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Identification of Ribonuclease Z Gene from an Outbreak of *Riemerella anatipestifer* Infection in Ducks of Bangladesh

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Abstract

Duck septicemia is a deadly disease of duck causing huge economic losses in duck rearing areas. Despite its importance, no study was devoted to unravel the *Riemerella* (*Pasteurella*) *anatepestifer* in Bangladesh. Therefore, a systemic investigation was carried out to identify the isolate targeting ribonuclease Z gene from an outbreak in 2013-2014. Samples were collected from sixty randomly selected dead ducks from three outbreaks areas of North East Bangladesh (Netrokona district). Histopathological, cultural and sugar fermentation test of all samples revealed the infectivity with *R. anatipestifer*. Polymerase chain reaction specific to *R. anatipestifer* identified the 421 bp fragment of ribonuclease Z gene in thirty seven cases. Identified gene of the isolate was sequenced (Accession No. KU992381) and phylogenetic lineage was analyzed for the first time in Bangladesh. The phylogenetic analysis showed that Bangladeshi isolate *R. anatipestifer* BD 2014 resembles highest similarity with the *R. anatipesrifer* isolated from ducks of china. This study provide evidence that duck septicemia could be the main culprit of mortality in growing ducks in the duck rearing areas. Further investigation is needed to identify secondary or co-infection state as well as the prevalence of this infection including the economic burdens in duck industry in Bangladesh.

Keywords: *R. anatipestifer*; Duck; PCR; Sequencing; Ribonuclease Z gene; Bangladesh

Introduction

The duck septicemia caused by the bacteria *R. anatipestifer* is a major disease of ducks throughout the world and causes significant economic losses due to high morbidity and mortality [1-3]. The *R. anatipestifer* is also pathogenic for turkeys, chickens, pheasants and waterfowl [4]. The disease was first described by Riemer in 1904 [5,6] and then reported by Hendrickson and Hilbert [7] who described it is a new serious and septicemic disease of duck. In Bangladesh, the disease was first reported by Mustafa et al. [8] and later by Haque [9] based on cultural and biochemical test.

There are >20 serotypes of *R. anatipestifer* and infection take place via the respiratory tract or through wounds of the skin particularly of the feet [1]. It causes the so-called *anatipestifer* syndrome or duck septicemia or new duck disease in ducks which is characterized by diarrhea, lethargy, respiratory (coughing, sneezing, nasal discharge) and nervous symptoms (ataxia, tremor of head and neck) [1,3]. Typically, ducklings of 1-8 weeks old are highly susceptible. Ducklings under 5-weeks old usually die 1 to 2 days after clinical signs appear; older birds may survive longer. Mortality rate may vary from 5 to 75% and morbidity is usually higher [3,10-12]. Stress factors, such as concomitant disease or adverse environmental conditions predispose ducklings to be infected with the disease [1,3].

Since few years, duck farming in the in North East Bangladesh (Netrokona district) facing problem with 35-65% mortality of ducks in 2013-2014 that was suspected as bacterial infection. Then, based on clinical signs and post mortem findings the disease was suspected as *R. anatipestifer* infection. Despite the importance of the disease and the considerable attention paid to it, the exact causes of mortality was not confirmed and we did not find a specific PCR assay and sequencing of specific gene of *R. anatipestifer* in Bangladesh. Therefore, a systemic investigation was carried out to diagnose the exact causes of morbidity and mortality of ducks or ducklings by analyzing the results of PCR and phylogenetic lineage, bacteriological, morphological, biochemical and histopathological properties.

Materials and Methods

Study design and collection of samples

Representative tissues from liver, spleen, trachea, lungs, brain and heart were collected from total 60 randomly selected sick and dead ducks from three outbreak areas (Kotwali, Khaliajuri and Kolmakanda Upazila) of Netrakona District, Bangladesh during July 2013 to June 2014. Laboratory tests were carried out in the Department of Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh. Samples were preserved in 10% formalin for histopathological analysis and at -80°C for DNA extraction and PCR detection of the *R. anatipestifer* bacteria and ribonuclease Z gene as well.

Culture and staining and biochemical tests for *R. anatipestifier*

Primary culture in Nutrient Broth and sub cultured on nutrient agar media, blood agar media, Eosin methylene blue (EMB) agar and Mac Conkey agar media were carried out [13]. The impressions

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smears were stained with Gram's stain and Leishman's stain according to standard techniques [13]. Retrieval of the culture was done at an interval of 30 days on blood agar medium for further characterization. The sugars fermentation tests included five basic sugars such as dextrose, sucrose, maltose, lactose, mannitol, glucose and dulcitol and other biochemical tests like Indole test, Catalase test, Methyl red (MR) and Voges Proskauer (VP) test were also carried out those were supportive to *R. anatipestifer* bacteria [13,14].

Histopathological study

Liver, spleen, heart, brain, lungs, kidney and trachea those preserved in 10% formalin were sectioned and stained with hematoxylin and eosin as per standard method [15]. The slides were observed under microscope at 530x magnification.

Genomic DNA extraction

20% suspension of affected liver was prepared for DNA extraction by crushing 20 mg of liver sample in liquid nitrogen and by adding PBS. Genomic DNA was extracted from prepared sample using Wizard® Genomic DNA purification kit (Promega BioSciences, LLC. San Luis Obispo, CA, USA) according to the manufacturer's instructions. The quantity of the extracted DNA was determined using a spectrophotomer. Briefly, 01 µL of extracted DNA was dissolved in 999 mL distilled water and the spectrophotomer analysis was carried out at 2600A and 2800A. The A260/A280 ratio more than 1.8 was considered reasonably pure DNA to use for this study.

PCR amplification

The forward (5′-TTACCGACTGATTGCCTTCTAG-3′) and reverse (5′-AGAGGAAGACCGAGGACATC 3′) primers previously used by Kardos et al. [16] were used in this study to amplify a 421 bp fragment of ribonuclease Z gene of *R. anatepestifer*. Isolated DNA was diluted in same concentration (60ng/ µL) for each sample. PCR program was carried out in a reaction mixture containing 5.0 µL (300ng) DNA template, 1.0 µL $MgCl_2$ (10 mmol), dNTP 1.0 µL (10 mmol), each primer 0.5 µL (20pmol)/reaction and 1.0 µL enzyme mix (1U/50 reaction). The PCR condition was optimized following different annealing temperature (50-60°C annealing temperature). The reaction was carried out in a thermal cycler (Eppendorf Mastercycler, Eppendorf, Germany) with an initial denaturation at 95°C for 5 min, followed by 44 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 3 min, and extension at 72°C for 2 min, and with a final extension step at 72°C for 7 min. Genomic DNA extracted from the pure bacterial culture of *R. anatipestifer* was used as known positive marker in every PCR reaction. The amplified PCR products were visualized by electrophoresis on a 1.8% agarose gel stained with ethidium bromide, illuminated by UV light in the image documentation system (Photo Doc, Labortek, Germany).

Sequencing and phylogenetic analysis of ribonuclease Z gene

The purified PCR products were sent to AIT biotech, Singapore, a commercial laboratory for sequencing. The amplified 421bp fragment of ribonuclease Z gene common to 37 isolates tested and appeared positive to PCR setting was sequenced. After sequencing, the sequences of Bangladeshi isolate of *R. anatipestifer* (*R. anatipestifer* BD 2014) were submitted to GeneBank (Accession No. KU992381). The sequences were analyzed using Basic local alignment system (BLAST) and deduced amino acid sequences were analyzed using Laser Gene software (DNASTAR, Madison, WI, USA). Homologues sequences of ribonuclease Z gene of other *R. anatipestifer* isolates were downloaded from the NCBI resources using Blast and phylogenetic analysis was carried out using MEGA5 software. The deduced amino acid sequences were aligned with other related sequences retrieved from the GenBank and percent identity and divergence were plotted.

Results

In this study, the infected ducks were 8-16 weeks of age with in-coordination, circling movement, head and neck tremor, diarrhea, ocular and nasal discharge and death in duck sheds or in low laying water bodies. At necropsy the predominant changes observed in carcasses were wide spread congestion and hemorrhages, grey color necrotic foci on liver, hemorrhages and congestion in trachea, lungs, spleen, brain, conical heart with sub-epicardial hemorrhages and enlarged kidney.

Cultural, staining and biochemical identification of *R. anatipestifier*

The bacteria showed diffused turbidity in nutrient broth; smooth, grey, glistening and dewdrop like colonies on nutrient agar; non hemolytic on blood agar plate but did not grow on MacConkey agar plate. The bacteria grown on EMB agar but did not produce metallic sheen. Gram's staining revealed the presence of single or in paired Gram negative short rod and Leishman's staining showed bipolar bacteria. Sugar fermentation test revealed that the bacteria ferment glucose, lactose, maltose, mannitol, dextrose, sucrose but did not ferment dulcitol. Catalase and MR tests were positive but Indole and VP test negative.

Histopathological study

Histopathological study revealed hemorrhages in all of the visceral organs. There was coagulative necrosis, deposition of fibrin, aggregation of reactive cells predominatly heterophils and proliferation of intrahepatic bile duct and bile duct epithelium in liver (Figure 1a). There was inflammed meningitis with leucocytic infiltration and fibrinous exudates. Infiltration of glial cells was present in the brain tissues (Figure 1b). In lungs, there were necrosis and filling of the airways and capillaries with edema fluid and fibrins. Multifocal aggregation of reactive cells and wide spread hemorrhages and congestion were predominant in lungs parenchyma (Figure 1c). Hemorrhagic tracheaitis with sloughed off mucosal epithelium and infiltration of leukocytes in submucosa were seen (Figure 1d).

Molecular detection and phylogenetic analysis

Results of PCR showed that out of 60 amplificon of 421 bp fragments of ribonuclease Z gene of *R. anatipestifer* of suspected cases, positive bands were produced in 37 cases (Figure 2). Nucleotide sequence similarities and divergence among the ribonuclease Z gene sequences of *R. anatipestifer* were showed in Figure 3. The sequence divergence within *R. anatipestifer* ribonuclease Z gene ranged from 1% to 5%. Homology or percent identity of Bangladeshi *R. anatipestifer* with other *R. anatipestifer* isolates is ranged from 93.2% to 98.2%. Phylogenetic analysis with the partial sequence of ribonuclease Z gene (*R/anatipestifer*/BD/2014) and similar gene sequences from GeneBank was carried out. Results of phylogenetic analysis of a fragment of ribonuclease Z gene revealed that the Bangladeshi isolate of *R. anatipestifer* is closely related with Chinese isolate and formed cluster with Chinese isolate (*R/anatipestifer*/Sichuan/China/2014) (Figure 4).

Mutation in ribonuclease Z gene of *R. anatipestifier*

The deduced amino acid sequences (ribonuclease Z gene) of *R.*

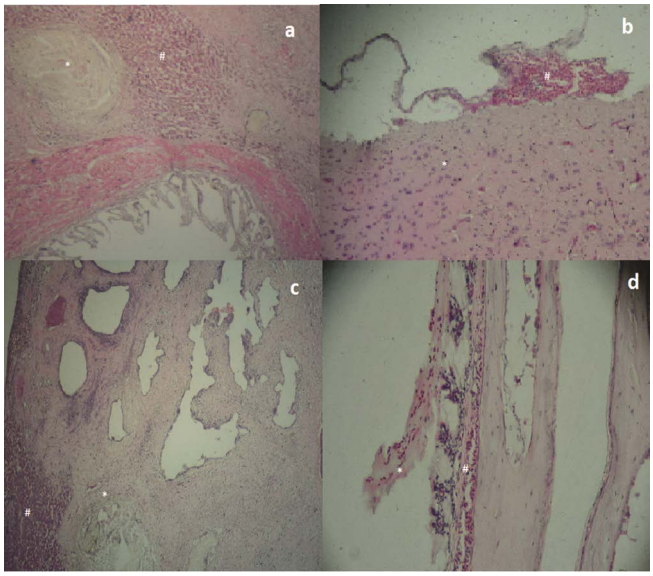

Figure 1: Histopathological findings: Tissue sections were stained with H&E stain and observed at 530x (a) liver section showing coagulative necrosis, deposition of fibrin* and aggregation of reactive cells predominating heterophils# in hepatic parenchyma. (b) Section of brain showing inflamed meninges with fibrinous exudates and leucocytes infiltration in the meninges# and increasing infiltration of glial cells in the brain tissues*. (c) Lung section showing the necrosis and filling of the airways and capillaries with edema fluid and fibrins* and multifocal aggregation of reactive cells in the necrotic area#. (d) Sloughed off trachea mucosal epithelium* with inflammatory cells in the submucosa# of inflammed trachea.

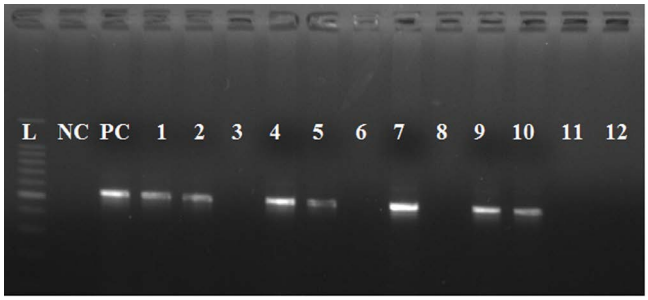

Figure 2: PCR detection of genomic DNA of *R. anatipestifer*. Lane L is for 100bp DNA ladder, lane NC containing PCR mixture without genomic DNA, lane PC containing known positive bacterial DNA obtained from the pure culture on agar medium. Lane 1 to 12 containing PCR amplicon onto the genomic DNA extracted from the liver of suspected ducks. Out of 60 selected ducks from the infected areas, *R. anatipestifer* specific 421 bp amplicon was found to generate in 37cases.

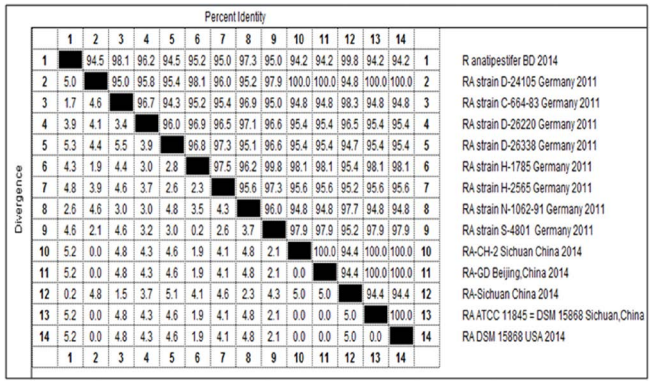

Figure 3: Divergence and percent identity of different strain of *Riemerella anatipestifer*.

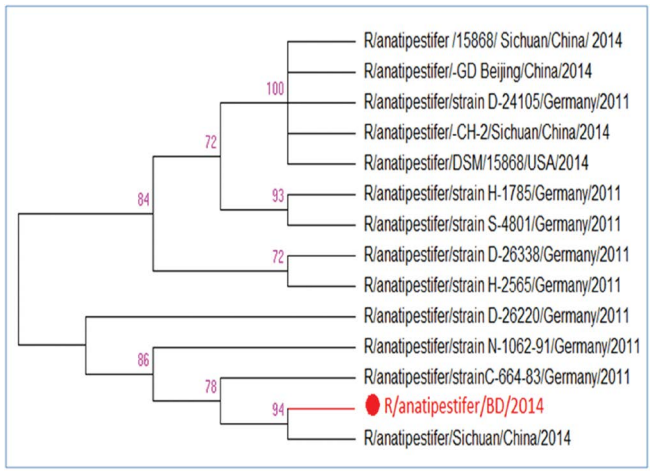

Figure 4: Phylogenetic tree of *Riemerella anatipestifer* based on nucleotide sequence. Maximum Likelihood evolutionary tree based on nucleotide sequence of *R. anatipestifer* organisms. Fourteen isolates were subjected to analysis including one Bangladeshi isolate. Bootstrap values (1000 replication) above 60% are shown next to the nodes.

Amino acid position	22	24	30	70	130	133	159
Consensus	M	K	S	A	M	G	V
Bangladeshi isolate	L	R	N	V	V	S	I
M: Methionine; L: Leucine; K: Lysine; R: Arginine; S: Serine; N: Asparagine; A: Alanine; V: Valine; G: Glycine; I: Isoleucine.							

Table 1: Specific Amino acid substitutions of *R anatipestifer* in different positions.

anatipestifer (Figure 5) revealed that, there were substitutions of amino acids in seven points (*R. anatipestifer* BD 2014) in relation with consensus sequence. Changes found in the amino acid positions 22, 24, 30, 33, 70, 133 and in 159. In amino acid position 22, M (Methionine) was replaced with L (Leucine), position 24 K (Lysine) with R (Arginine), position 30 S (Serine) with N (Asparagine), position 70 A (Alanine) with V (Valine), position 130 M (Methionine) with V (Valine), position 133 G (Glycine) with S (Serine) and at position 159 amino acid V (Valine) was replaced with I (Isoleucine) (Table 1).

Discussion

Outbreak of duck mortality in the duck rearing areas of Bangladesh caused a huge economic loss of duck farmers. It was a tremendous need to confirm the specific organism involved in this outbreak. Therefore, we proceed in a systemic way for definitive diagnosis of the disease which includes isolation of the organism suspecting *R. anatipestifer* and characterization of ribonuclease Z gene that is specific for all isolate of the *R. anatipestifer*. The result revealed maximum morbidity and mortality during June to July at the 8-10 weeks of age group where, earlier report suggested *R. anatipestifer* infection occur during summer [9] and 7-10 weeks of age of ducks [3,7,8]. Nervous signs observed in this study are suggestive to infection with *R. anatipestifer* [10,17,18]. At necropsy, grey color necrotic foci onto the surface of swollen, congested and hemorrhagic liver was characteristics and typical lesions for infection with either *R. anatipestifer* [8,19,20] or *P.*

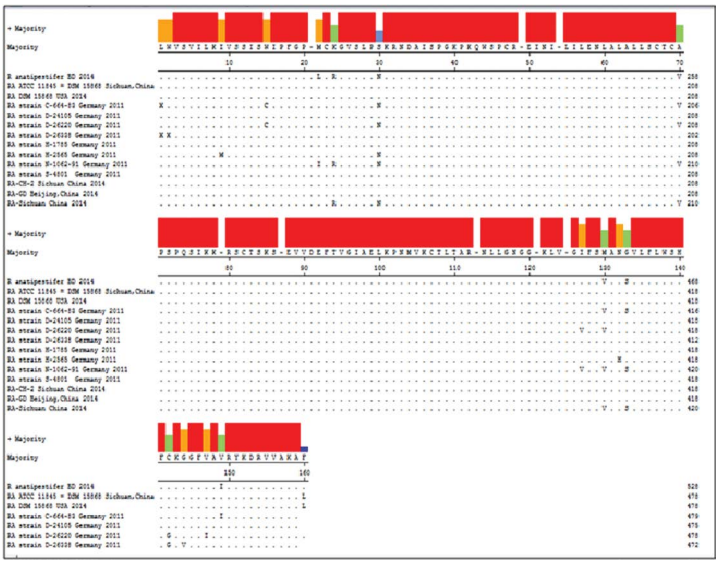

Figure 5: Alignment of deduced amino acid sequence of ribonuclease Z gene. Residues identical to the consensus are indicated by dots.

multocida (duck cholera) [21,22]. In this study, we also found all these similar morphological, cultural and staining characteristics which are the indicator of presence of *Riemerella* genus [3,5,11]. Results of biochemical tests of the present study showed that the bacteria ferment five basic sugars (dextrose, glucose, maltose, lactose and sucrose) but do not ferment dulcitol; positive catalase test and MR test, but negative Indole and VP test. Literature available indicated that *P. multocida* does not ferment lactose but positive in indol test, where *R. anatipestifer* ferment lactose and negative Indole test [23]. Results of the present study showed that the bacteria ferment five basic sugars including lactose and negative Indole test thus differentiated *R. anatipestifer* from *P. multocida*.

Histopathological changes observed in this study are also suggestive for *R. anatipestifer* infection. Liver lesions are similar with the finding of Pickrell [24] which revealed coagulative necrosis, heterophil infiltration, and hydropic degeneration of liver paranchymal cells. Similar airways and capillaries changes like necrosis, firinous and edematous swelling with multifocal aggregation of inflammatory cells in lung parenchyma were also reported by Graham et al. [11] in *R. anatipestifer* infection in earlier study. Similar changes in the central nervous system predominantly fibrinous meningitis with leukocytic infiltration in the meninges in ducks were reported [18,25] and suggested infectivity with to *R. anatipestifer* bacteria.

However, due to the presence of pathological, morphological, or biochemical similarity of *R. anatipestifer* and *P. multocida* and both can simultaneously be present in fowl stocks, the above tests are not sufficient to identify and differentiate specific infection. Hence, the bacterial isolation and identification of specific gene was carried out to identify the infectious etiology. PCR detection of *R. anatipestifer* bacteria and sequence analysis were therefore, carried out for the first time in Bangladesh to confirm the etiology of duck mortality. The PCR was carried out targeting ribonuclease Z gene of *R. anatipestifer* and absence of *P. multocida* organisms. The PCR protocol successfully generated 421 bp amplicon of 37 samples out of 60 samples tested and the studied bacteria were truly *R. anatipestifer*. Results of phylogenetic analysis showed that the Bangladeshi isolate of *R. anatipestifer* *R. anatipestifer* bearing similarity with the Chinese isolate. The free living migratory birds could have played pivotal role towards disseminating the bacteria in water fowls of Bangladesh and China. Analysis of deduced amino acid sequences of ribonuclease Z gene revealed that Bangladeshi isolate acquired mutation in amino acid positions 22, 24, 30, 33, 70, 133 and in 159. The sequence divergence within ribonuclease Z gene of *R. anatipestifer* ranged from 1% to 5% and percent identity of Bangladeshi *R. anatipestifer* with other *R. anatipestifer* isolates is ranged from 93.2% to 98.2%.

Conclusion

From the above study, it can be concluded that *R. anatipestifer* is a cause of major outbreak of duck mortality in duck rearing areas of Bangladesh. Further investigation is needed to design preventive and control strategy, identify secondary or co-infection state as well as the prevalence of this infection including the economic burdens in duck industry in Bangladesh.

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