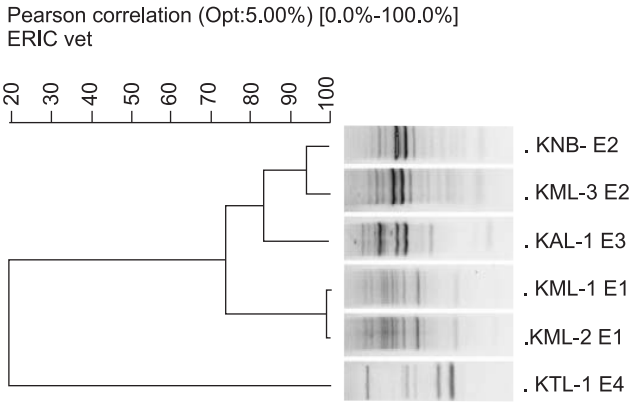


Fig. 2. Dendrogram of ERIC-PCR amplified products depicting the relationship between each isolate of *R. anatipestifer.*

Pioneers working in the field reported that among the 3 different repetitive elements based PCRs, *Salmonella* Enteritidis repetitive element (SERE)-PCR and BOX-PCR, did not produce patterns suitable for phylogenetic analysis of *R. anatipestifer* strains, whereas ERIC-PCR produced discriminative fingerprints (Kiss *et al.* 2007). Hence in this study, the authors selected ERIC-PCR to determine the genetic relatedness of the isolates. PCR amplification of bacterial genomic DNA with ERIC-PCR primer pair resulted in highly reproducible and unique banding patterns for different genomes. The variant E1 consisted of KML-1 and KML-2 with 98% similarity and these were isolated from the same flock aged 1–2 months. They exhibited clinical signs like dullness, ocular and nasal discharge, weight loss, photophobia, swollen limbs and died 2 days after the signs were shown. Fibrinous pericarditis and perihepatitis could be observed on gross examination of the birds. The isolates KNB-1 and KML-3, with 94 % similarity, constituting the variant E2, were obtained from geographically related areas of Kerala. The genetic homogeneity among these isolates suggested that they have a common source. Both the flocks exhibited clinical signs like greenish diarrhoea with tremors of head and neck whereas the gross lesions included marked congestion of meninges and fibrinousperihepatitis. The third variant E3 contained only 1 isolate which was 84 % similar to E2 which was collected from a postmortem case from Ambalapuzha, presented to Avian Disease Diagnostic Laboratory, Thiruvalla, Kerala. The affected flock comprising 2–3 month-old ducks showed nervous symptoms. Severe congestion of meninges was observed on postmortem examination. E2 and E3 variants showed 74 % similarity to the variant E1. KTL-1 fingerprint pattern was entirely different from all other isolates and included in variant E4 which was collected from an organized instructional farm in central Kerala. The affected flock consisted of 36 day-old ducklings showing severe fibrinous pericarditis. By comparing the different outbreaks which occurred in

geographically distinct areas of Kerala, it could be hypothesized that the variants obtained could be of different pathotypes resulting in infection with different degree of severity and clinical symptoms. Hence it can be concluded that the different variants obtained by the dendrogram of ERIC-PCR amplified products showed different clinical picture as well.

ERIC-PCR is used widely for typing of bacterial genomes based on the strain-specific fingerprints obtained (de Bruijn 1992). The DNA fragments in the bands of ERIC-PCR profiles are used to develop strain-specific probes or PCR primers for specific detection of bacteria in clinical or environmental samples (Giesendorf *et al*. 1993). Kardos and Kiss (2005) conducted molecular epidemiology investigations on fowl cholera and reported that the results of ERIC-PCR were concordant with the serotyping results. Extensive diversity among *R. anatipestifer* isolates was confirmed by serological typing (Sandhu and Harry 1981). But many strains could not be serotyped or produced cross reactions while typing (Rimler and Nordholm 1998). Molecular methods offer powerful, reliable and widely accessible tools to characterise *Riemerella* spp isolates (Kiss *et al.* 2007).

With the aid of molecular fingerprinting technique, isolates originating from the same flocks were found to be genetically homologous, and isolates originating from geographically different and distant regions of Kerala in India showed different fingerprints. The results are suggestive of the fact that genetic heterogeneity exists among *R. anatipestifer* infecting ducks of India. The variants obtained from commercial farms in Kerala were genetically more diverse compared to the variant associated with outbreak in organized farm. This interesting finding tempts the authors to speculate that the intense antibiotic use in commercial farms where flocks are routinely being monitored and treated for the prevailing diseases in the area, might have led to the emergence of newer types of the organism. More efforts are required to evaluate in-depth the molecular epidemiology of this pathogen in India and its impact in the duck industry. At present no vaccination strategies or preventive measures are available in India to protect the birds from the emerging New Duck disease. The information about diverse genetic make-up of *R. anatipestifer* would facilitate development of proper prevention and control strategy against the emerging New Duck disease in India.

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