RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 2: 211-217 (February 2023)

Isolation and Molecular Characterization of *Riemerella anatipestifer* from Domesticated Ducks of Assam, India

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10.18805/IJAR.B-4295

ABSTRACT

Background: *Riemerella anatipestifer* (*R. anatipestifer*) is a gram negative, microaerophilic, non-motile, bipolar bacteria. High genetic diversity and molecular differentiation were reported among field isolates. Although the bacterium causes one of the most economically important duck diseases in the north-eastern region of India, little work has been done on isolation, identification and molecular characterization of the bacteria. Hence, the present investigation was undertaken with a view to characterize the *R. anatipestifer* isolates from ducks of Assam.

Methods: Phenotypic and molecular identification of *R. anatipestifer* isolates from domesticated ducks of Assam, India were carried out during the period from February, 2019 to January 2020. A total of 624 samples (Ocular swab, throat swab, liver, spleen, kidney, brain, heart, lung) from ducks comprising of apparently healthy, ailing and dead ducks were collected from five districts of Assam, India were processed to isolate and identify the bacteria. The tentative identification of the bacteria was done based on phenotypic characteristics *viz*., colony morphology, growth characteristics and biochemical reactions. All the phenotypically positive isolates were further subjected to molecular identification based on PCR assay targeting *16S rRNA* gene and ERIC sequence.

Result: The bacteria could be isolated from different field samples. The highest percentage of the samples that yielded the bacteria are from brain (76%) followed by spleen (74%) of dead ducks and less number of ocular swab (33%) from apparently healthy ducks were found positive. Sequencing of the amplified product of the selected *R. anatipestifer* isolates targeting *16S rRNA* gene revealed homology percentage of 96.5-100%. Further, sequences representing five geographical locations were submitted to NCBI gene bank. Phylogenetic studies of the isolates indicated that there is prevalence of at least two genetically different strains of *R. anatipestifer* in the study area. The study suggested that the *R. anatipestifer* infection is endemic in Assam causing varying rate of morbidity (39%) and mortality (53%) and molecular based confirmation is necessary besides phenotypic identification.

Key words: *16S rRNA,* Isolation, Molecular characterization, PCR, *Riemerella anatipestifer.*

INTRODUCTION

Riemerella anatipestifer (*R. anatipestifer*) is a gram negative, microaerophilic, non-motile, bipolar bacteria under the family *Flavobacteriaceae* of the phylum Bacteroidetes (Sandhu, 2008; Gong *et al*., 2020). High genetic diversity and molecular differentiation was reported among the *R. anatipestifer* field isolates (Tsai *et al*., 2005; Yu *et al*., 2008). To date, more than 21 serotypes of *R. anatipestifer* have been identified worldwide with no effective cross-protection between them (Chang *et al*., 2019). Different serotypes show wide variations in virulence factors (Huang *et al*., 2002) and involvement of multiple serotypes has been reported in the same farm flock (Pala and Radhakrishnan *et al*., 2014).

The transmission of the disease occurs primarily through respiratory tract and skin abrasions (Sandhu 2003). The infection is highly contagious and produces fibrinous pericarditis, airsacculitis and perihepatitis in the infected ducks (Sandhu, 2008). It causes acute, subacute and chronic diseases in wide range of hosts, *viz*. domesticated duck, wild waterfowl, chicken and pig (Li *et al*., 2011). In ducklings, it causes severe, subacute infection while in adult duck, it causes chronic to subclinical infection (Sandhu 2003). The infection is more frequent and fatal in ducklings below 5 weeks of age than in adult ducks (Yu *et al*., 2008). Infection with *R. anatipestifer* results in huge economic losses to the duck industry due to reduced egg production, weight loss and mortality (Ryll *et al*., 2001; Sarver *et al*., 2005). During the recent times, frequent outbreaks of the disease have been recorded in many parts of the duck populated countries including India (Shome *et al*., 2004;

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How to cite this article: Doley, M.K., Das, S., Sharma, R.K., Borah, P., Sarma, D.K., Buragohain, L., Hazarika, R. and Baruah, N. (2023). Isolation and Molecular Characterization of *Riemerella anatipestifer* from Domesticated Ducks of Assam, India. Indian Journal of Animal Research. 57(2): 211-217. doi: 10.18805/IJAR.B-4295.

Submitted: 26-08-2020 **Accepted:** 23-03-2021 **Online:** 12-04-2021

Priya *et al*., 2008; Hazarika *et al*., 2020). The disease can cause morbidity as high as 100 per cent in ducklings and mortality ranging from 5 to 75 per cent (Zhong *et al*., 2009).

Diagnosis of the *R. anatipestifer* infection based on clinical signs and symptoms and phenotypic characteristics are often difficult due to resemblance of the organism with many other bacterial species such as *Pasteurella multocida*, *Salmonella* and *E. coli* (Sandhu 2003). For any disease control programme, proper isolation, identification and molecular characterization is a must for better understanding of disease pattern, pathogenesis and most importantly the prophylactic measures to be adapted. Molecular detection and characterization of *R. anatipestifer* infection targeting different genes such as *gyrB* gene, conserved *16SrRNA* gene, Ribonuclease Z gene *etc*. are highly specific and less time-consuming than the conventional methods (Tsai *et al*., 2005; Wang *et al*., 2012; Sarker *et al*., 2017). Although the bacterium causes one of the most economically important duck diseases in the north-eastern region of India, little work has been done on isolation, identification and molecular characterization of the bacteria. Hence, the present investigation was undertaken with a view to characterize the *R. anatipestifer* isolates from ducks of Assam.

MATERIALS AND METHODS

Collection of samples

Samples were collected from unorganized as well as organized duck farms from five districts of the state having the highest duck population (As per 19th Livestock Census, 2012), *i.e*. Kamrup, Dibrugarh, Sivasagar, Dhubri and Cachar. During the study, some relevant epidemiological data like number of outbreaks, duck population at risk, age group, number of ducks affected, morbidity and morbidity rate were also recorded. The study was carried out during the period of February 2019 to January 2020, in the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati.

A total of 27 field outbreaks in the five selected districts of Assam suspected of *R. anatipestifer* infection were attended and representative samples were collected. The samples (throat and ocular swabs) from apparently healthy as well as ailing ducks showing typical clinical signs and symptoms were collected in Hiculture sterile swab (Himedia) with or without 0.85 per cent saline. The dead ducks collected from the field outbreaks were subjected to post mortem examination and the gross lesions were recorded. Tissue samples, *i.e.* spleen, liver, kidney, lung, heart and brain were collected aseptically in sterile vials without adding any preservative and transported to the laboratory in ice pack.

Samples processing and Isolation

For isolating the bacteria, samples were inoculated into different growth media, *viz*. Blood agar (Brain Heart Infusion agar with 10% sheep defibrinated blood), MacConkey agar and BHIA agar following all aseptic measures and incubated at 37°C for 24 hrs in microaerobic condition created by candle jar method. Single suspected colonies were further subcultured to obtain pure bacterial cultures. The isolated bacteria were preserved at -20°C in 20 per cent glycerol for future use.

Identification of the bacteria

The preliminary identification of the bacteria was done based on phenotypic characteristics, *viz*. staining, cultural, morphological and biochemical characteristics following standard methods (Quinn *et al*., 2002).

Molecular confirmation of the organism was done by polymerase chain reaction (PCR) targeting amplification of *16S rRNA* (16S rRNA-PCR) specific for *R. anatipestifer* and Enterobacterial Repetitive Consensus (ERIC) fragments (ERIC-PCR), respectively as described by Cha *et al*. (2015) and Kardos *et al*. (2007) with slight modification. The amplified products of *R. anatipestifer* specific *16S rRNA* gene were sequenced and their identity was compared using NCBI-BLAST. The genomic bacterial DNA was extracted by hot cold lysis method and used as a template for PCR assay. Briefly, 700 µl of overnight grown pure colony in BHI were centrifuged at 10000 rpm for 10 minutes. The pellet was then collected and washed twice by suspending in 100 µl PBS followed by centrifugation at 10000 rpm for 10 minutes. The final pellet was suspended in 100 µl nuclease-free water and kept in a heating block at 100°C for 10 minutes. The content was transferred immediately into -20°C for 10 minutes and thawed. Finally, it was centrifuged at 10000 rpm for 10 minutes and the clear supernatant containing the genomic DNA was collected in a clean microcentrifuge tube without disturbing the pellet. Purity of the extracted DNA was checked by BioSpectrometer (Eppendorf) and stored at -20°C for future use. Two sets of reference primers (Table 1) were used for amplification of the targeted genes of *R. anatipestifer*. The annealing temperatures of the primers were standardized using a gradient thermocycler (Techne, UK).

Both the PCR assays were performed in 25 µl reaction mixture containing 12.5 µl master mix (DreamTaq, Thermoscientific), 1 µl (10 pmol/µl) each of forward and reverse primers, 6 µl of template DNA (60 ng/µl) and 4.5 µl of nuclease-free water under optimum conditions. To monitor contamination, a negative control was included without adding template DNA during PCR. The amplification for *16S rRN*A gene was performed with initial denaturation at 95°C for 5 min followed by 35 cycles at 94°C for 30 s, 54°C for

Table 1: Details of primers used for detection of *R. anatipestifer* isolates.

Primer gene	Primer sequence (5'-3')	Amplicon size	References
16S rRNA	F-GTATTGAAAGCTCTGGCGG	654 bp	Cha *et al.,* 2015
	R-TCGCTTAGTCTCTGAACCC		
ERIC sequence	F-TTACCGACTGATTGCCTTCTAG	546 bp	Kardos *et al*., 2007
	R-AGAGGAAGACCGAGGACATC		

40 s and 72°C for 30 s and a final extension cycle at 72°C for 7 min. Similarly, for ERIC-PCR, the amplification was carried out with an initial denaturation at 94°C for 5 min followed by 44 cycles of 94°C for 1 min, 61°C for 3 min and 72°C for 2 min and final extension at 72°C for 7 min. The amplified PCR products were separated by electrophoresis on 1.8% agarose gel. The gel was visualized and documented in a gel documentation system (Labonet International).

The amplified products of the *16S rRNA* gene were sent to 1st Base Apical Scientific Sdn Bhd, Malaysia for sequencing.

Pairwise sequence identity and distance of *16s rRNA* gene at the nucleotide level

Sequence Demarcation Tool (SDT) v1.2 (Muhire, 2014) was used to estimate the pairwise identity. The pairwise distance of *the 16S rRNA* gene of *R. anatipestifer* was estimated in MEGA X software. For estimation of pairwise sequence distance and identity of partial *16s rRNA* gene at the nucleotide level, except for the sequence of *R. columbina*, the same twenty sequences used in the phylogenetic analysis were taken into consideration.

Phylogenetic analysis based on *16S rRNA* gene

Fifteen reference nucleotide sequences of *16S rRNA* gene belonging to *R. anatipestifer* were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). Five partial nucleotide sequences of *16s rRNA* gene (654 bp) of *R. anatipestifer* isolates randomly selected from among the isolates obtained from different locations of Assam and submitted to NCBI with Accession nos. MN918292.1, MN918293.1, MN918294.1, MN918295.1 and MN918296. 1 were also included in phylogenetic analysis. One *16s rRNA* gene sequence of *R. columbina* was included as an outlier. In total, 21 nucleotide sequences were included in the phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) based on the Kimura 2-parameter substitution model (Kimura, 1980) and statistical significance of the tree was tested by bootstrap analysis (Felsenstein, 1985) of 1000 pseudo-replicates. Finally, the deduced phylogenetic tree was displayed using the online program iTOL (Letunic and Bork, 2019).

RESULTS AND DISCUSSION

During the study period, 38 villages of the five selected districts of Assam were surveyed based on reports of duck population and occurrence of clinical cases. Some epidemiological data like number of outbreaks, duck population at risk, age group, number of ducks affected, morbidity and morbidity rate were also recorded (Table 2). The overall mortality and morbidity were found to be 39% and 53%, respectively with variability in different age groups of the ducks. Higher mortality was observed in ducklings (57%) than adult ducks (19%). In a previous study, it was showed that the overall morbidity and mortality of duck may vary and upto 75% mortality were recorded depending on the age and level of stress factors (Subramaniam *et al*., 2000; Crasta *et al*., 2002). The mortality rate in ducklings below 8 weeks of age was found to be higher than adult ducks (Yu *et al*., 2008, Phonvisay *et al*., 2017). Various factors predispose susceptible ducklings to the disease such as concurrent disease, adverse climate conditions and nutritional status (Vandamme *et al*., 2006).

R. anatipestifer causes multisystemic, septicemic disease resulting in huge economic losses to duck farming (Ryll *et al*., 2001; Sarver *et al*., 2005). The clinical signs and symptoms during outbreaks include greenish-white diarrhoea, ocular and nasal discharges, off fed, paddling movement, torticollis, often accompanied with the tremor of head and neck, incoordination of movement (Priya *et al*., 2008; Deif *et al*., 2015). Diarrhoea along with neurological disturbances are often common symptoms in affected ducks (Sarker *et al*., 2017). The gross lesions observed on post mortem mostly consist of fibrinous perihepatitis, pericarditis, airsaculitis, congestion of lung, enlarged kidney, necrotic foci on liver and enlargement and congestion of spleen (Deif *et al*., 2015).

Isolation of *R. anatipestifer* is very difficult due to non-availability of selective media and special growth requirements accompanied by varying phenotypic characteristics (Hinz *et al*., 1998; Hess *et al*., 2013). Mostly the bacteria could be isolated from brain samples followed by spleen and least from ocular swab. According to Gooderham (1996), the most suitable tissue sample for isolation is the brain due to its less chance of contamination

Table 2: Some epidemiological data relating to *Riemerella anatipestifer* outbreaks.

Name of the District	Outbreaks attended (no. of Village)	Ducks at risk	Ducks affected (% morbidity)	No. of ducks died (% mortality) at different age groups			Total no. of ducks died (% mortality)
				Duckling (0-8 wks)	Grower (8-20 wks)	Adult (>20)	
Kamrup	7 (10)	1358	573 (42.19)	193 (61.66)	69 (19.48)	51 (16)	313 (55)
Sivasagar	5 (8)	2136	964 (45)	234 (50)	132 (28)	106 (22)	472 (49)
Cachar	5 (6)	1410	532 (38)	219 (66)	68 (21)	43 (13)	330 (62)
Dhubri	6 (7)	1280	474 (37)	159 (59)	62 (23)	47 (18)	268 (57)
Dibrugarh	4 (5)	1570	438 (28)	94 (45)	64 (31)	49 (24)	207 (47)
Total	27 (38)	7718	2981 (39)	899 (57)	395 (25)	296(19)	1590 (53)

Figures in parenthesis indicate percentage in the table.

with other bacterial species. In the present study, bacteria could be isolated equally from apparently healthy and ailing ducks which may be due to the reason that the bacterium is found as a commensal in the upper respiratory tract of healthy ducks (Ryll *et al*., 2001). Cha *et al*. (2015) reported isolation of the organism 69.6% of pharyngeal swab samples. Pathanasophon *et al*. (1994) isolated the bacteria from liver, lung, spleen and brain tissue samples. Bisgaard (1995) opined that ovary could be a suitable tissue sample for isolation as it causes chronic salpingitis in adult duck and geese.

The diagnosis of *R. anatipestifer* infection based on clinical signs and symptoms and phenotypic characteristics (cultural, biochemical, staining) is very difficult and inconclusive (Hinz *et al*., 1998). Hence, the tentatively identified bacteria were further subjected to confirmation by PCR targeting amplification of 16*S rRNA* gene and fragment of Enterobacterial Repetitive Consensus of the bacteria (ERIC-PCR). The PCR assay targeting *R. anatipestifer* specific *16S rRNA* gene segments indicated that it could amplify the gene in phenotypically positive pure RA field isolates with annealing temperature at 54°C for 40 seconds and the anticipated product (654 bp) was obtained following agarose electrophoresis (Fig 1,2). Sequencing of the amplified products revealed that all the isolates belonged to *R. anatipestifer* with an identity of 96.5-100% with similar sequences of the organism in GenBank. Similarly, other workers (Cha *et al*., 2015; Tsai *et al*., 2005) used the specific *16S rRNA* gene segments for detection and characterization of *R. anatipestifer* from wild waterfowl followed by sequencing. Qu *et al*., (2006) designed species-specific primers for detection of *R. anatipestifer* through multiple alignment of *16S rRNA* among *R. anatipestifer*, *E. coli*, *P. multocida*, *S. enteritidis*, *S. gallinarum* and opined that the primers could be used specifically detect the bacteria from clinical samples. The overall PCR positive sample (28) among the phenotypically identified positive samples (36) was recorded to be 78 per cent (Table 3). The highest number PCR positive samples was obtained from the brain (91%) followed by spleen and lung (87%) and ocular swab (69%).

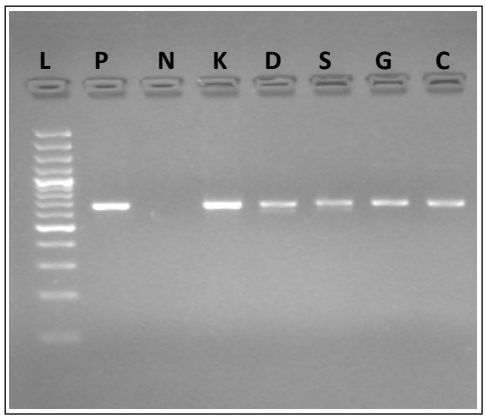

Fig 1: Electrophoresis analysis of PCR product of species specific *16S rRNA* gene (654 bp) of *R. anatipestifer*. L: 100 bp ladder; P: Positive control; N: Negative control; K, D, S, G, C field isolates.

The *16S rRNA* gene is considered to be highly conserved among the bacterial species; hence it is used for the detection of pathogenic bacteria (Kuhn *et al*., 2011). However, due to the low mutation rate (1% change in 5000 million years), it is sometimes difficult to discriminate between closely related bacterial species (Wang *et al*., 2012). The comparative study of application of *16S rRNA*-PCR and ERIC-PCR assay on bacterial isolates from different field samples showed similar positivity. The annealing temperature for ECRIC-PCR was standardized at 61°C by gradient PCR and obtained the anticipated product size of 546 bp (Fig 2). In contrast to our findings, several workers (Christensen and Bisgaard, 2010; Wang *et al*., 2012) opined that *R. anatipestifer* detection by PCR assay targeting *16S rRNA* gene segments show a false positive result due low genetic evolutionary nature of the gene among related bacterial species. The time required

Table 3: Detection of *R. anatipestifer* by PCR assay targeting *16Sr RNA* gene from phenotypically positive isolates.

Sample type		Phenotypically positive RA/No. of samplestested (% positive)	PCR positive samples/phenotypically positive samples (% positive)
Healthy ducks	Ocular swab	33/96 (34)	23/34 (68)
	Throat swab	49/96 (51)	37/49 (76)
	Subtotal (A)	41/96 (43)	30/41 (73)
Ailing ducks	Ocular swab	38/90 (42)	26/38 (68)
	Throat swab	40/90 (44)	29/40 (73)
	Subtotal (A)	39/90 (43)	30/39 (77)
Dead ducks	Liver	30/42 (71)	23/30 (77)
	Spleen	31/42 (74)	27/31 (87)
	Kidney	22/42 (52)	18/22 (82)
	Brain	32/42 (76)	29/32 (91)
	Heart	28/42 (67)	23/28 (82)
	Lung	23/42 (55)	20/23 (87)
	Subtotal (A)	28/42 (67)	23/28 (82)
	Total (A+B+C)	36/76 (47)	28/36 (78)

Figures in parenthesis indicate percentages rounded off to the nearest value.

for run PCR assay was found to be quicker (2.45 hrs) for amplification of *16S rRNA* gene, whereas it was 4 hrs for ERIC-PCR assay with similar positivity of samples. Thus, PCR assay targeting *R. anatipestifer* specific *16S rRNA* gene could be used for early diagnosis in face of disease outbreak without further sequencing in compared to ERIC-PCR. From the study of it can be concluded that the *R. anatipestifer* bacteria is prevalent among the duck population of Assam causing heavy morbidity and mortality. It could be further opined that the diagnosis of the infection based on phenotypic characteristics of the isolate has little value and molecular based PCR identification is must for confirmatory diagnosis of the disease.

Pairwise sequence identity and distance

The pairwise sequence identity analysis done by using SDT tool showed that the identity of *16s rRNA* gene sequences among the *R. anatipestifer* isolates were between 96.5-100% (Fig 4) Similarly, divergence ranged from 0 to 3.5% among the *16s rRNA* gene sequences of *R. anatipestifer* strains. Based on pairwise sequence identity, it was observed that isolates ASC/AAU/RA5, ASDi/AAU/RA4, ASS/AAU/RA3 and ASK/AAU/RA1 formed one molecular sub-group (Fig 4; highlighted in red box) whereas isolate ASD/AAU/RA2 was far from this molecular group. Among all the stains of *R. anatipestifer*, the largest divergence (3.5%) was exhibited by the isolate ASD/AAU/RA2. There were no significant divergence among ASC/AAU/RA5, ASDi/AAU/RA4, ASS/AAU/RA3 and ASK/AAU/RA1, whereas ASD/AAU/RA2 showed 1.7% divergence when compared with other four isolates from Assam. These findings additionally support the phylogenetic analysis which is suggestive of circulation of at least two different strains in Assam.

Phylogenetic analysis

The *16S rRNA* gene is the most conserved region among various ribosomal genes and considered as “evolutionary

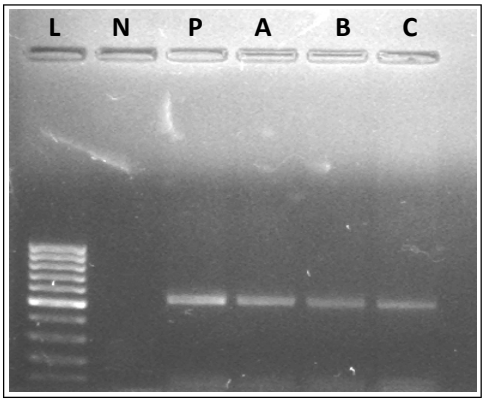

Fig 2: Electrophoresis analysis of ERIC-PCR product of RA (546 bp) of *R. anatipestifer*. L: 100 bp ladder; N: Negative control; P: Positive control; A, B, C field isolates.

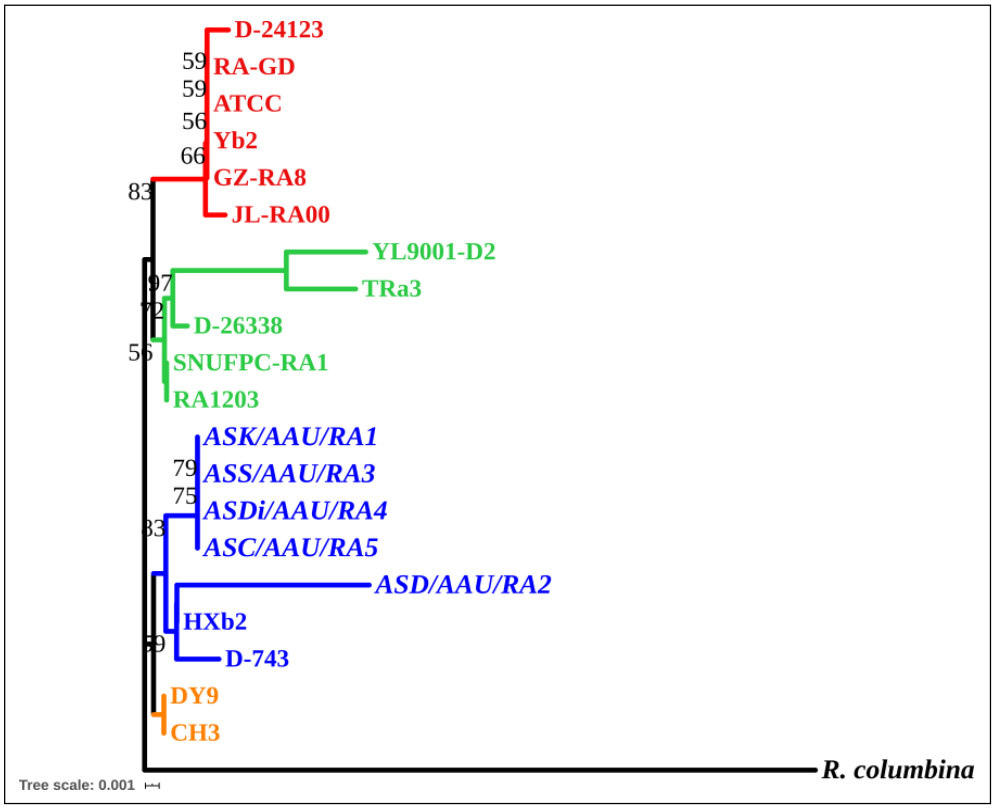

Fig 3: Phylogenetic tree of *16S rRNA* gene sequences of *R. anatipestifer* strains. Numbers along the branches refer to the bootstrap value (percentage of confidence). The five *R. anatipestifer* isolates of Assam used in this analysis were highlighted with blue italics letter.

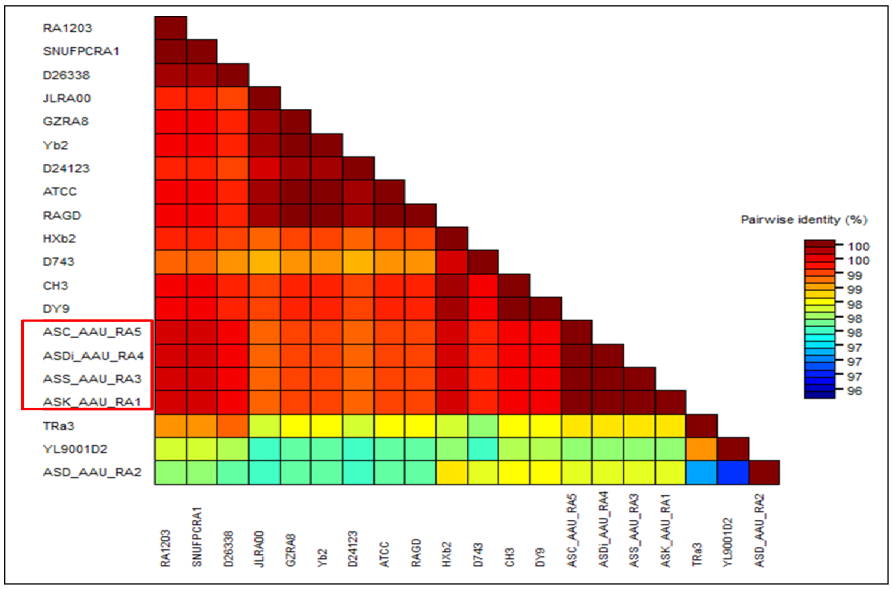

Fig 4: Pairwise identity matrix of *16S rRNA* gene sequences of various *R. anatipestifer strains*. A colour-coded pairwise identity matrix was generated using SDT program. Each coloured cell represents a percentage of identity score between two sequences (one indicated horizontally the other vertically). Four *R. anatipestifer* isolates of Assam forming a sub-group are demarcated by red box.

clock" (Woese, 1987). The phylogenetic tree constructed by the Neighbour Joining method using *16s rRNA* gene sequences of *R. anatipestifer* depicted four distinguished clades (red, green, orange and blue) (Fig 3,4). It was observed that all the five *R. anatipestifer* isolates from Assam recovered in the present study formed a single clade (blue, highlighted in italics) with two reference strains HXb2 and D-743 of China. Moreover, within the clade, it was observed that isolates ASC/AAU/RA5, ASDi/AAU/RA4, ASS/AAU/RA3 and ASK/AAU/RA1 formed one sub-clade, whereas ASD/AAU/RA2 formed another sub-clade with HXb2 and D-743 strains. The analysis revealed that *R. anatipestifer* circulating in Assam is closely related to the Chinese strains of the organism and at least two different strains are prevalent in the study area. The genetic similarity observed with the Chinese isolates may be due to spread of the bacteria through migratory waterfowl along with sharing of international boundary between the countries. In a similar study, Sarker *et al*. (2017) found that the Bangladeshi isolates of the bacteria has similarity with Chinese isolates.

CONCLUSION

The results of the present study suggested that *Riemerella anatipestifer* infection is highly prevalent (39%) among the domesticated duck population in Assam, India causing high morbidity and mortality among domesticated ducks. The different diagnostic approaches suggested that the diagnosis of the infection based on isolation and other phenotypic characteristics are often difficult, time-consuming and inconclusive and hence, molecular confirmation of the pathogen by PCR may be advocated. Phylogenetic studies of the isolates indicated that there is prevalence of at least two genetically different strains of *R. anatipestifer* in the study area. However, as the sample size in the present study was limited, there is a need of an in-depth systemic study to ascertain the molecular characteristics of the pathogen prevalent in the state so as identify suitable vaccine candidates for prevention and control of the disease.

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