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Characterization of *Pasteurella multocida* and *Riemerella anatipestifer* of Ducks in Assam, India

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ABSTRACT

Background: Among the various bacterial pathogens *P. multocida* and *R. anatipestifer* cause varying degrees of mortality and morbidity leading to huge economic losses among the duck farming community. Considering the lack of information regarding the characterization of *P. multocida* and *R. anatipestifer*, a study was undertaken to establish the prevalence of these diseases in ducks from different parts of Assam, together with the capsular and virulence-associated gene characterization and antimicrobial resistance.

Methods: A total of 235 samples comprising lungs, tracheal swabs, livers, heart blood and spleens collected from 47 diseased ducks, were screened for *P. multocida* and *R. anatipestifer*. The capsular and virulence-associated genes were amplified in isolated strains. The resistance pattern towards commonly used antimicrobial agents was studied by the disc diffusion method.

Result: The prevalence of duck septicemia and new duck disease was confirmed in ducks of Assam, India. Molecular tools were found to be highly specific besides phenotypic-based approaches in the detection, confirmation and differentiation of both bacteria due to phenotypic similarity. The antibiotic resistance patterns of both bacteria revealed a varying degree of sensitivity toward different antibiotics

Key words: Antibiotic resistance, *Pasteurella multocida*, *Riemerella anatipestifer*, Virulence genes.

INTRODUCTION

Duck is the second most important poultry species after chicken, reared for table egg production in India. Despite the natural resistance, ducks may be affected by many diseases prevalent in poultry. Among the various bacterial diseases, *Pasteurella multocida* associated duck pasteurellosis (fowl cholera) has been recognized as one of the prime causes of duck mortality (Amonsin *et al*. 2002), while Duck septicemia, caused by *Riemerella anatipestifer* is another important bacterial disease. In India, the disease has been reported in ducks from Meghalaya, Kerala and Assam (Priya *et al*. 2008, Hazarika *et al*. 2020). Due to their phenotypic similarity, species-specific molecular tests are recommended to differentiate *P. multocida* from *R. anatipestifer* (Ryll *et al*. 2001). Several host and pathogen-specific determinants are associated with the infections caused by *P. multocida*, among which, the capsular and virulence-associated genes are important (Katsuda *et al*. 2013). Despite the serious economic losses, the virulence factors associated with the pathogenesis of *R. anatipestifer* are yet to be explored.

Considering the increased popularity of duck farming in the North-Eastern part of the country, all-around supports are essential to raising disease-free duck farming by identifying the most common duck pathogens. Keeping the above facts in view, an investigation was attempted to characterize *P. multocida* and *R. anatipestifer*, prevalent in ducks from different parts of Assam.

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MATERIALS AND METHODS

The present investigation was initiated with 235 samples, comprising lungs, tracheal swabs, livers, heart blood and spleens collected from 47 diseased ducks. The clinical materials were obtained aseptically from the post-mortem section, College of Veterinary Science, Assam Agricultural University, as well as from the local markets and private duck farms in and around Guwahati, Assam during the period of January 2021-March 2022. The clinical samples of apparently healthy and diseased/dead ducks were subjected to bacteriological screening for *P. multocida* and *R. anatipestifer*.

The morphologically positive isolates were confirmed by simplex Polymerase Chain Reaction (s-PCR), targeting the species-specific (*kmt*1) gene (460 bp) and *R. anatipestifer-specific* Ribonuclease Z gene of 546 bp size. The genomic DNA (gDNA) was extracted from the respective isolates by the snap chill method. The *P. multocida-specific* PCR (PM PCR) was conducted as described by Deka *et al*. (2017). Similarly, the amplification of *the R. anatipestifer-specific* gene (RNAse Z gene**)** was done as reported by (Hazarika *et al*. 2020). The *kmt1-positive* isolates were subjected to molecular screening of capsular-specific gene(s) for capsular typing. The simplex capsular specific PCR (cap-PCR) was carried out, following the previously described protocol (Deka *et al*. 2017) with capsular specific primers for the *hyaD-hyaC* (type A), *bcbD* (type B) and *dcbF* (type D) gene. The reaction was performed with previously mentioned cycling conditions, followed by electrophoresis in a 1.5% agarose gel at 80 volts for 1 hr. The amplified products were visualized in a Gel documentation system (MiniLumi, DNR Bio-Imaging System, Israel). The identified capsular types of *P. multocida* were further subjected to molecular screening for distribution of certain virulence-associated gene(s), *viz*. the *ompH*, *ompA*87, *fim*A and *nan*B by standard s-PCR procedures (Tang *et al*. 2009) with the respective primers. An attempt was also made to characterize the *R. anatipestifer* isolate, for the *ompA* gene (1119 bp). The molecular screening was carried out with reported primer sequences and PCR conditions, as described by Yu *et al*. (2008).

Randomly selected isolates representing the respective capsular types of *P. multocida* along with *R. anatipestifer* recovered from duck were screened for their outer membrane protein (OMP) profile. The OMP fraction was extracted from the selected isolates, following the standard protocol (Wheeler *et al*. 2009). The sample mixtures were subjected to electrophoresis in stacking (5.0%) and resolving (12.0 %) gels to determine the protein profile, as per the protocol described by Laemmli *et al*. (1970). A pre-stained molecular weight marker was also run along with the samples.

In vitro drug resistance of *P. multocida* and *R. anatipestifer* isolates were tested against a panel of antimicrobial agents, *viz*., penicillin G (10 unit), sulfamethoxazole/trimethoprim (23.75/1.25 µg), doxycycline (30 µg), sulfadiazine (300 ìg), amoxicillin (10 µg), cloxacillin (10 µg), ampicillin (10 µg), erythromycin (10 µg), tetracycline (30 µg), ofloxacin (5 µg) and cefotaxime (30 µg) (Hi-Media, Mumbai) by using the disc diffusion method as per the performance standards M31-A3 of the Clinical and Laboratory Standards Institute.

RESULTS AND DISCUSSION

Isolation and molecular characterization of *P. multocida* and *R. anatipestifer* isolates

The screening of 235 clinical samples comprising of lungs, tracheal swabs, liver, heart blood and spleen of diseased/ dead ducks with involvement of respiratory and nervous system revealed *P. multocida* in 32(13.62%) samples. Recovery of *R. anatipestifer* was possible only in one tracheal swab (0.43%) of a duck showing severe nervous symptoms (Table 1). Among the *P. multocida* isolates, the highest recovery was observed in tracheal swabs (10), followed by lung and heart blood (9 each) and liver (4), while the affected spleens could not reveal *P. multocida*. Demonstration of species-specific *KMT*1 (460bp) gene could further confirm all the 32 isolates to be *P. multocida* (Fig 1A). Similarly, the lone *R. anatipestifer* isolate exhibited the species-specific *RNAse Z* (546 bp) gene (Fig 2A). Further screening of the confirmed isolates of *P. multocida* of clinically affected ducks revealed 18 (56.25%) isolates as capsular type A (Table 1), bearing capsular type-specific *hyaD-hyaC* gene of 1044bp size (Fig 1B), while 5(15.63%) were found to exhibit the type D specific *dcbF* gene of 657bp amplicon size (Fig 1C). The remaining 9 isolates were recognized as untypable (UT). Exploring for certain virulence-associated genes in the *P. multocida* isolates could indicate the distribution of the *ompH* gene (1000bp) along with the *ompA87* gene (838bp) in all the capsular type A and D isolates (Fig 1D and 1E). Demonstration of the *fimA* gene (866 bp) was possible in all the type D isolates, while 14 of the type A isolates could exhibit the *fimA* gene (Fig 1F). None of the capsular types A and D could reveal the *nanB* gene. All the untypable isolates were also found to bear the *ompH* gene, while five of the untypable isolates could exhibit the *ompA87* gene (Table 2). The untypable isolates were found to be lacking in the *fimA* and *nanB* genes. The only isolate of *R. anatipestifer* recovered from a diseased duck

Table 1: Isolation of *P. multocida* and *R. anatipestifer* from clinically affected duck.

Nature of sample	No. of samples examined	No. of isolates positive for *R. anatipestifer* specific RNAse *Z* gene	No. of isolates positive for *P. multocida* specific *KMT1* gene	*P. multocida* Capsular type		
				A	D	UT*
Lung	47	0	9	5	0	4
Liver	47	0	4	0	0	4
Spleen	47	0	0	0	0	0
Heart blood	47	0	9	3	5	1
Tracheal swab	47	1	10	10	0	0
Total	235	1 (0.43)	32 (13.62)	18 (56.25)	5 (15.63)	9 (28.13)

*UT= Untypable.

could exhibit the *OmpA* gene of 1119bp size (Fig 2B). Contrary to the low percentage of recovery in our study, Mbuthia *et al*. (2008) reported the isolation of *P. multocida* from 25.9% of apparently healthy ducks. Based on the observation of the *kmt*1 as a marker gene, Deka *et al*. (2017) opined that PM-PCR is a rapid, robust and highly specific confirmatory method for *P. multocida,* irrespective of serotypes. The capsular type A, followed by D and F have been established as the most prevalent capsular type of *P. multocida* in Indian duck. Like the Indian reports, the predominance of capsular type A, followed by type D was also previously recorded in ducks (Eldin *et al*. 2016). Recovery of untypable strains of *P. multocida* was found to be common in avian hosts (Arumugam *et al*. 2011). The loss of capsules following sub-cultivation might be a probable explanation for rendering circulated *P. multocida* in animal environments untypable (Dziva *et al*. 2008). Among the available literature from India, a low recovery rate of *R. anatipestifer* from suspected cases of duck septicemia was reported by Surya *et al*. (2016). A previous investigation, considered to be the first information from Assam on the prevalence of duck septicemia-like disease revealed recovery of *R. anatipestifer* from ocular and pharyngeal swabs of clinically affected ducks (Hazarika *et al*. 2020). Their study could demonstrate both the *R. anatipestifer* species-specific *RNAse Z* (564 bp) and *gyr*B(162 bp) genes as a suitable marker in the identification process. Contrary to the present observation, a recent study from Assam, India could record a higher recovery rate of *R. anatipestifer* from the brain (76%) and spleen (74%) of suspected duck

Table 2: Distribution of virulence-associated gene(s) in *Pasteurella multocida* isolates.

Capsular type	No of positive isolates positive	Isolates carrying virulence-associated genes			
		ompH	*ompA87*	*nanB*	*fimA*
A	18	18	18	0	14
D	5	5	5	0	5
UT	9	9	5	0	0
Total	32	32	28	0	19

*UT= Untypable.

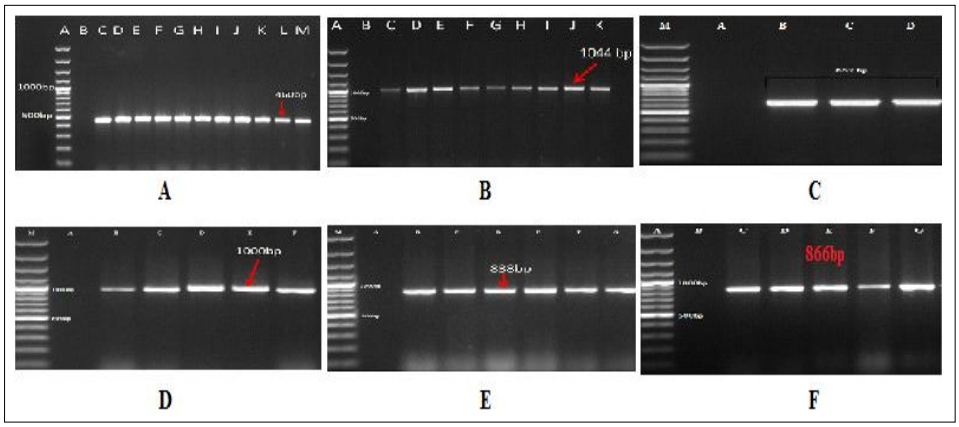

Fig 1: Amplified gene products in capsular type A and D *Pasteurella multocida*. A=Species specific *KMT1* gene (460bp), B= Type A specific *hyaD-hyaC* gene (1044 bp), C= Type D specific *dcbF* gene (657 bp), D= *ompH* gene (1000 bp), E= *ompA87* gene (838 bp), F= *fimA* gene (866 bp) in the field isolates.

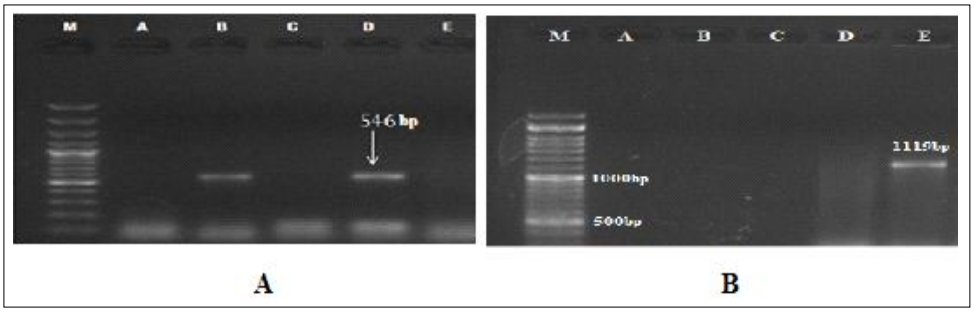

Fig 2: Amplified gene products in *Riemerella anatipestifer* isolate; A= species-specific RNAse Z gene (546 bp), B= *ompA* gene (1119 bp).

septicemia cases. Phylogenetic studies of the isolates revealed at least two genetically different strains in the study areas and suggested the *R. anatipestifer* infection as endemic in Assam with variable morbidity and mortality (Doley *et al*. 2021).

Outer membrane protein profiling of the *P. multocida* and *R. anatipestifer* isolates

The OMP fractions of representative isolates of the *P. multocida* type A and D revealed an almost similar protein profile. Both the isolates exhibited a total of 14 polypeptide bands with approximate molecular weight (MW), within the range of 14 to 63 kDa and above (Fig 3A). Among those visible protein bands, 14, 35, 37, 42 and 48 kDa proteins, shared by both types appeared as major proteins. A total of 10 polypeptide bands could be visible in the OMP fraction of a randomly selected UT strain of *P. multocida*, of which proteins of approximately 14, 32 and 42 kDa MW exhibited distinct bands. Among the five visible polypeptides in the OMP fraction of the lone *R. anatipestifer* isolate, a band of an approximate size of 40kDa was recognized as the predominant protein (Fig 3B). The available data on the prevalence of virulence-associated gene(s) in *P. multocida* of avian origin reflected the consistent distribution of *ompH* and *omp*A in capsular types A and D of *P. multocida*. However, there may be extensive molecular mass heterogeneity in the OmpA and OmpH proteins (Deka *et al*. 2017). Among the adhesion-associated genes, the *fimA* was identified in association with the pathogenic strains of *P. multocida* (Ewers *et al*. 2006). Contrary to the present observation, the distribution of the *nanB* gene was recorded previously in both type A and D isolates of *P. multocida,* even in the un-typable strains. Among the scanty information exploring virulence-associated gene(s) of *R. anatipestifer*, a study by Ahmad *et al*. (2017) could establish the utilization of *omp*A as a useful component in the field of diagnosis and control strategies for new duck disease. The *omp*A gene was found highly conserved among the *R. anatipestifer* isolates.

During a proteomic study on Indian isolates (type B) and the vaccine strain of *P. multocida*, the 32, 37, 72 and 89 kDa proteins appeared as the immunogenic OMPs (Somshekhar *et al*. 2014). The predominance of the 31, 33 and 37 kDa proteins was also previously reported from India in the OMP fraction of *P. multocida* serotype B: 2 (Tomer *et al*. 2002). However, the nucleotide sequence analysis of the *ompA* gene in the duck septicemia-associated *R. anatipestifer* isolate, the encoded protein of 387 amino acids with a molecular mass of 42 kDa as the major predominant, species-specific antigen in *R. anatipestifer* (Subramaniam *et al*. 2000).

Antimicrobial resistance pattern of the isolates

All the *P. multocida* isolated from suspected cases of duck pasteurellosis revealed penicillin G, sulfamethoxazole/ trimethoprim, sulfadiazine, cloxacillin, erythromycin and tetracycline resistant (Table 3), while resistance towards ampicillin and doxycycline was recorded in 76.0 and 68.0% of the isolates, respectively. Amoxicillin and ofloxacin were found to be effective antimicrobial agents for all the *P. multocida* isolates. Contrary to the *P. multocida* isolates, the single isolate of *R. anatipestifer* was found to be sensitive to most of the antimicrobial agents under test. However, the isolate was found to be resistant to penicillin G, sulfadiazine, ofloxacin and cefotaxime. An absolute resistance against sulfadiazine was previously demonstrated among 123 Indian isolates of *P. multocida* from different avian species; while chloramphenicol might be a more effective antibiotic for the treatment of *P. multocida* infection. The study could also reveal the emergence of multidrug-resistant strains of *P. multocida* among Indian poultry (Shivachandra *et al*. 2004). Development of resistance towards enrofloxacin, ciprofloxacin, ofloxacin, gentamicin, amikacin, ampicillin and penicillin were seen in a majority of avian *P. multocida*

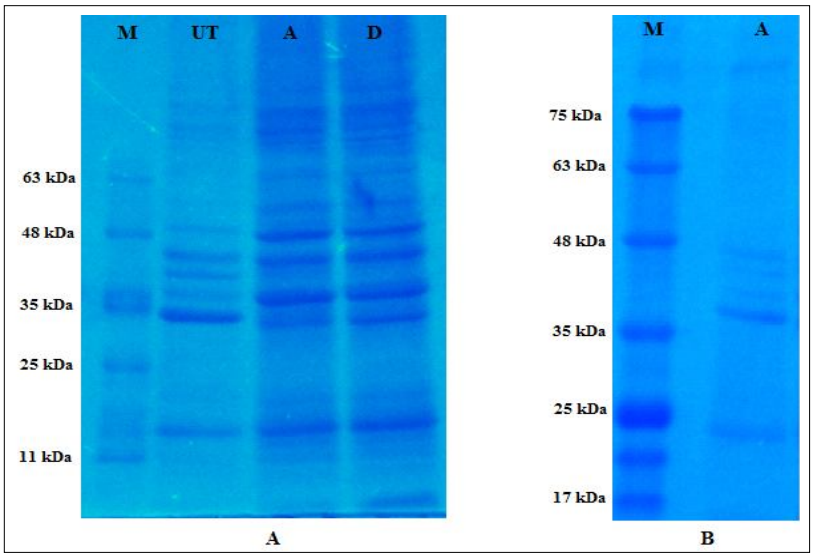

Fig 3: Outer Membrane protein profile of (A) *Pasteurella multocida* (Type A, D and untypable) and (B) *Riemerella anatipestifer*.

Table 3: Antimicrobial Resistance profile of *Pasteurella multocida* and *Riemerella anatipestifer*.

Name of Antibiotics	No. of *P. multocida* isolates showing resistance (%)	No. of *R. anatipestifer* isolates showing resistance (%)
Penicillin G	100.00	100.00
Sulfamethoxazole/trimethoprim	100.00	0.00
Doxycycline	68.00	0.00
Sulfadiazine	100.00	100.00
Amoxicillin	0.00	0.00
Cloxacillin	100.00	0.00
Ampicillin	76.00	0.00
Erythromycin	100.00	0.00
Tetracycline	100.00	0.00
Ofloxacin	0.00	100.00
Cephotaxime	100.00	100.00

isolates and recognized as a major hurdle for the Indian poultry industry (Balakrishnan *et al*. 2012).

The available literature on resistance patterns in the Indian isolates of *R. anatipestifer* could reveal most of the isolates are resistant to penicillin G, trimethoprim, ampicillin, amoxicillin and tetracycline (Hazarika *et al*. 2020). Due to the regular use in the treatment or as feed additives, the gradual development of sulfadiazine resistance among the *R. anatipestifer* isolates was also recorded. Ofloxacin and cefotaxime were also previously recorded as ineffective for *R. anatipestifer* isolates (Surya *et al*. 2016).

CONCLUSION

Based on the results obtained during the present study on the characterization of *P. multocida* and *R. anatipestifer* of duck origin, the prevalence of duck septicemia and new duck disease was confirmed in Assam, India. The present study also revealed the presence of concurrent infection of both *R. anatipestifer* in *P. multocida* in the same host. Molecular tools were found to be highly specific besides phenotypic-based approaches in the detection, confirmation and differentiation of both bacteria due to phenotypic similarity. The antibiotic resistance patterns of both bacteria revealed a varying degree of sensitivity towards different antibiotics. Hence, one must be careful in choosing a specific antibiotic regime in the face of a disease outbreak. However, as the sample size in the present study was limited, there is a further need for an in-depth systemic study on epidemiology for the prevention and control of duck diseases.

Conflict of interest: None.

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