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**Dermisha S**
M.Sc ( Applied Microbiology) student, Dept of Veterinary Microbiology, College of Veterinary and Animal Sciences (CVAS), Mannuthy, Thrissur, Kerala, India

**Priya PM**
Assistant Professor, Dept of Vety. Microbiology, CVAS, Mannuthy, Thrissur, Kerala, India

**Sheikh Moin Ahmad**
PhD Scholar, Dept of Vety. Microbiology, Madras Veterinary College, Vepery, Chennai, Tamil Nadu, India

**Rinsha Balan**
PhD Scholar, Dept of Vety. Microbiology, CVAS, Mannuthy, Thrissur, Kerala, India

**Mini M**
Professor and Head, Dept of Vety. Microbiology, CVAS, Mannuthy, Thrissur, Kerala, India

**Correspondence**
**Priya PM**
Assistant Professor, Dept of Vety. Microbiology, CVAS, Mannuthy, Thrissur, Kerala, India

# Association between biofilm formation and antibiotic resistance of *Riemerella anatipestifer* isolates of Kerala

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

**Abstract**
Riemerellosis, a bacterial disease causing huge economic loss among ducks of Kerala. Persistent infections were noticed in several infected farms and hence the role of biofilm production was analysed. One evaluating the minimum inhibitory concentration and minimum bactericidal concentration against enrofloxacin and sulpha-trimethoprim, twice the values of planktonic cells were obtained for biofilm formers. This indicates the biofilm formers are more resistant than planktonic cells and it correlated well with the field condition experienced earlier.

**Keywords:** Biofilm formation antibiotic resistance, *Riemerella anatipestifer*

## Introduction
Ducks are commonly reared in many parts of Kerala, especially in the water logged areas, both as backyard and typical farming system. They are a regular source of income as well as nutrition to the weaker section of the society. Duck farming of Kerala is experiencing great economic loss due to riemerellosis from 2008 onwards. *Riemerella anatipestifer* (RA) is the causative agent, which is a gram negative, non- sporulating and non-capsulated rod, which infects mainly domestic ducks. 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, six representative isolates of *Riemerella anatipestifer* (RA1, RA2, RA3, RA4, RA5 and RA6) were tested for its ability to form biofilm by tube (qualitative) and tissue culture plate (TCP) as quantitative method. It was identified that three of the isolates (RA1, RA5 and RA6) were biofilm formers. Patel (2005) [9] suggest that antimicrobials often fail to penetrate into the biofilms. In field condition, even with high doses of enrofloxacin, no complete recovery of the flock from infection was noticed. The reason might be that a fraction of cells survives in biofilms as persistence (Broun *et al.*, 2000) [2] and the antibiotic could kill only the planktonic cells. Once the antibiotic was removed, the persistent cells present inside the biofilm grow and multiply, that could cause a second round of infection and may lead to an outbreak. Secondary infection also, produces antibiotic tolerant persisters as well as antibiotic-sensitive cells. The infection is perpetuating in this manner in spite of prolonged therapy. Keeping these in mind, a study was conducted to evaluate the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of biofilm producers against sulpha-trimethoprim and enrofloxacin in comparison with planktonic culture.

## Materials and Methods
### Revival of bacterial isolates
Six representative isolates of *R. anatipestifer* maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, as lyophilized form were utilized in the present study. The Brain heart infusion agar (BHIA) (HiMedia Laboratories, Mumbai), prepared as per manufacturer's instructions was rehydrated and supplemented with sterile defibrinated bovine blood at five to ten per cent level and this blood agar (BA) were used for the revival of the isolates.

### Estimation of MIC and MBC
The procedure of Grenier *et al.* (2009) [5] and few modifications as per CLSI (1998) [3] were employed to find out the MIC and MBC of enrofloxacin and sulpha- trimethoprim. Enrofloxacin (Ex) and Sulpha-trimethoprim (STM) were procured from Hi-media, Mumbai.

100 mg of Enrofloxacin and 120 mg of Sulpha-trimethoprim was dissolved separately to 1 ml of distilled water, respectively and used as stock solution.

**MIC and MBC detection for planktonic cultures**

The identified biofilm formers (RA1, RA5 and RA6) based on the tube adherence and microtitre plate method (Dermisha *et al*., 2018) [4], were subjected to estimation of MIC and MBC with positive and negative control**.**

The MIC and MBC of planktonic cells were determined using U-bottom micro titre plates for enrofloxacin and sulpha trimethoprim separately. Antibiotic stock solution (100 µl) was added to the first wells of five columns (A1, B1, C1, D1, and E1). Fifty microliter of sterile brain heart infusion broth (BHIB) was added from 2nd to 12th wells in five rows (B2-B12; C2-C12; D2-D12; E2-E12). Two fold serial dilutions is done by transferring 50 µl of antibiotic from the stock solution taken in first well, to the second well which contains sterile broth. Mixed it and again 50 µl of solution were transferred from the second well to the third well and repeat it till the 12th well. Discarded 50 µl of solution from the 12th well. Thus the enrofloxacin was serially diluted in such a way that the first well contains 10, 000 µg; second well 5000 and serially diluted up to 4.8 in 12th well. Added 50 µl of *Pseudomonas* overnight culture adjusted to 0.5 McFarland units to each of the wells of the first row. Second row is coated with DH5α followed by RA1 in 3rd, RA5 in 4th and RA 6 in 5th row respectively. The culture plates were incubated in microaerophilic condition with 5-10 per cent CO2 for 24 hrs at 37 $^0$C.

To determine the MBC, turbidity of microtitre plates were checked after 24 hrs of incubation. The wells which were clear indicate no visible growth of organism. Turbidity of wells are due to bacterial growth which indicate that an antibiotic concentration of those wells were neither inhibitory nor bactericidal. It was not known whether the organisms present in the clear wells, have died (bactericidal concentration) or their growth was merely inhibited (inhibitory concentration). These tubes contain either dead organisms or viable growth inhibited organisms. The next step was centrifuge the content of clear wells. The supernatant containing the antibiotic was poured off and fresh broth were added and further incubated for 24 hrs. If the wells were turbid, it indicates the presence of viable organisms, which was under antibiotic inhibition earlier; but now due to removal of antibiotic, they were grown.

The highest dilution of antibiotic (lowest concentration) which produce clear wells were considered as MIC and the clear wells produced after antibiotic removal were considered as MBC. The same procedure was followed to find out the MIC and MBC of the organism against sulpha-trimethoprim except the starting concentration was 12,000 µg in the first well and up to 6 µg in the 12th well.

**MIC and MBC detection for biofilm formers**

Micro titre plates containing 24 hours preformed biofilm in each well was taken. Column1 contain *Pseudomonas* followed by DH5α, RA1, RA5 and RA6 respectively. After aspiration of culture supernatant, 100 µl of fresh culture broth containing two fold serial dilutions of antibiotic were added to each well and plates were incubated in micro-aerophilic condition with 5-10 per cent CO2 for 24 hrs at 37 $^0$C. The same procedure of planktonic cells for estimating MIC and MBC were followed for biofilm formers also.

**Results and Discussion**

**Revival of bacterial isolates**

The six representative isolates of *R. anatipestifer* designated as RA1, RA2, RA3, RA4, RA5 and RA6 were revived by culturing on BA. The inoculated petri plates were incubated at 37 ºC for 24 to 48h in a candle jar. They were subcultured and were maintained on BA plates for further studies.

**MIC and MBC**

The biofilm formers of *R. anatipestifer* isolate (RA1, RA5 and RA6) detected by microtitre plate method were subjected to determine MIC and MBC against enrofloxacin and sulfa-trimethoprim which are the commonly used antibiotics for RA infection in field condition (Fig 1 & 2). Organism with broth was taken as positive control and broth with antibiotic was considered as negative control.

The value obtained for MIC and MBC of planktonic and biofilm cultures were shown in table 1 to 4.

In the assay on RA1 and RA6 planktonic, the contents of well 9 and10 which showed clear were pipetted out, centrifuged and the supernatant containing antibiotic (enrofloxacin) was removed. After adding fresh BHIB to the same wells, on further incubation for 24 hrs well 9 were found to be clear and 10 as turbid. Similarly for RA5 planktonic, on removal and further incubation from wells 8 and 9, turbidity was noticed in the 9th well whereas well 8 was clear.

Biofilmed cells of RA1 and RA6 seemed to be clear in the 8th and 9th well. The content was pipetted out, centrifuged and supernatant that contain antibiotic were removed. It was again incubated for another 24 hrs after adding fresh BHIB to same wells. The 8thwells were found to be clear and the 9thas turbid. RA5 was found to be turbid at 7th and 8th well, after centrifugation and further incubation turbidity was noticed in the 8th well and 9th well was clear.

Different values were observed in the determination of MIC and MBC using sulfa trimethoprim. Planktonic cells of RA1 and RA6 were clear up to 9thwell. The content of 8th and 9th well were pipetted, centrifuged and fresh BHIB was added after discarding the antibiotic containing supernatant. After incubation for 24 hrs, the 9th well appeared turbid and 8th remain clear. Similarly for RA5 planktonic, on removal and further incubation from wells 7 and 8, turbidity was noticed in the8thwell, whereas well 9 were clear.

Biofilmed cells of RA1and RA6 seemed to be clear in the 7th and 8th well. The contents were pipetted out, centrifuged and supernatant were removed. After the addition of fresh BHIB, the plates were incubated for 24 hrs. The 7th well was found to be clear and the 8th was turbid. RA5 was found to be turbid at 6th and7thwell. After centrifugation and further incubation the 6th well was found to be clear and the 7thwell show turbidity.

**Table 1:** Value obtained for MIC and MBC for planktonic cells of *R. anatipestifer* against enrofloxacin

| | Enrofloxacin | +ve control | | -ve control | | RA1 | | RA5 | | RA6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| wells | Concentration (µg) | Organism+ broth | | Broth +A/B | | | | | | | |
| | | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs |
| 1 | 10000 | T | T | C | C | C | C | C | C | C | C |
| 2 | 5000 | T | T | C | C | C | C | C | C | C | C |
| 3 | 2500 | T | T | C | C | C | C | C | C | C | C |
| 4 | 1250 | T | T | C | C | C | C | C | C | C | C |
| 5 | 625 | T | T | C | C | C | C | C | C | C | C |
| 6 | 313 | T | T | C | C | C | C | C | C | C | C |
| 7 | 156 | T | T | C | C | C | C | C | C | C | C |
| 8 | 78 | T | T | C | C | C | C | C | C | C | C |
| 9 | 39 | T | T | C | C | C | C | C | T | C | C |
| 10 | 20 | T | T | C | C | C | T | T | T | C | T |
| 11 | 10 | T | T | C | C | T | T | T | T | T | T |
| 12 | 5 | T | T | C | C | T | T | T | T | T | T |

**Table 2:** Value obtained for MIC and MBC for biofilmed cells of *R. anatipestifer* against enrofloxacin

| | Enrofloxacin | +ve control | | -ve control | | RA1 | | RA5 | | RA6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| wells | Concentration (µg) | Organism + broth | | Broth +A/B | | | | | | | |
| | | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs |
| 1 | 10000 | T | T | C | C | C | C | C | C | C | C |
| 2 | 5000 | T | T | C | C | C | C | C | C | C | C |
| 3 | 2500 | T | T | C | C | C | C | C | C | C | C |
| 4 | 1250 | T | T | C | C | C | C | C | C | C | C |
| 5 | 625 | T | T | C | C | C | C | C | C | C | C |
| 6 | 313 | T | T | C | C | C | C | C | C | C | C |
| 7 | 156 | T | T | C | C | C | C | C | C | C | C |
| 8 | 78 | T | T | C | C | C | C | C | T | C | C |
| 9 | 39 | T | T | C | C | C | T | T | T | C | T |
| 10 | 20 | T | T | C | C | T | T | T | T | T | T |
| 11 | 10 | T | T | C | C | T | T | T | T | T | T |
| 12 | 5 | T | T | C | C | T | T | T | T | T | T |

**Table 3:** Value obtained for MIC and MBC for planktonic cells of *R. anatipestifer* against sulpha-trimethoprim

| | STM | +ve control | | -ve control | | RA1 | | RA5 | | RA6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| wells | Concentration (µg) | Organism + broth | | Broth + A/B | | | | | | | |
| | | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs | 24 hrs | 48 hrs |
| 1 | 12000 | T | T | C | C | C | C | C | C | C | C |
| 2 | 6000 | T | T | C | C | C | C | C | C | C | C |
| 3 | 3000 | T | T | C | C | C | C | C | C | C | C |
| 4 | 1500 | T | T | C | C | C | C | C | C | C | C |
| 5 | 750 | T | T | C | C | C | C | C | C | C | C |
| 6 | 375 | T | T | C | C | C | C | C | C | C | C |
| 7 | 188 | T | T | C | C | C | C | C | C | C | C |
| 8 | 94 | T | T | C | C | C | C | C | T | C | C |
| 9 | 47 | T | T | C | C | C | T | T | T | C | T |
| 10 | 23 | T | T | C | C | T | T | T | T | T | T |
| 11 | 12 | T | T | C | C | T | T | T | T | T | T |
| 12 | 6 | T | T | C | C | T | T | T | T | T | T |

**Table 4:** Value obtained for MIC and MBC for biofilmed cells of *R. anatipestifer* against sulpha-trimethoprim.

| | STM | +ve control | | -ve control | | RA1 | | RA5 | | RA6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Wells | Concentration (µg) | Organism + broth | | Broth + A/B | | | | | | | |
| | | 24 hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs | 24hrs | 48hrs |
| 1 | 12000 | T | T | C | C | C | C | C | C | C | C |
| 2 | 6000 | T | T | C | C | C | C | C | C | C | C |
| 3 | 3000 | T | T | C | C | C | C | C | C | C | C |
| 4 | 1500 | T | T | C | C | C | C | C | C | C | C |
| 5 | 750 | T | T | C | C | C | C | C | C | C | C |
| 6 | 375 | T | T | C | C | C | C | C | C | C | C |
| 7 | 188 | T | T | C | C | C | C | C | T | C | C |
| 8 | 94 | T | T | C | C | C | T | T | T | C | T |
| 9 | 47 | T | T | C | C | T | T | T | T | T | T |
| 10 | 23 | T | T | C | C | T | T | T | T | T | T |
| 11 | 12 | T | T | C | C | T | T | T | T | T | T |
| 12 | 6 | T | T | C | C | T | T | T | T | T | T |

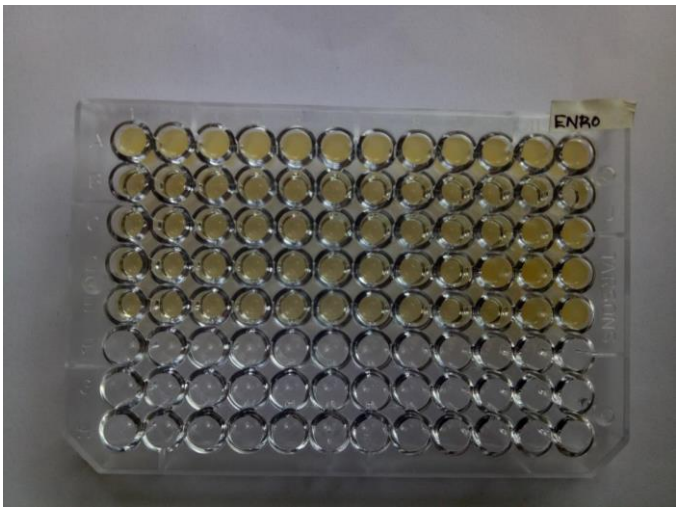


**Fig 1:** MIC and MBC for *R.anatipestifer* isolates against enrofloxacin.

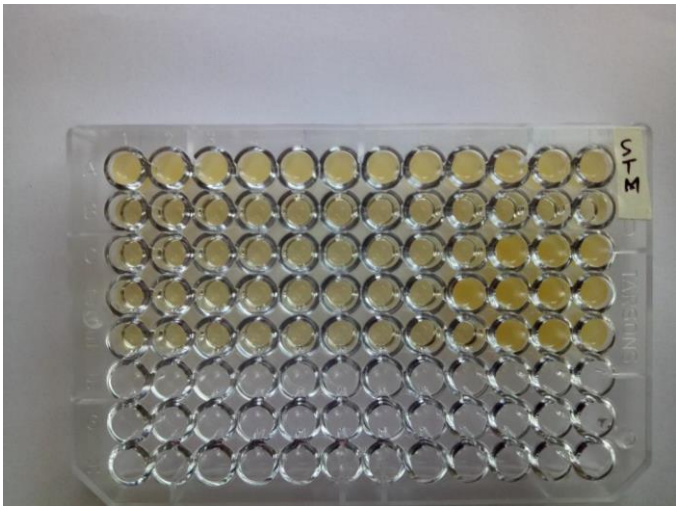


**Fig 2:** MIC and MBC for *R. anatipestifer* isolates against sulpha-trimethoprim.

The lowest concentration of antibiotic that prevent the visible growth of a microorganism after overnight incubation is defined as minimum inhibitory concentration (MIC). The lowest concentration of antibiotic that prevents the growth of an organism in antibiotic free media is known as minimum bactericidal concentration (MBC).

Broth dilution was one of the most basic methods for testing antimicrobial susceptibility. Two-fold dilutions of antimicrobial agent was prepared in a liquid growth medium distributed in tubes containing a minimum volume of 2 mL (macro dilution) or with smaller volumes (micro dilution) using 96-well microtiter plate. Microbial inoculum is prepared after dilution of standardized microbial suspension which was adjusted to 0.5 McFarland scale. Each tube or well is inoculated with this inoculum. After well-mixing, the inoculated tubes or the 96-well microtiter plate are incubated under favourable conditions depending on the test microorganism (Balouiri *et al.*, 2016) [1].

For calculating MIC and MBC of planktonic *R. anatipestifer* isolates with enrofloxacin, atleast 2 wells with higher concentration from that of turbid wells were clarified and after removal of antibiotic, fresh media was poured and incubated further. The above steps were followed in the study for well 9th and10th wells. On further incubation, tube 9 was found to be clear and10 was turbid indicates that the clearness of tube10 earlier was due to the effects of antibiotic. Once the antibiotic was removed the organism has grown and hence the antibiotic concentration of table 1 (20µg) is considered as MIC and value of well 9$^{th}$ (39µg) was arrived as MBC for RA1 and RA6, whereas for RA5 the MIC and MBC were 39µg and 78µg, respectively.

Similarly for sulpha-trimethprim, the MIC and MBC values of planktonic RA isolates were estimate as 47µg and 94µg, respectively.

The MIC and MBC value derived for enrofloxacin and sulpha-trimethprim for biofilm formers were double than that of the planktonic cells. i.e., the MIC and MBC values of enrofloxacin were 39µg and 78µg for RA1 and RA6 and a value of 78µg and 156µg were calculated for RA5.

The same methods of calculation were employed earlier by Islam *et al.* (2008) [8] and Holla (2012) [6].

In this study double the values were obtained for biofilm formers against enrofloxacin and sulpha-trimethprim, whereas Hu *et al.* (2011) [7] estimates that the biofilm formers give a value of 5-61 times more than that of planktonic cells.

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