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OUTER MEMBRANE PROTEIN A (OmpA) BASED POLYMERASE CHAIN REACTION (PCR) FOR THE DETECTION OF *Riemerella anatipestifer* INFECTION IN DUCKS OF KERALA, INDIA

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Abstract: *Riemerella anatipestifer* is the causative agent of New Duck Disease affecting ducks worldwide. It is difficult to differentiate *R. anatipestifer* from *Pasteurella multocida* based on conventional identification techniques. *Riemerella anatipestifer* contains several outer membrane proteins (omp), one of which designated as ompA has been found common to all serotypes. The present study describes PCR for the identification of *R. anatipestifer* with in-house designed primers for the ompA gene which is conserved in all serotypes of *R. anatipestifer*. Eight isolates obtained from different parts of Kerala were subjected to PCR and all gave positive results. The assay was found to be highly sensitive and rapid in detecting *R. anatipestifer*.

Keywords: OmpA PCR, Riemerella anatipestifer, OmpA, New duck disease.

Introduction

Riemerella anatipestifer, a Gram-negative, non-motile, non-spore forming, rod-shaped bacterium, is the causative agent of duck septicaemia (Segers *et al*., 1993). It has been identified as a member of the family *Flavobacteriaceae* in rRNA superfamily V, based on 16S rRNA gene sequence analyses (Subramaniam *et al*., 1997). It causes a contagious septicaemic disease primarily affecting ducks (Fulton and Rimler, 2010), less frequently geese (Pierce & Vorhies, 1973) and turkeys (Helfer & Helmboldt, 1977). The disease is presented as fibrinous perihepatitis, pericarditis, airsaculitis and meningitis (Leibovitz, 1972; Sandhu *et al.,* 2003). Twenty-one serotypes of *R. anatipestifer* have been identified so far (Loh *et al*., 1992; Pathanasophon *et al*., 1994) and serotypes 1 and 2 have been found to be most prevalent pathogenic agents responsible for outbreaks. In India, *R. anatipestifer* infection in ducks has been reported from Meghalaya and Kerala (Shome *et al*., 2004 and Priya *et al*., 2008). The definitive diagnosis of *R. anatipestifer* infection requires isolation and

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identification of bacteria and is identified on the basis of cultural, morphological and biochemical characteristics. However, it is characterized more by the absence than by the presence of specific phenotypic properties (Hinz *et al*., 1998). The outer membrane of the gram negative bacteria comprises of proteins (50% mass) and the major proteins present in the outer membrane are ompA and porins. The ompA of *R. anatipestifer* has been found common to all isolates with an Open Reading Frame of 1164 bp gene encoding 387 amino acids protein product of 42kDa (Subramaniam *et al.,* 2000).Outer membrane protein A gene is found to be conserved in all the serotypes of *R. anatipestifer* and it can be utilized in the control and diagnostic strategies. The current study was designed to develop the ompA based PCR as a diagnostic tool.

Materials and Methods

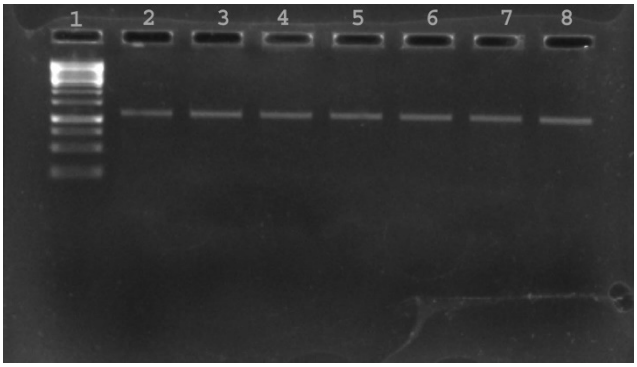
Eight isolates maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala were used in the study and they were isolated from the outbreaks of the disease in different parts of Kerala. Lyophilized isolates were revived by growing them on 10 per cent bovine blood agar plates at 37^{o}C in an atmosphere enriched with 5-10 per cent CO_2 for 24 h. Identification of isolates was based on the cultural, morphological and biochemical properties. After identification, colonies were inoculated into 5 ml brain heart infusion broth (BHIB) and incubated overnight at 37^{o} C in 5-10 percent CO_2 rich atmosphere. Genomic DNA was isolated from the pellet using DNA extraction kit (Macherey Nagel). The isolates were also identified by species-specific PCR assay (Kardos *et al.*, 2006) to confirm the accuracy of biochemical tests. The *R. anatipestifer* ompA sequence was acquired from GeneBank (http://www.ncbi.nih.gov) and primers were designed in-house for the PCR. In addition to nucleotide sequences of ompA, two restriction sites for *Hind* III and *Bam* HI were also added to the 5′ end of primer sequences for further research in molecular cloning and expression. The primer sequences used in this study are as follows: Forward (5′TTGGATCCATGGGTAAAGAATTTATGTTG) and Reverse (5′ TGAAGCTTTTTTCTTTTCTTTTTTACTAC)and were synthesized by Sigma Aldrich.

PCR was performed using a 25 µl reaction mixture that consisted of 12.5 µl of 2XPrimeSTAR Max DNA polymerase premix (2 mM Mg^{2+}, 0.4 mM each dNTP)(Takara), 2 µl (10 pmol) each of forward and reverse primer and 50-200ng of template DNA. The PCR assay was performed in thermal cycler (Bio-Rad, CA, USA) initially, with the gradient temperatures to standardize the assay and then the following conditions were selected on the basis of gradient results: 30 cycles of denaturation at 98^{o} C for 10s, followed by annealing at

58° C for 5s, extension at 72° C for 10s. All the isolates which were found positive on biochemical tests and *R. anatipestifer* species- specific PCR were selected in the study. The PCR products were run in 1per cent agarose gel (Axygen) using horizontal submarine electrophoresis apparatus (Hoefer, CA, USA) and visualized in gel documentation system (Bio-Rad, CA, USA).

Results and discussion

DNA concentrations and purity of all the isolates was determined using Nanodrop 2000 (Thermo Scientific) and the DNA having purity in proximity of 1.8 were used in PCR assay. We used PrimeSTAR Max DNA polymerase in our study which possesses fastest extension speeds, extremely high accuracy, high sensitivity, high specificity and high fidelity. Among the different annealing temperatures tested, 58° C gave the best results. Using optimal PCR conditions, the reaction produced approximately 1.2kb product in all the *R. anatipestifer* isolates that were used in the study and could be clearly visualized in gel after running the PCR product in 1per cent agarose(Fig.1)



(Fig. 1) 1.100 bp DNA ladder, **2-8**. PCR products of *R. anatipestifer isolates*.

Discussion

Identification of *R. anatipestifer* by traditional microbiological methods is time consuming. Several methods have been developed for efficient identification of *R. anatipestifer*, like fluorescent antibody technique (Guo *etal*., 1982) which is sensitive but still time consuming because of the need to prepare fluorescent labelled antibodies. That leaves the room for development of other molecular diagnostic tools. Several molecular techniques have been used for detection of *R. anatipestifer* such as real time PCR and loop- mediated isothermal amplification (LAMP) which are sensitive but tedious and expensive.

The 16S rRNA gene is considered as a stable fragment on the chromosome of all prokaryotes (Tsai *et al*., 2005). This gene is typically applied to the determination of phylogenetic

relationship among bacteria and has been widely used as a molecular marker to detect pathogenic bacteria (James, 2010; Kühn*et al.,*2011). The 16s rRNA based PCR has been employed to detect *R. anatipestifer* (Tsai *et al*., 2005; Pala *et al.*, 2013, Somu, 2014). However, due to low mutation rates of 16s rRNA, it has been difficult to differentiate closely related species (Wang *et al.*, 2012). Species-specific PCR assays represent valuable tools in identification and differentiation of pathogens by exploiting a common fragment of DNA. By identifying a sequence that is conserved in all the isolates of *R. anatipestifer,* species-specific assay was designed that could detect *R. anatipestifer* and differentiate it from pastuerella species, since both are morphologically similar and could cause a false alarm of fowl cholera (Kardos *et al.,* 2006).

Outer membrane proteins play an important role in virulence and elicit a strong immune response in host. One of the outer membrane proteins designated as ompA has been characterized as highly immunogenic and the gene encoding the ompA has been found highly conserved among all the isolates of *R. anatipestifer*. By exploiting this highly conserved fragment in *R. anatipestifer,* we conducted a study for developing a diagnostic strategy to curtail the *R. anatipestifer* infection in the ducks of Kerala. After optimizing the PCR, we amplified a PCR product of approximately 1.2kb from 8 clinical isolates. The PCR assay proved to be rapid (1 hour) than the species- specific PCR.

In conclusion, ompA based PCR could be used to detect *R. anatipestifer* efficiently and is faster than the other PCR based diagnostic techniques. The assay proved to be highly sensitive but, further research is needed to check the specificity. In addition to this, in-house designed primers can be utilized in recombinant DNA technology, to produce ompA based vaccine against *Riemerella* infections in ducks.

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