Biofilm detection of *Riemerella anatipestifer* isolates of Kerala

Dermisha S, Priya PM, Sheikh Moin Ahmad, Rinsha Balan and Mini M

Abstract

*Riemerella anatipestifer*, causative agent of Riemerellosis, is a gram negative, non-sporulating and non-capsulated rod, which infects mainly domestic ducks. Kerala is experiencing great economic loss due to riemerellosis from 2008 onwards. It is very hard to control the infection in the field even after antibiotic therapy at right time. Resistant infection noticed among infected farms might have a link with biofilm forming capacity of the causative agent. Hence, in this study, formation of biofilm was estimated by tube (qualitative) and tissue culture plate (TCP) as quantitative method, using six representatives isolates of *Riemerella anatipestifer* (RA1, RA2, RA3, RA4, RA5 and RA6). It was identified that three of the isolates (RA1, RA5 and RA6) were biofilm formers and TCP method was considered as gold standard assay to detect the biofilm formation.

**Keywords:** Riemerellosis, *Riemerella anatipestifer*, biofilm, tube method, tissue culture plate method

Introduction

*Riemerella anatipestifer* (RA) infection also known as riemerellosis is identified as one of the disease which curbs down the duck industry of Kerala, since the past ten years (Priya et al., 2008). Considering its impact on duck industry, several researches has been undertaken in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy to diagnose and control the infection. Once the RA infection hits a farm, even after the administration of a course of selected antibiotic, reoccurrence of infection were noticed in some farms resulting in continuous losses among different batches of ducks. It might be due to the persistent nature of the organism. Many clinical infections are ascribed to biofilms. Hence, a study is needed to detect whether the RA isolates of Kerala has the ability to form biofilm or not. Keeping these in mind, a study was framed with the objective of determining the ability of RA isolates to form biofilm using tube adherence test and microtitre plate assay.

Materials and Methods

Revival of bacterial isolates

The six lyophilised isolates of *R. anatipestifer* (RA1, RA2, RA3, RA4, RA5 and RA6) maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy were dissolved in distilled water and were streaked on to the blood agar (BA) plates and incubated for 24 to 48 hours at 37°C in a candle jar. They were confirmed as *R. anatipestifer* as per the method described by Surya et al. (2016), *Pseudomonas aeruginosa* and DH5α maintained in the department were used as positive and negative control for biofilm producers, respectively.

Detection of biofilm formation by tube adherence method

The method described by Christensen et al. (1982) was followed. Organisms isolated from fresh agar plates were inoculated into 15 ml of BHIB with 1.5 ml of foetal calf serum. Culture with two different concentration (1.0 and 0.1 OD at 600 nm respectively) were checked for biofilm formation at 24 hrs and 48 hrs after inoculation. From this 5 ml of BHIB was transferred into two sterile test tubes. The tubes were incubated in a candle jar at 37°C. The positive control (*Pseudomonas aeruginosa*) and the negative control (DH5α) were incubated separately at 37°C. After incubation the tubes were washed with PBS (pH 7.2) for three times. The tubes were then stained with Crystal violet (0.1 per cent) for twenty minutes. Excess stain is washed out with deionized water.
The tubes were dried and scoring was done according to result of control strain. Biofilm production in tube method was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. After the examination of tube, the amount of biofilm formed was scored as 0- none, 1- weak, 2-moderate and 3-high/strong (Deka, 2014) [7].

Detection of biofilm formation by tissue culture plate method

The assay was done as per the method of Christensen et al. (1985) [6]. Organisms isolated from fresh agar plates were inoculated into 15 ml of BHIB with 1.5 ml of foetal calf serum. Culture with two different concentration (1.0 and 0.1 OD at 600 nm respectively) where checked for biofilm formation at 24 hrs and 48 hrs after inoculation. 200 µL of OD adjusted broth were added to individual wells of two sterile 96 well flat bottom polystyrene tissue culture plates in triplicates. The positive control and negative control organisms were also added in triplicates to the tissue culture plates. The plates were incubated in candle jar at 37°C. After incubation, the contents of wells were removed by gentle tapping. The wells were washed three times with PBS. After washing 250 µL of methanol is added to each well to fix the biofilm. The wells are decanted and stained with crystal violet for 20 min. The wells are again decanted and dried. OD value of each wells are measured at 600 nm using an ELISA plate reader. Biofilm formation is considered positive when the mean value is equal to the twice the mean value of negative control.

Results and Discussion

Riemerellosis is a disease capable of causing acute and chronic infections, which was a serious global problem for domestic duck, wild water fowl, turkeys and other birds (Sandhu, 2008) [22]. The disease was reported in Kerala from 2008 onwards and has caused a set back the duck industry of the state. The biofilm formation by microorganisms is a mechanism that allows them to become insistent colonisers, exchange genetic materials, resist clearance by the host immune system and enhance the antibiotic resistance (Donlan and Costerton, 2002) [9]. In this study, formation of biofilm by RA isolates isolated during outbreaks was characterised.

Revival and identification of bacterial isolates

For the revival of R. anatipestifer, BHIA supplemented with sterile defibrinated bovine blood at five to ten per cent level were used. Frommer et al. (1990) [10], Priya et al. (2008) [20], Surya (2011) [28], Sabnann (2015) [21] and Shancy (2015) [24] also employed bovine BA as enriched media for primary isolation of the organism from clinical samples. Based on the assessment of morphology, cultural characteristics and biochemical reactions, purity of the isolates were checked. The colonies on BA were smooth, convex, grayish-white, translucent and butyrous, measuring one to three millimetres in diameter. Similar colony characters were observed by Smith et al. (1987) [26], Segers et al. (1993) [23] and Songer and Post (2005) [27]. All the isolates (RA1 to RA6) were Gram negative, non-motile and morphology varied from coccobacilli, short rods to filamentous forms, grew micro aerobically and did not grow on MCA. They were non-haemolytic on BA. All the isolates were catalase and oxidase positive and unreactive to O-F test. The findings were in accordance with Baba et al. (1987) [2], Leavitt and Ayroud (1997) [14], Carter and Wise (2004) [4] and Surya (2011) [28]. 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, 2010) [18].

Detection of biofilm formation of R. anatipestifer

There are various methods for biofilm detection (Christensen et al., 1985; Vroom et al., 1999; Donlan et al., 2001; Vasudevan et al., 2003 Jain et al., 2008; Hu et al., 2010) [6, 31, 8, 29, 13, 12].

Tube Method

Tube method described here was a qualitative method for biofilm formation. Using this method, biofilm formation were detected by earlier researchers in different bacteria (Christensen et al., 1982; Mack et al., 1996; Mulder et al., 1998; Baqui et al., 2008; Deka, 2014 and Veena et al., 2015) [5, 15, 13, 7, 30]. They employed bacterial cultures with an OD of 0.1 after 24hrs of incubation at different nanometre depending on the spectrophotometer and the organism they used. As RA is slow growing and fastidious based on the literature, OD at 0.1 and 1.0 at 600 nm on 24and 48 hrs were recorded.

It was noticed that OD at 600nm at 24hrs, all the RA isolates except RA6 were positive. Pseudomonas was scored as negative. On 48hrs of incubation, Pseudomonas had given a score of 2 and RA4 as 2. At OD of 1 at 600nm on 24hrs, Pseudomonas scored 3 and rest of the tubes as 2 and DH5α as 1 (RA3 and RA6). This indicates that Pseudomonas was not forming biofilm at 24hrs and it formed on further incubation and hence recorded as 2 on 48hrs of incubation. Also Pseudomonas recorded maximum score at high concentration of organism (OD of 1). Similarly RA also scored more on further incubation. Scoring of tube method was done according to the result of control strains as mentioned by Hassan et al. (2011) [11].

Tissue culture plate method

In 0.1OD on 24 hrs incubation, RA1, RA5, RA6 were recorded as positive and on further incubation (48 hrs), RA1 and RA6 shown as positive. At OD 1, both at 24 and 48 hrs of incubation, RA1, RA5, and RA4 were calculated as positive.

| Table 1: OD values of Riemerella anatipestifer at 600 nm (0.1 OD culture) on 24 hrs. Score- RA1, RA5 and RA6- positive; Others-Negative |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Pseudomonas | DH5α | RA1 | RA2 | RA3 | RA4 | RA5 | RA6 |
| 0.068114 | 0.083763 | 0.63711 | 0.080888 | 0.055113 | 0.05907 | 0.083736 | 0.08479 |
| 0.035853 | 0.050191 | 0.067383 | 0.077985 | 0.09007 | 0.055091 | 0.082881 | 0.089732 |
| 0.064782 | 0.058667 | 0.056248 | 0.074238 | 0.078703 | 0.057417 | 0.079563 | 0.235396 |
| 0.063826 | 0.064207 | 0.25358 | 0.077703 | 0.074628 | 0.057193 | 0.08206 | 0.136639 |
Mathur et al. (2006) [16]; Hassan (2011) [11] considered the quantitative test described by Christensen et al. (1985) [6] as the gold-standard method for biofilm detection. Hence in this study also it was taken as standard and concluded that the RA isolates, RA1, RA5, and RA6 were biofilm formers and other isolates as non-biofilm formers. Our results had good correlation with the persistent nature of infection noted on farms from where the isolates obtained (Surya, 2011) [28].

Table 2: OD values of Riemerella anatipestifer at 600nm (0.1 OD culture) on 48 hrs. Score-RA1 andRA6- positive; Others-Negative

<table>
<thead>
<tr>
<th>Pseudomonas</th>
<th>DH5u</th>
<th>RA1</th>
<th>RA2</th>
<th>RA3</th>
<th>RA4</th>
<th>RA5</th>
<th>RA6</th>
</tr>
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<td>0.062165</td>
<td>0.102621</td>
<td>0.091103</td>
</tr>
</tbody>
</table>

Table 3: OD values of Riemerella anatipestifer at 600 nm (1.0 OD culture) on 24 hrs. Score-RA1, RA5 and RA6- positive; Others- Negative

<table>
<thead>
<tr>
<th>Pseudomonas</th>
<th>DH5u</th>
<th>RA1</th>
<th>RA2</th>
<th>RA3</th>
<th>RA4</th>
<th>RA5</th>
<th>RA6</th>
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</tbody>
</table>

Table 4: OD values of Riemerella anatipestifer at 600 nm (1.0 OD culture) on 48 hrs. Score-RA1, RA5 and RA6- positive; Others- Negative

<table>
<thead>
<tr>
<th>Pseudomonas</th>
<th>DH5u</th>
<th>RA1</th>
<th>RA2</th>
<th>RA3</th>
<th>RA4</th>
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References


