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Isolation and Molecular Identification of *Riemerella anatipestifer* from Ducks in Assam

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A B S T R A C T

Considering the highest duck population in the eastern part of India, a preliminary study was carried out in Assam to investigate duck septicaemia like disease condition and the association of *Riemerella anatipestifer* in ducks. Among the 98samples collected from ducks (34) comprising healthy ducks (10), ducks with clinical symptoms (19), suggestive of duck septicaemia and dead ducks (5); 13 samples revealed isolation of *R. anatipestifer*. Molecular confirmation of the isolates, using *R. anatipestifer* specific PCR assay (564 bp), as well as *gyrB*-based PCR (162 bp) revealed both the genes to be suitable molecular markers for identification of the isolates as *R. anatipestifer*. Kirby-Bauer disc diffusion tests were used to analyze the antibiotic resistance of 13*R. anatipestifer* isolates. The present study is the first report of molecular detection of *R. anatipestifer* from ducks in Guwahati, Assam and suggests that the PCR assay can facilitate fast and accurate identification of *R. anatipestifer* infection in ducks during disease outbreaks.

Introduction

Among the states of India, Assam is considered to be the second-highest duck populated (7.21 million) state. Despite the natural resistance, ducks may be affected by many diseases prevalent in poultry. Out of the bacterial diseases, New Duck disease or duck septicaemia caused by *Riemerella anatipestifer*, a gram-negative, non-motile, nonspore-forming, rod-shaped bacterium, is considered to be an economically important disease throughout the world, resulting in high morbidity and mortality rates in ducks. It was reported and described by Riemer, for the first time in 1904 (Segers *et al.*, 1993). Kardos *et al.*, (2007) developed a novel PCR assay proved to be specific for *R. anatipestifer* and capable of correctly identifying it from pure cultures as well as

clinical samples from birds. In a recent study, Udayan *et al.,* (2019) reported the universal gyrase B-encoding gene (*gyr*B), a type II DNA topoisomerase as a more accurate, sensitive and specific marker for *R. anatipestifer* identification. *R. anatipestifer* infection can be treated and controlled by judicious use of suitable antibiotics. Therefore, the antibiotic sensitivity test is a must to ensure maximum drug effectiveness by minimizing the needless use of antibiotics to which *R. anatipestifer* is resistant.

Materials and Methods

Sample collection

During the months of March to June 2019, heavy mortality in ducklings (4-8 weeks of age) was recorded from Guwahati, Assam and its surrounding areas (Fig. 1) with notable signs of respiratory distress and neurological signs, such as trembling of head and neck, paddling of legs and ataxia (Fig. 2). None of the ailing and dead ducks, subjected to the Dept. of Microbiology, College of Veterinary Science, Khanapara, Guwahati (Assam) could reveal duck plague infection, confirmed by PCR based detection of duck plague specific DNA directed DNA polymerase (UL-30) gene. For the bacteriological investigation, a total of 98 nos. of clinical samples, comprising of ocular and pharyngeal swabs, liver, lungs, kidney, brain, spleen and heart blood were collected from apparently healthy ducks (10), clinically affected ducks (19) and dead ducks (5) (Table 1). Samples were collected aseptically and immediately processed for bacteriological examination.

Isolation and identification

Collected samples were processed for isolation of *Riemerella anatipestifer* by inoculating into 10 ml of Brain Heart Infusion (BHI) broth (Hi-media) with overnight incubation at 37°C under a micro-aerophilic condition in a candle jar. Blood agar (BA) plates were inoculated with inoculum from overnight BHI broth culture and further incubated for 24 hrs in micro-aerophilic condition at 37°C inside a candle jar. Based on colony morphology and staining reaction, exhibiting characteristic Gram-negative bipolar short rods were tentatively identified as *R. anatipestifer* and were considered for molecular confirmation.

Molecular confirmation of *R. anatipestifer*

All the morphologically identified *R. anatipestifer* isolates were considered for molecular confirmation by Polymerase Chain Reaction (PCR). Template DNA was prepared from each tentatively identified *R. anatipestifer* isolates by hot cold lysis method (Titball *et al.,* 1989) and assessed for molecular confirmation by *R. anatipestifer* species-specific PCR (546 bp) and *gyrB*gene (162 bp) based PCR.

Riemerella anatipestifer species-specific PCR

DNA extracted from all the tentatively identified field isolates were singly determined for *Riemerella anatipestifer* by species-specific PCR assay (Kardos *et al.,* 2007), with the primer sequence mentioned in Table 2. Simplex PCR was carried out with reaction mixture, comprising of 3.0 µl (100-150 ng) of respective template DNA, 12.5 µl of 2 X Dream Taq master mixture (Fermentas), 0.7 µl (10 pmol conc.) of each forward and reverse primers and sufficient Nuclease-Free Water (NFW) to make the final vol. of 25.0 µl. One additional negative control without template DNA was included to monitor any contamination.

PCR was carried out in thermocycler (Techne, USA) with cycling conditions, mentioned by

Sarker *et al.,* (2017) with slight modification (Table 3). The amplified PCR products were electrophoresed in 1.5 % agarose gel stained with ethidium bromide for 1 hr at 50V and were visualized by UV light in the gel documentation system (DNR Bioimaging System Minilumi, Sigma). The amplified DNA was sequenced from Eurofins Genomics India Pvt. Ltd. Bengaluru, Karnataka, India and sequence result was validated by performing a sequence alignment with *R. anatipestifer* specific gene sequences in GenBank, using genetic analysis software.

Molecular Detection of *gyrB* gene

Extracted DNA of respective *R. anatipestifer* isolates were further tested for the presence of a *gyrB*gene (162 bp) by simplex PCR, as mentioned by Udayan *et al.,* (2019) with slight modifications. Details of primers sequences for *the gyrB* gene are tabulated in Table 1. The composition used in 25.0 µl reaction mixture was the same as that of *Riemerella anatipestifer* species-specific PCR assay except for an additional 0.5 µl of $MgCl_2$ (25mM). PCR programme for amplification of the *gyr*B gene was done with reported thermocycling condition as shown in Table 3.

Antimicrobial Resistance pattern of *Riemerella anatipestifer* isolates

Riemerella anatipestifer isolates recovered during the study were characterized in respect to their resistance pattern towards few commonly used antimicrobials (HiMedia Lab, Mumbai), *viz.,* ciprofloxacin (5 mcg/disc), enrofloxacin (10 mcg/disc), ofloxacin (5 mcg/disc), streptomycin (10 mcg/disc), neomycin (30 mcg/disc), lincomycin (15 mcg/disc), gentamicin (30 mcg/disc) and cefazolin (30 mcg/disc) by Kirby-Bauer disk diffusion susceptibility test (Bauer *et al*.,1966).

Results and Discussion

Bacteriological examination of98 clinical samples revealed 41 samples to be bacteriologically positive, yielding an equal no. of isolates under micro-aerophilic environment. Isolates with a typical non-haemolytic colony, gram-negative and bipolar staining reaction and non-motile short rods were tentatively identified as *R. anatipestifer*. Similar cultural, morphological and staining characteristics were also reported by Surya *et al.,* (2016). However, they also recorded one isolate to be haemolytic.

Riemerella anatipestifer species-specific PCR assay

Screening of all 41 morphologically identified isolates for PCR based identification could confirm 13 isolates to be *R. anatipestifer*. All confirmed isolates were found to exhibit the desired amplicon size of 546 bp for *R. anatipestifer* species-specific PCR assay (Fig.3). Majority of the *R. anatipestifer* isolates were recovered from ocular swabs (6) and pharyngeal (5) of clinically infected ducks, while only two isolates from infected liver of dead ducks were confirmed to be *R. anatipestifer* positive. However, *R. anatipestifer* species-specific PCR assay could not reveal the presence of *R. anatipestifer* in any of the suspected isolates from lungs, kidneys, spleen, brain and heart blood of clinically infected ducks. Amplified product with band size 546 bp, suggestive of *R. anatipestifer* species-specific gene was confirmed by sequencing and validating with the NCBI website (Table 3). PCR based identification affirmed all the samples of apparently healthy ducks to be negative for *R. anatipestifer*.

Considering the sporadic duck population in India, a very little in-depth study could be traced out from available literature in respect

to the duck disease of bacterial origin. An *R. anatipestifer* like infection in the duck population, as reported by Sarma *et al.,* (1985) may be considered to be the earliest report from Assam, India. Similarly, Shome *et al.,* (2004) observed an outbreak in ducks of Meghalaya, India. However, they identified the outbreak of the infections, based on clinical manifestation and pathological alteration at necropsy. Priya *et al.,* (2008) could also record a similar type of infection from Kerala for the first time in duck. Association of *Riemerella anatipestifer* with all these previous studies from India were based on conventional isolation, identification and biochemical characterization, which proved to be tedious and problematic in differentiating with *Pasteurella multocida.*

Table.1 Collection of samples for isolation of *Riemerella anatipestifer* isolates

Nature of samples	**No. of samples screened for *Riemerella anatipestifer***	**No. of isolates positive for the *Riemerella anatipestifer* specific gene**	**No. of isolates positive for *Riemerella anatipestifer* specific *gyr*B gene**	**Samples confirmed for *Riemerella anatipestifer***
1. Apparently Healthy Birds (10)				
Ocular Swabs	10	0	0	0
Pharyngeal Swabs	10	0	0	0
Total	20	0	0	0
2. Clinically Affected Birds(19)				
Ocular Swabs	19	6	6	6
Pharyngeal Swabs	19	5	5	5
Total	38	11	11	11
3. Dead Birds(5)				
Ocular Swabs	5	0	0	0
Pharyngeal Swabs	5	0	0	0
Liver	5	2	2	2
Lungs	5	0	0	0
Kidney	5	0	0	0
Spleen	5	0	0	0
Brain	5	0	0	0
Heart blood	5	0	0	0
Total	40	2	2	2
Grand total	**98**	**13**	**13**	**13(13.3%)**

Table.2 Primers used to characterize the *Riemerella anatipestifer* isolates

Primer	Sequence (5′-3′)	Target	Product size(bp)	Reference
***Riemerella anatipestifer*-F**	TTACCGACTGATTGCCTTCTA	*Riemerella anatipestifer*s pecies-specific gene	546	Kardos *et al*., (2007)
***Riemerella anatipestifer*-R**	AGAGGAAGACCGAGGACATC			
***gyr*B-F**	GGCTAAGGCAAGACAAGCTG	*gyr*B *gene*	162	Udayan *et al*., (2019)
***gyr*B-R**	GCAGTTCCTCCTGCAGAGTC			

Table.3 Thermocycling conditions for molecular detection of *Riemerella anatipestifer* species-specific and *gyr*B genes

Target gene	Conditions	Temperature	Duration	No. of Cycles
***Riemerella anatipestifer* species-specific gene (Sarker *et al*., 2017)**	Initial Denaturation	95˚C	5	
	Denaturation	94˚C	1	44
	Prime Annealing	61˚C	3	
	Extension	72˚C	2	
	Final Extension	72˚C	7	
***gyr*B *gene* (Udayan *et al*., 2019)**	Initial Denaturation	95˚C	4	
	Denaturation	95˚C	1	35
	Primer Annealing	55˚C	1	
	Extension	72˚C	1	
	Final Extension	72˚C	7	

Table.4 Detail of BLAST output for the sequenced PCR products

Target gene	Description of the top result	Gen Bank accession	Max identity (%)	*E*-value
***R.anatipestifer specific gene (Riemerella anatipestifer** strain D-26220 RNAse Z and xanthosine triphosphatepyrophosphatase genes, partial cds).*	*Riemerella anatipestifer* strain D-26220 RNAse Z and xanthosine triphosphate pyrophosphatase genes, partial cds.	**JN578235.1**	96.74	0

Figure.1 Geographic origin of the samples collected for this study

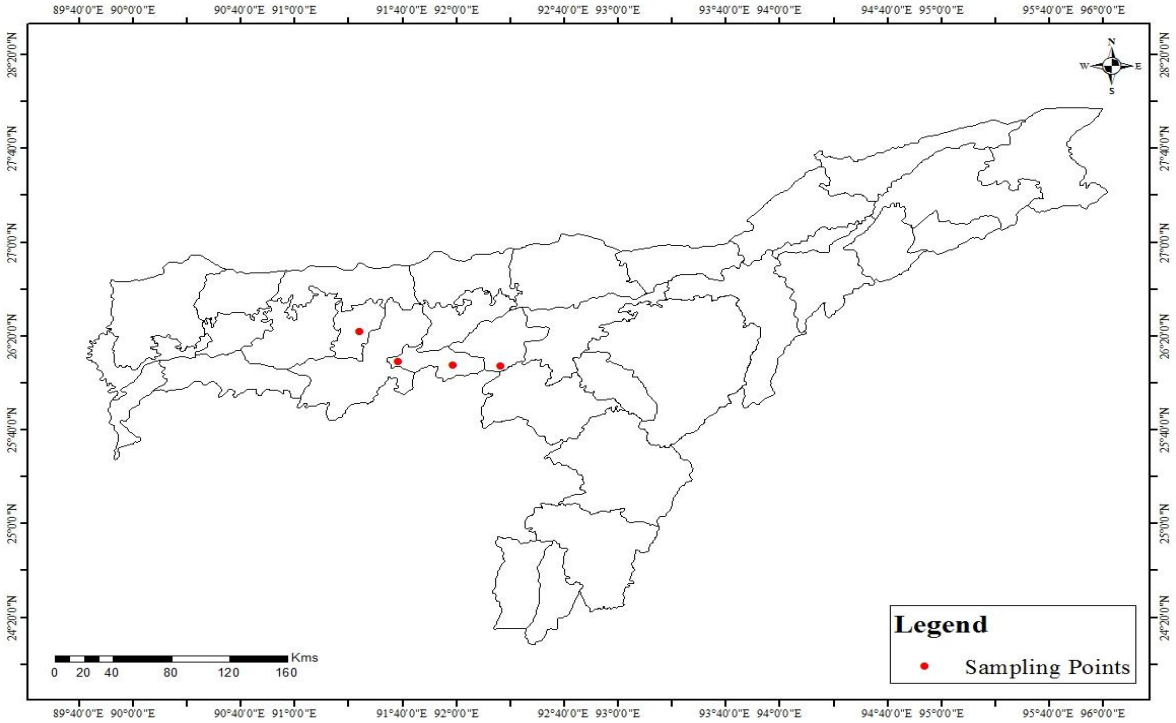
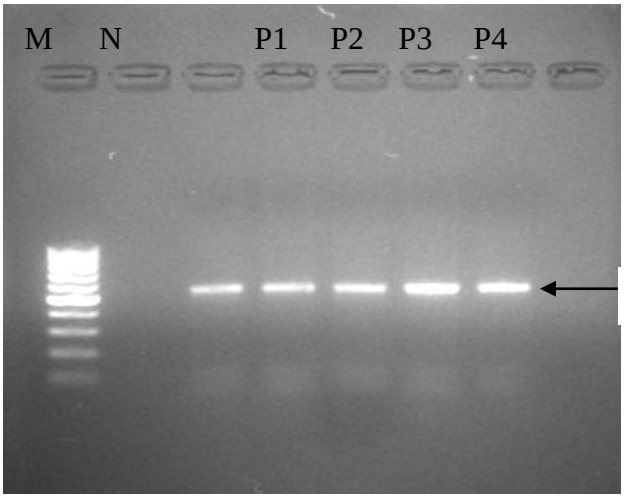


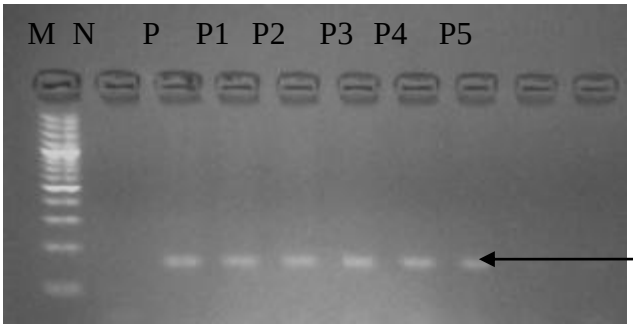
Figure.2 Clinically affected ducks

Figure.3 Detection of *Riemerella anatipestifer* species-specific PCR assay in the field isolates



M=Marker, Lane N= Negative control, Lane P to P4= field isolates positive for *R. anatipestifer*species-specific gene (546 bp).

Figure.4 Detection of *Riemerella anatipestifer* specific *gyr*B gene in the field isolates by PCR



M=Marker, Lane N= Negative control, Lane P to P5= field isolates positive for *R. anatipestifer* species-specific gene (162 bp).

Considering the phenotypic similarity with *P. multocida* and importance of *R. anatipestifer* infection, Kardos *et al.,* (2007) successfully presented a suitable molecular-based method with designed primers for proper identification of *R. anatipestifer* from cultures. They recorded 546 bp PCR amplicon size in all the 72 clinical isolates of *R. anatipestifer*. Contrary to present observation, Sarker *et al.,* (2017) could confirm 37 out of 60 suspected *R. anatipestifer* isolates, based on visible 421 bp fragment of ribonuclease Z gene, using the same primer sequence of Kardos *et al.,* (2007) for 546 bp size gene. In support of the 421 bp amplicon size, instead of 546 bp, they opined for a probable mutation in the amino acid sequence of isolates from Bangladesh. Confirmation of two suspected *R. anatipestifer* isolates, based on detection of a species-specific gene with an amplicon size of 546 bp was also reported by Shancy *et al.,* (2018).

Molecular detection of *gyrB* gene

Exploring for the universal gyrase B-encoding gene could reveal the presence of the *gyr*B gene in all the 12 isolates that were found

positive for *R. anatipestifer* species-specific PCR assay. All the isolates could exhibit a band size of 162bp (Fig.4). Considering the limitation of 16S rRNA as molecular marker to differentiate closely related bacteria, because of low mutation rate, the DNA gyrase, subunit B associated gene **(***gyr*B) was identified as an equally good marker sequence for the classification of bacteria at the species and subspecies level (Kumar *et al.,* 2006 and Takeda *et al.,* 2010). In a comparative study, Wang *et al.,* (2012) could confirm gyrB-based PCR to be more consistent and was more specific (100.0%) for detection of *R. anatipestifer* strains in comparison to the 16S rRNA based PCR (63.6%). In similar comparison with a pair of PCR primers designed for *gyr*B gene sequence of *R. anatipestifer,* Udayan *et al.,* (2019) concluded the *gyr*B-based PCR to be more accurate than 16S rRNA sequence-based PCR with confirmation of *R. anatipestifer* in a record 93.3% of throat swabs. They also opined for a faster evolutionary rate of the *gyr*B gene as main criteria behind its selection and application.

Antimicrobial Resistance pattern of *Riemerella anatipestifer* isolates

Among the 8 different antimicrobial agents used, the isolates were found to be highly sensitive to enrofloxacin, ciprofloxacin, ofloxacin and neomycin; intermediately sensitive to streptomycin and lincomycin, and resistant to gentamicin as well as cefazolin. Upon comparing the results of the present study with that of previous works (Zhong *et al.,* 2009 and Surya *et al.,* 2016) conducted on antibiotic sensitivity of *R. anatipestifer*, the antibiotic sensitivity of *R. anatipestifer* is found to vary with time, thus it is best to perform antibiotic sensitivity test before prescribing and administering the best choice of antibiotic for treatment of *R. anatipestifer* infection in ducks.

In conclusion, the above findings represent the presence of *Riemerella anatipestifer* in the ducks during a disease outbreak. The present study is the first report of molecularly confirmed isolation of *R. anatipestifer* in ducks of Guwahati, Assam. The pathway of *R. anatipestifer* transmission was not established in this outbreak study. Hence, further studies are necessary to contribute to developing knowledge in *R. anatipestifer* infection from ducks of Assam.

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