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RAPID DETECTION OF *Riemerella anatipestifer* ISOLATES USING 16SrRNA BASED PCR AND SPECIES- SPECIFIC PCR ASSAY

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Abstract: Even after regular vaccination against duck plaque and duck pasteurellosis, reports of high mortality were noticed by duck owners in Kerala. On detailed investigation, *Riemerella anatipestifer*, a Gram- negative rod shaped, non-motile, non-sporulating bacterium was isolated as the causative agent. They were characterized based on morphological, cultural and biochemical tests. The isolates were also confirmed by molecular assays like 16S rRNA based PCR and *R.anatipestifer* specific PCR, which would help for rapid confirmation of the disease at the time of an outbreak.

Keywords: *Riemerella anatipestifer*, 16S rRNA based PCR, *R.anatipestifer* specific PCR, Kerala.

INTRODUCTION

Due to known and unknown global environmental changes, several new diseases emerged which could target ducks. One such disease is the new duck disease in Kerala, reported since 2008 (Priya *et al*., 2008). It is an enzootic, contagious, often primary septicemic disease of domesticated ducklings (Fulton and Rimler, 2010). In addition to ducks, it also infects geese, turkey, chicken, wild birds and domestic pigs (Segers *et al*., 1993). In young ducklings, it results in a mortality rate as high as 75 per cent and in adult birds; it ranges from 20 to 40 per cent. The causative agent is *Riemerella anatipestifer* (RA), a Gram- negative rod shaped, non-motile, non-sporulating bacterium. The main gross lesions were fibrinous pericarditis, perihepatitis and air-sacculitis with severely congested liver and spleen. At the time of

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disease outbreak, these clinical signs and gross lesions resembled the one noticed for duck pasteurellosis or salmonellosis or colibacillosis. Though the detection of *R. anatipestifer* from clinical samples by culture and identification method is considered as the gold standard test, it is time- consuming, labour- involving and costly. Now-a-days, molecular assays like polymerase chain reaction (PCR) have replaced the traditional cultural methods. Hence, a study was planned to identify the RA isolates by 16S rRNA based PCR and species- specific PCR assay. This would help for the rapid and reliable identification of the isolates at the time of outbreak.

MATERIALS AND METHODS

Bacterial isolates and their revival

Among the different isolates of *Riemerella anatipestifer*, one each of predominant and lesser predominant isolates maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy were revived and the colonies were subcultured on blood agar plates and they were designated as RA1 and RA2. Based on the assessment of morphology, cultural characteristics and biochemical reactions, purity of the isolates were checked.

Isolation of genomic DNA and testing the purity

The genomic DNA of the two isolatesof RA (RA1 and RA2) was extracted based on the method suggested by Sambrook and Russell (2011). By measuring the OD values at 260 nm and 280 nm, the purity of the DNA was checked. For PCR, the preparation with a value (260/280) of 1.8 or above was used.

Identification by 16S rRNA Based PCR

The extracted DNA from the two isolates was identified by16S rRNA gene based PCR which is suitable for screening of *R. anatipestifer* isolates. Two primers based on the conserved region of the 16S rRNA gene designed by Tsai *et al*. (2005) were used.

Forward (F) 5´ CAGCTTAACTGTAGAACTGC 3´

Reverse (R) 5´ TCGAGATTTGCATCACTTCG 3´

PCR was performed as per the protocol reported by Pala *et al*. (2013) as follows.

The composition used in 25 µL single reaction mix for amplification contains EmeraldAmp® GT PCR master mix (12.5 µL), 2.0 µL each of forward primer (10 pM/µL) and reverse primer (10 pM/µL), 2.0 µL of DNA and 6.5 µL of Nuclease free water. PCR programme for the amplification of 16S rDNA sequence was carried out in a thermal cycler (Biorad, USA) with an initial denaturation of 95 ºC for 5 min., followed by 25 cycles of denaturation at 94

ºC for 30 s, annealing at 54 ºC for 50 sec. and extension step at 72 ºC for 1min. with final extension of 72 ºC for 7 min.

Identification by *Riemerella anatipestifer* specific PCR

A PCR assay that is a valuable tool for the rapid and species-specific identification of *R. anatipestifer* from bacterial culture, were performed for the further identification and confirmation (Kardos *et al*., 2007).

Primer used is shown below:

Forward (F) (5´-TTACCGACTGATTGCCTTCTA-3´)

Reverse (R) (5´-AGAGGAAGACCGAGGACATC-3´)

The composition used in 25 µL single reaction mix was the same as that of 16S rRNA PCR except the template DNA (1 µL) and nuclease free water (7.5 µL). PCR programme was done out with an initial denaturation of 95 ºC for 4 min., followed by 35 cycles of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min. and extension step at 72 ºC for 1min. with final extension of 72 ºC for 7 min.

Confirmation of PCR products by gel electrophoresis

The PCR products generated were confirmed for their expected size in one per cent agarose gel in Tris borate EDTA buffer using horizontal submarine electrophoresis apparatus (Bangalore GeNei, India). The gel was electrophoresed at 50 V for 1 hour, after that it was analysed in a Gel Documentation System (Bio-Rad, USA).

RESULTS AND DISCUSION

Bacterial isolates and their revival

R. anatipestifer infection is often confused with duck pasteurellosis, and the accurate and early diagnosis of the infection is important to avoid heavy loss by mortality. *R. anatipestifer* isolates already maintained in the Department of Veterinary Microbiology was successfully revived and purified.

Purity checking of the isolates

First stage of biochemical tests

On BA plate, RA1 produce confluent, gray, convex, entire, butyrous, non-haemolytic dewdrop colonies was noticed after 24h incubation (Table. 1). No growth was noticed on SDA, BHIA and MCA, as recorded by Surya *et al*. (2016).

The RA1 isolate was non-haemolytic on BA whereas the RA2 produced a clear zone of haemolysis after 48h of incubation. Among 123 field strains of *R. anatipestifer*, Hinz *et al*.

(1998b) recorded that 25 strains showed β-haemolysis on blood agar after 24h to 48h incubation.

The primary revival and the preparation of the organism were carried out in five to ten per cent bovine BA. According to Rimler *et al*. (1998) no selective and/ or indicative media had been used for the isolation. Higgins *et al*. (2000) opined that it was some time difficult to isolate the organism from clinical samples due to overgrowth of other organism. Chocolate agar (Leavitts and Ayroud, 1997), bovine BA at ten per cent level (Crasta *et al*., 2002; Priya *et al*., 2008) have been reported to be useful for the primary isolation of the organism.

The incubation carried out in a candle jar with mild CO_2 tension at 37ºC for 48 h was found to be optimum for the culture of *R. anatipestifer*. Smith *et al*. (1987) reported that the organism grown under microaerophilic environment. Segers *et al*. (1993) suggested that the 35-37ºC was the best growing temperature of the organism in a CO_2 enriched atmosphere. The finding in the present study is in agreement with the observations made by earlier workers. On Gram's staining, the organism revealed Gram negative coccobacilli, short rods to filamentous forms. Similar observation were made by Baba *et al*. (1987) and Leavitts and Ayroud (1997). No growth on MCA, positive reaction for oxidase, catalase and unreactivity to O-F test observed in the present study was recorded earlier by Carter and Wise (2004).

Second stage of biochemical tests

The second stage biochemical reactions used for characterization of *R. anatipestifer* (Segers *et al*., 1993) were almost identical for RA1 and RA2 isolates (Table 2 & 3). Variations were observed only on urease test and fermentation of sugars. Similar findings have been reported by Pillai *et al*. (1993), Vancanneyt *et al*. (1999), Bernardet *et al*. (2002) and Shome *et al*. (2004).

R. anatipestifer can be differentiated from *P. multocida* based on the inability of the former to produce indole and ornithine decarboxylase and its ability to liquefy gelatin (OIE, terrestrial manual, 2008).

With regards to the fermentation of sugars, both the isolates failed to ferment dextrose, galactose, lactose, fructose, xylose, mannose, maltose, mannitol, sorbitol, dulcitol, adonitol, inositol, salicin, insulin, arabinose, trehalose, melibiose, cellobiose, rhamnose and raffinose. The RA1 isolate did not ferment sucrose and raffinose while the RA2 isolate ferment these two sugars. These results agreed with the observations of Baba *et al*. (1987) Charlton *et al*. (1993) Pillai *et al*. (1993). Pathanasophon *et al*. (1991) Hinz *et al*., (1998a) and Bernardet *et al*. (2002).

Both the isolates showed negative result for indole, methyl red, Voges Proskaur, citrate and nitrate tests. The RA1 gave positive for urease whereas RA2 were negative. Similar observations were recorded by Surya *et al*. (2016) and Soman *et al*. (2014).

Confirmation of isolates by PCR

R. anatipestifer is characterized more by the absence than the presence of specific phenotypic properties (Hinz *et al*., 1998a). Based on cultural and biochemical characteristics identification is time consuming and laborious, identification based only on phenotypic characteristics alone is difficult (Soman *et al*., 2014).

16S rRNA based PCR

According to Tsai *et al*. (2005), the 16S rRNA gene based PCR was able to amplify all 18 Taiwanese strains and 10 reference strains and the identity of 16S rRNA sequence of these strains and seven other sequences yielded a product size of 665 bp retrieved from GENEBANK was 95.0 to 100.0 per cent. Phylogenetic analysis based on the 16S rRNA gene showed that all the *R. anatipestifer* strains grouped into a single cluster. Hence, the revived RA1 and RA2 isolates were subjected to specific 16S rRNA based PCR, using primer designed by Tsai *et al* (2005). Analysis of the electrophoresed gel revealed the presence of 665 bp fragment in both of the isolate and thus confirmed as *R. anatipestifer* (Fig. 1). The same product size was obtained by Tsai *et al* (2005). They observed that among the annealing temperature tested, 67.2ºC produced the best result whereas in this present study the annealing temperature was optimized at 54ºC. Ninety nine per cent sequence similarity between the 16S rRNA genes of two *R. anatipestifer* like strains were observed by Ryll *et al*. (2001). A PCR assay was developed for detection of a conserved region of the 16S rRNA of *R. anatipestifer* and successfully detected the organism from clinical specimens. Qu *et al*. (2006). James (2010), Kuhn *et al*. (2011) and Shonima (2012) studied on 16S rRNA gene for detection of pathogenic bacteria and opined that it was used as molecular marker.

***Riemerella anatipestifer* specific PCR**

The species specific PCR assay developed by Kardos *et al*. (2007), revealed the presence of 546 bp of amplicon size from both the isolates (Fig.2). Soman *et al*. (2014) also obtained the same product size and opined that the PCR assay could easily differentiate between *R. anatipestifer* and *P. multocida* and this method could easily replace the traditional method of differentiation.

CONCLUSION

R. anatipestifer species specific PCR assay represent a valuable tool in the identification and differentiation of pathogen. Micro-environmental conditions like availability of nutrient or growth condition influence the pathogen and hence the traditional methods like biochemical tests are not sensitive. Moreover, performing biochemical test with large number of samples was difficult. It might also be important that PCR based assay provide more suitable result, faster than the traditional biochemical methods.Hence the novel PCR assay could help to differentiate between *R. anatipestifer* from *P. multocida*, which could be difficult and time consuming with traditional methods.

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Table1. First stage biochemical test

TESTS	RA1	RA2
Grams's reaction	Gram negative	Gram negative
Morphology	Coccobacilli/Short rods/ filaments	Coccobacilli/Short rods/ filaments
Motility	-	-
Growth microaerobically	+	+
Growth aerobically	+	+
Growth on MCA	-	-
Haemolysis on BA	-	+
Catalase	+	+
Oxidase	+	+
O- F test	Unreactive	Unreactive

Table 2. Second stage of biochemical tests

TESTS	RA1	RA2
Indole production	-	-
Methyl- red test	-	-
Voges-Proskauer test	-	-
Urease	+	-
H_2S production	-	-
Nitratereduction	-	-
Citrate utilization	-	-
Gelatinliquefaction	+	+
Ornithinedecarboxylase	-	-

Table 3: Sugar fermentation tests

Sugar fermentation	**RA1**	**RA2**
Dextrose	-	-
Galactose	-	-
Lactose	-	-
Fructose	-	-
Sucrose	-	+
Xylose	-	-
Mannose	-	-
Maltose	-	-
Mannitol	-	-
Sorbitol	-	-
Dulcitol	-	-
Adonitol	-	-
Inositol	-	-
Salicin	-	-
Inulin	-	-
Arabinose	-	-
Trehalose	-	-
Melibiose	-	-
Cellobiose	-	-
Rhamnose	-	-
Raffinose	-	+

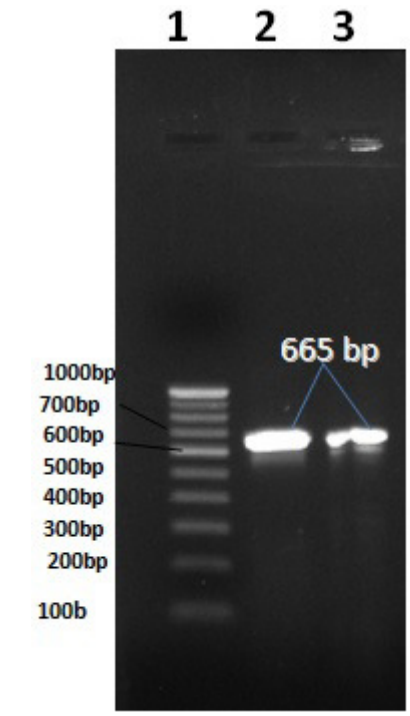

Fig.1. 16S rRNA based PCR

Lane 1- 100 bp ladder

Lane 2- RA1 isolate

Lane 2- RA 2isolate

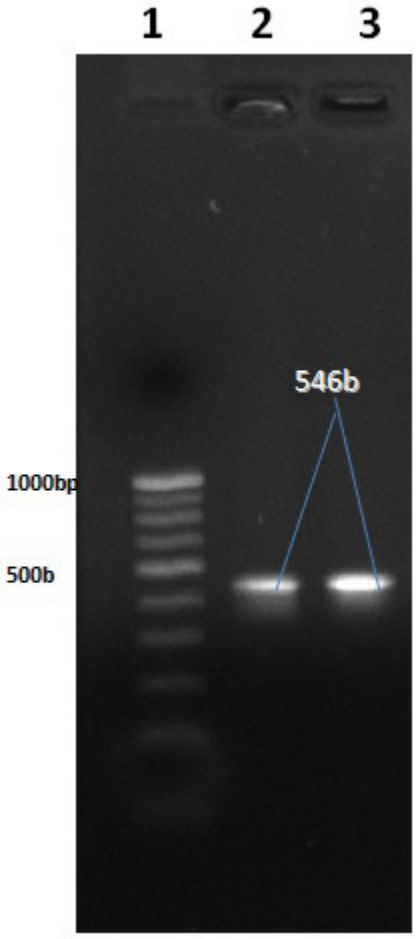

Fig.2 *Riemerella anatipestifer* specific PCR

Lane 1- 100 bp ladder

Lane 2- RA1 isolate

Lane 2- RA 2isolate