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RESEARCH ARTICLE
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Isolation and polymerase chain reaction-based identification of *Riemerella anatipestifer* from ducks in Kerala, India

Manju Soman, Sreeja R. Nair, M. Mini, Binu K. Mani and Siju Joseph

Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India.
Corresponding author: Manju Soman, e-mail: manjuso1993@gmail.com, SRN: drsreejarnair@gmail.com, MM: drmmini@yahoo.co.in, BKM: binukmani@yahoo.com, SJ: siju96@gmail.com
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Abstract

Aim: The aim was to isolate and characterize *Riemerella anatipestifer* organisms from disease outbreaks in ducks in Kerala.

Materials and Methods: Ducklings, suspected of *Riemerella* infection, were sacrificed and subjected to post-mortem examination. Heart blood smears and impression smears from liver and spleen were examined for the presence of pathogenic organisms. Heart blood, lung, liver, and spleen collected aseptically from the birds were subjected to isolation trials in brain heart infusion agar and 10% bovine blood agar. The isolates were characterized based on morphology, cultural characteristics and biochemical tests, and their identity were confirmed by polymerase chain reaction (PCR) and the PCR amplified DNA was sequenced. The antibiotic sensitivity testing of the isolates were carried out using six antibiotics viz ciprofloxacin, chloramphenicol, enrofloxacin, amoxycillin, cotrimoxazole, and gentamicin.

Results: Colonies suggestive of *Riemerella* organisms could be isolated on blood agar. Biochemical characterization and PCR confirmed the identity of isolates as *R. anatipestifer.* The nucleotide sequence of the PCR product showed 99% homology to the *R. anatipestifer* sequences in the NCBI. The antibiogram revealed that the organisms were sensitive to ciprofloxacin, enrofloxacin, and gentamicin.

Conclusion: The present study suggests that the PCR assay can facilitate fast and proper identification of *R. anatipestifer* infection in ducks. The assay can also differentiate between *R. anatipestifer* and *Pasteurella multocida* and can replace the traditional methods of differentiation which are cumbersome and time-consuming.

Keywords: antibiogram, ducks, isolation, polymerase chain reaction, *Riemerella anatipestifer.*

Introduction

Riemerella anatipestifer is a major bacterial pathogen of avian species, causing severe economic loss in commercially important poultry worldwide. The disease is commonly referred as "new duck disease" and variously known as infectious serositis, duck septicaemia and anatipestifer syndrome. Affected ducks, usually 1-7 weeks of age, often develop ocular and nasal discharges, mild coughing and sneezing, tremors of the head and neck, and incoordination. In typical cases, affected ducklings may lie on their back with paddling movement of legs. Necrotic dermatitis on the lower back or around the vent may also be observed.

Mortality is usually 2-50%. Fibrinous polyserositis, pericarditis, perihepatitis, and airsacculitis are commonly observed as the gross post-mortem lesions. Infection of the central nervous system can result in fibrinous meningitis. Birds with chronic form of the disease may develop mucopurulent or caseous salpingitis leading to loss of egg production [1]. The large scale mortality inflicted by this disease has become a cause of major concern in commercial duck farmers in Kerala.

The present study reports the isolation, identification, and antibiogram of *R. anatipestifer* from different incidence of disease outbreak in Kerala.

Materials and Methods

Ethical approval

All the procedures have been carried out in accordance with the guidelines laid down by Institutional Ethics Committee and in accordance with local laws and regulations.

During the months of April and May 2013, heavy mortality in ducklings of age group of 4-8 weeks was reported from Central Kerala. The ducklings received for examination were designated as D1, D2, and D3. They showed signs of respiratory distress and neurologic signs such as trembling of head and neck, ataxia, and heavy mortality.

The ducklings were sacrificed and subjected to post-mortem examination. Heart blood smears and impression smears from liver and spleen were stained by Leishman's stain and examined for the presence of pathogenic organisms. Samples of heart blood, lung, liver, and spleen collected aseptically from the birds were used for isolation of the organism. The clinical samples were directly streaked on MacConkey agar, Brain heart infusion agar (BHIA) and 10% bovine blood agar. MacConkey agar and BHIA were incubated for 24 h at 37°C under ordinary conditions while

blood agar was incubated at 37°C for 24 h in an atmosphere enriched with 5% CO_2. The samples were also inoculated on Sabouraud's dextrose agar (SDA) and incubated at room temperature for 7 days.

Characterization of the isolate was done based on morphology, cultural characters, and biochemical tests [2].

Ducklings in the age group of 9-10 days and mice in the age group of 6 weeks were subjected to pathogenicity testing using the isolates [3]. Mice were injected with 0.2 ml of inoculum with a concentration of 3×10^8 organisms/ml intraperitoneally. The ducklings were given 1 ml of inoculum with the same concentration of organism by intramuscular route. Two control animals were kept for each experiment. The mice and birds were observed for the development of clinical signs or death for a period of 2 weeks. Re-isolation of the organism was attempted from heart blood, lung, liver, spleen, and brain in 10% bovine blood agar.

Polymerase chain reaction (PCR) was performed to confirm the identity of the isolates as per Kardos *et al*. [4] with minor modifications. Template DNA was extracted by the method of heat lysis of the bacterial culture. Pure culture in 5 ml of phosphate buffered saline (PBS) was centrifuged at 3000 ×*g* for 10 min at 4°C, and the pellet was washed twice in PBS. The final pellet was resuspended in 100 µl of triple distilled water. It was boiled for 10 min and chilled in ice for 30 min. After centrifugation at 3000 ×*g* for 5 min at 4°C, the supernatant was collected and used as template DNA. PCR was performed in a 25 µl reaction mixture. *Riemerella* specific primers, forward primer 5′-TTACCGACTGATTGCCTTCTAG-3′ and reverse primer 5′-AGAGGAAGACCGAGGACATC-3′ were used in the study. To each PCR tube, 20 µl of master mix containing 20 pmol of each primer, 200 µM of each dNTP, one unit of Taq DNA polymerase in a PCR reaction buffer with 50 mM KCl, 10 mM Tris HCl, 1.5 mM MgCl2, and 5 µl of template DNA were added. One negative control without template DNA was included to monitor contamination.

PCR was carried out in Applied Biosystems thermal cycler with an initial denaturation at 95°C for 4 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by a final extension of 72°C for 7 min The amplified PCR products were electrophoresed in 1% agarose gel for 1 h at 50 V.

The amplified DNA was sequenced from SciGenom, Cochin, Kerala, India.

The antibiotic sensitivity test, of the isolates, was performed using six antibiotics *viz* ciprofloxacin, chloramphenicol, enrofloxacin, amoxycillin, co-trimoxazole, and gentamicin [5].

Results

Post-mortem lesions of fibrinous polyserositis, fibrinous pericarditis, perihepatitis, and airsacculitis were observed in D1 and D2. In addition to this, D3 revealed the presence of esophageal corrugations suggestive of duck plague.

Heart blood smears and impression smears from liver and spleen revealed the presence of bipolar stained organisms.

The blood agar plates yielded confluent, grey, moist, convex, entire, butyrous, transparent, non-hemolytic dew drop colonies in 24 h (Figure-1). No growth was observed on BHIA, MacConkey agar and SDA. Gram's staining of the colonies revealed small, Gram-negative organisms with a morphology varying from cocco-bacillary, short rods to filamentous forms. The organisms were found to be nonmotile, and were catalase and oxidase positive. Results of biochemical tests showed that the isolate was negative for indole, methyl red, Voges–Proskauer, citrate and nitrate tests and positive for urease test.

The mice subjected to pathogenicity tests, remained healthy even after a period of 14 days and *R. anatipestifer* could not be re-isolated from these animals. All the duckling subjected to pathogenicity tests, died within 12-48 h while the control duckling remained healthy. The gross lesions observed were enlargement and congestion of the liver and enlarged spleen. Heart blood and liver impression smears revealed bipolar organisms when stained with Leishman's stain. *R. anatipestifer* could be re-isolated from heart blood, lung, liver, and spleen of the dead duckling's on 10% bovine blood agar and its identity was confirmed by PCR.

The PCR products revealed an amplicon size of 546 bp when viewed under gel documentation system (Figure-2).

Nucleotide sequence of the amplified product showed 99% homology to that of *R. anatipestifer* sequences deposited in the GenBank, NCBI (Figures-3 and 4).

Antibiotic sensitivity test revealed that the isolates were sensitive to ciprofloxacin, enrofloxacin, and gentamicin and were resistant to chloramphenol, amoxycillin, and co-trimoxazole.

Figure-1: Blood agar plate showing *Riemerella anatipestifer* isolates.

Discussion

R. anatipestifer infection is often confused with duck pasteurellosis, and hence an accurate and early diagnosis of this infection is important to avoid heavy loss by mortality. Identification based on cultural and biochemical characteristics is time-consuming and laborious and as *Riemerella* is characterized by the absence of certain specific phenotypic properties, identification by phenotypic properties or biochemical characteristics alone is difficult. The PCR is a rapid, sensitive and highly specific assay for detection of microbial infections. In this study, we used PCR to confirm the identity of the isolates obtained. The same could be used to detect *Riemerella* organisms directly on clinical specimens, and thus help in implementing early treatment and control strategies. In this study, a co-infection of duck plague and *R. anatipestifer* was detected in one of the ducklings. An immunosuppressive state induced by duck plague may explain the presence of secondary infections of *Pasteurella multocida*, *R. anatipestifer* and *Escherichia coli* in natural outbreaks of duck plague in ducklings [1].

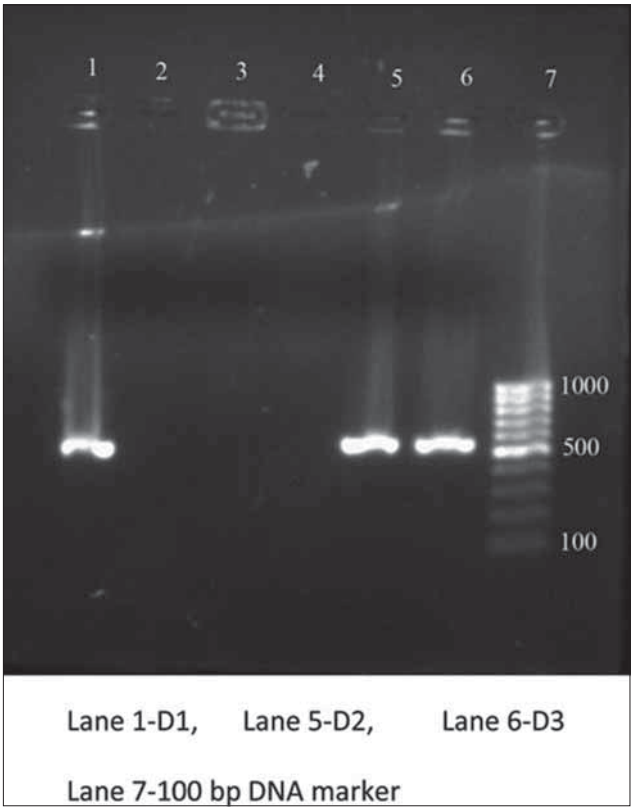

Figure-2: *Riemerella anatipestifer*-Polymerase chain reaction amplified DNA on agarose gel.

The primers used in the study were selected [4]. A 700 bp fragment of DNA, common to 16 *Riemerella* isolates, was amplified using enterobacterial repetitive intergenic consensus PCR (ERIC PCR), sequenced and used to design a primer for a PCR which could amplify a 546 bp conserved sequence of *R. anatipetifer* [4]. They reported that the PCR assay was specific for *R. anatipestifer* and was capable of correctly identifying the organism from pure culture as well as from clinical samples and could differentiate between *R. anatipestifer* and *P. multocida*. A PCR assay for detection of a conserved region of the 16S rRNA gene of *R. anatipestifer*, was developed [6], which could successfully detect the organism in clinical specimens. A new *Riemerella*-specific PCR assay was designed by Rubbenstroth *et al*. [7] who also conducted matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry fingerprinting of whole bacterial cells to identify *Riemerella* isolates. In a study conducted to determine the gene profile of *R. anatipestifer* organisms, [8] detected a total of 48 genes in the *Riemerella* genome of which eight were novel genes which were thought to be potent virulence factors. A multiplex PCR assay for simultaneously detecting four pathogenic bacteria in ducks viz, *P. multocida, Salmonella enterica, R. anatipestifer,* and *E. coli* was developed using target genes KMT1 of *P. multocida*, the invasion protein gene of *S. enterica*, 16S rDNA of *R. anatipestifer*, and the alkaline phosphatase gene of *E. coli*. The assay could detect the four bacteria in laboratory as well as field samples [9]. In the present study PCR could successfully amplify *R. anatipestifer* DNA and this was confirmed by nucleotide sequencing.

More than 20 different serotypes of *R. anatipestifer* have been reported to cause infection in

Figure-3: Sequence analysis of the isolate of *Riemerella anatipestifer* with *R. anatipestifer* RA-CH-2, complete genome, published in NCBI.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Riemerella anatipestifer RA-CH-2, complete genome	925	925	99%	0.0	100%	CP004020.1
Riemerella anatipestifer ATCC 11845 = DSM 15868, complete genome	925	925	99%	0.0	100%	CP003388.1
Riemerella anatipestifer RA-GD, complete genome	925	925	99%	0.0	100%	CP002562.1
Riemerella anatipestifer DSM 15868, complete genome	925	925	99%	0.0	100%	CP002346.1
Riemerella anatipestifer strain D-24105 RNAse Z and xanthosine triphosphate pyrophosphatase genes, partial cds	892	892	95%	0.0	100%	JN578232.1

Figure-4: The NCBI blast search analysis of the sequenced DNA of field isolate of *Riemerella anatipestifer* with the published sequences.

field cases. A comparative genomic analysis of three *Riemerella* isolates demonstrated the genetic diversity between the isolates [10]. Inactivated bacterins prepared from different serotypes of *R. anatipestifer* do not confer cross-protection to other serotypes. This lack of cross-protection limits the usefulness of vaccination against this disease. Consequently, chemotherapy is very important aspect in the treatment of ducks infected with *R. anatipestifer* [11]. A study conducted [12], showed levamisole to be a useful adjuvant in inactivated *Riemerella* vaccine and that it could enhance the immune response of ducklings and also alleviate local injection lesions by reducing the dose of the vaccine. A trivalent inactivated vaccine of *R. anatipestifer*, including serotype 1, 2, and 10 showed 100% protection in experimental animals from challenge with any of the three serotypes [13]. *In vitro* and *in vivo* antimicrobial susceptibilities of 50 field duck isolates of *R. anatipestifer*, to ceftiofur and 16 other commonly used antimicrobials was carried out [11]. They found that the *Riemerella* organisms were most sensitive to penicillin, ceftiofur, cephalothin, chloramphenicol, flumequine, and kanamycin. In another study conducted [14], *R. anatipestifer* was reported to be sensitive to enrofloxacin, chloramphenicol, lincomycin, streptomycin, and neomycin but resistant to penicillin G, ampicillin, tetracycline, trimethoprim-sulfamethoxazole, kanamycin, and gentamycin. Clonal spread of multi-drug resistant *R. anatipestifer* isolates among ducks in farms in China has been reported [15]. In the present study, the isolates were found to be sensitive to ciprofloxacin, enrofloxacin, and gentamicin and were resistant to amoxicillin, chloramphenicol, and co-trimoxazole.

Conclusion

The present study suggests that PCR assay will facilitate fast and proper identification of *R. anatipestifer* infection in ducks. Furthermore, the assay can easily differentiate between *R. anatipestifer* and *P. multocida*, and can replace the traditional methods of differentiation which is difficult and time-consuming. The ability of PCR assay to detect *Riemerella* organisms directly in clinical specimens is an added benefit. Confirmatory identification of *R. anatipestifer* infection in ducks in Kerala has laid the foundation for the development of an effective vaccine for this disease and in effect could provide economic relief to the commercial duck farmers in Kerala.

Authors' Contribution

MS and SRN carried out the study. MS drafted and revised the article. MM, BKM and SJ participated in scientific discussion. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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