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RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL RESISTANCE OF BIOFILM FORMS OF AVIAN *SALMONELLA GALLINARUM* TO FLUOROQUINOLONES

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Abstract

Objective: Antimicrobial resistance is a growing concern worldwide. The indiscriminate use of antibiotics for a period of time has led to the emergence of antibiotic resistance in pathogenic bacteria. The present study was designed to evaluate the antibacterial efficacy of fluoroquinolone drugs, ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin against avian *Salmonella gallinarum* bacterial biofilms.
Methods: The study parameters, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and biofilm elimination concentration (BEC) were determined on days 1, 3, 7, 10, 14 and 20 post inoculation for the planktonic (free) and biofilm cells of *S. gallinarum* by macro broth dilution method. The MIC and MBC values determined on days 1, 3, 7, 10, 14 and 20 for each of the fluoroquinolone drugs against the planktonic and biofilm forms of avian *S. gallinarum* were found to be non-significant.
Results: BEC values determined against the biofilm forms of *S. gallinarum* during the study period were found to be non-significant among the tested fluoroquinolones.
Conclusion: The results of the present study demonstrated that fluoroquinolone drugs were effective *in vitro* against both the planktonic and biofilm forms of avian *S. gallinarum.*
Keywords: Antibiotic resistance, biofilm, biofilm elimination concentration (BEC), fluoroquinolones, minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC), *S. gallinarum.*

INTRODUCTION

Antibacterial agents are commonly used as growth promoters in poultry and animal husbandry. Usage of antibiotics at sub therapeutic levels for therapeutic and prophylactic use can mediate the development of antimicrobial resistance in bacterial pathogens. Bacterial pathogens were gradually transformed to 'biofilm forms' and eventually more resistant to common antimicrobial drugs[1]. Under electron microscopy, biofilm revealed a pattern of colonization of bacterial cells in multiple layers[2,3]. The bacterial cells bind firmly to the surface by producing exopolysaccharide glycocalyx polymers, forming a matrix inside which microcolonies develop. As the size and number of the adherent microcolonies increases, they coalesce to form biofilms[4]. Bacterial biofilms are bacterial colonies adhering to a substrate, encased within the synthesized extracellular matrix of carbohydrate polysaccharide glycocalyx moiety[5] and thus protected from various antagonistic agents including antibiotics[6]. Fowl typhoid is a common infectious disease in poultry caused by *Salmonella gallinarum*. This dreadful disease produces persistent and recurrent morbidity and mortality in poultry. Poultry processing waste can act as reservoirs of transferrable drug-resistant *Salmonella sp.*[7] and contributed for the development of multiple drug resistance[8]. The virulence – associated plasmid of strains of *S. gallinarum* contributes toward virulence in fowl typhoid[9]. Fluoroquinolones are synthetic antibacterial agents used in veterinary/human medicine because of their high potency and rapid bactericidal action[10,11]. The target site for fluoroquinolones is the A subunit of DNA gyrase enzyme, which mediates the ATP-dependent crossing of one DNA duplex through a transient enzyme-bridge the double standard break in another DNA segment[12,13]. For *E. coli* and other Gram

negative bacteria, the concentration of quinolone that inhibits supercoiling of plasmid DNA or DNA synthesis by 50 per cent correlates well with the MIC[14,15,16.] Biofilm infections are of considerable importance in therapeutics. Since ciprofloxacin, a drug of the fluoroquinolone group, was effective in treating biofilm infections, the present study was carried out to evaluate the antibacterial efficacy of fluoroquinolone drugs against planktonic and biofilm forms of avian *S. gallinarum*.

MATERIALS AND METHODS

The present study was carried out in Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore, India.

Culture

The present study was conducted using Type I culture of *S. gallinarum* obtained from the Institute of Animal Health and Veterinary Biologicals (IAH and VB), Bangalore, India. Standard staining procedures and biochemical tests were carried out for confirmation of the organisms[17].

Antimicrobial drugs

The fluoroquinolone drugs, ciprofloxacin, moxi-floxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin were procured from Astrazeneca Pharmaceuticals Pvt. Ltd., Bangalore, India and enrofloxacin was obtained from Vetcare, Bangalore, India.

Antimicrobial sensitivity test

Antimicrobial susceptibility of *S. gallinarum* was determined for the fluoroquinolone drugs, ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin by antimicrobial sensitivity test method[18] using antimicrobial sensitivity test discs (Hi Media laboratories, Mumbai, India).

Preparation of free form of *S. gallinarum*

S. gallinarum culture grown in tryptic soya broth was harvested on days 1, 3, 7, 10, 14 and 20 after inoculation. Free form of *S. gallinarum* were then quantified by the Miles and Misra[19] method and expressed as colony-forming units per milliliter (CFU/ml).

Preparation of biofilm form of *S. gallinarum*

Growth medium

To 0.16% tryptic soya broth, 0.3% w/v bentonite clay powder was added and mixed well. This medium was autoclaved and checked for sterility by incubating at 37°C.

Procedure

To the biofilm growth medium, *S. gallinarum inoculum* containing 10^9 cells/ml was added and incubated at 37°C. The biofilm on the bentonite clay was harvested on days 1, 3, 7, 10, 14 and 20 after inoculation. The biofilm cells were quantified by sedimenting the biofilm cells colonized on bentonite clay at 1000 rpm for 5 minutes. The bacterial biofilm sediment was retained and the supernatant was discarded. The pellet was washed thrice with phosphate buffered saline (pH 7.4); later 10 ml. of sterile PBS was added to pellet and vortexed vigorously for 3 minutes. Biofilm cells released in supernatant were quantified by the Miles and Misra method[19] and expressed as colony forming unit (CFU/ml). Similarly, viable counts were determined on days 1, 3, 7, 10, 14 and 20 post inoculation[20].

Estimation of minimum inhibitory concentration (MIC, µg/ml) by macrobroth dilution method for planktonic and biofilm cells of *S. gallinarum*

A two-fold serial dilution of fluoroquinolone antibacterial drug in tryptic soya broth was prepared. One ml of planktonic *S. gallinarum inoculum* at a concentration of 10^6 CFU/ml was added to one ml of each dilution of fluoroquinolone drug preparation. Then the tubes were incubated at 37°C for 18 to 24 hours. The MIC values were then noted as the least amount of antimicrobial drug that resulted in complete inhibition of growth of planktonic/biofilm cells of *S. gallinarum*. The MIC values for planktonic and biofilm forms of *S. gallinarum* were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

Estimation of minimum bactericidal concentration (MBC, µg/ml) by macrobroth dilution method[21] for planktonic and biofilm cells of *S. gallinarum*

A two-fold serial dilution of fluoroquinolone drug in tryptic soya broth was prepared. To one ml of each dilution of an antimicrobial preparation, one ml of planktonic/biofilm inoculum of *S. gallinarum* at a concentration of 10^6 CFU/ml was added. The test tubes were then incubated at 37°C for 18 to 24 hours. After this inhibitory phase of the test was completed, 10µl from each tube was subcultured on a nutrient agar plate. The plates were then incubated overnight and the MBC was determined as the lowest concentration of antimicrobial agent, subculture of which was lethal to 99.9 per cent of the original inoculum. The MBCs for planktonic and biofilm forms of *S. gallinarum* were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

Estimation of biofilm elimination concentration (BEC, µg/ml) for biofilm cells of *S. gallinarum*

To one ml of *S. gallinarum* biofilm inoculum containing 10^6CFU/ml, one ml of each antimicrobial drug preparation prepared in tryptic soy broth (TSB) was added. The tubes were incubated for 18 to 24 hours at 37°C and at the end of the incubation period, each tube was vortex mixed for five minutes and 10µl from each tube was dropped on to the surface of nutrient agar plate. The biofilm elimination concentration was the minimum amount of antibiotic concentration required to eliminate 99.9 per cent cells in the biofilms. The biofilm elimination concentrations were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

Statistical analysis

The paired t-test was used to assess the significance of the difference of two means whereas one-way ANOVA was employed to compare all the groups. The values were expressed as mean±SE, n=6. The computer software Graph Pad Prism version IV was used to analyze the data.

RESULTS

Antimicrobial sensitivity test

In the present study, the antimicrobial sensitivity test revealed that *S. gallinarum* was found to be sensitive to all the fluoroquinolone drugs tested such as ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin. The results were represented in Table 1.

Minimum inhibitory concentration (MIC, µg/ml)

The minimum inhibitory concentrations of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the planktonic and biofilm forms of *S. gallinarum* determined on days 1, 3, 7, 10, 14 and 20 were compared by paired t-test.

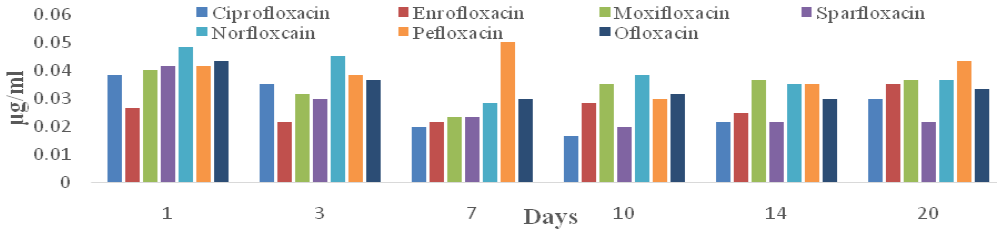

Figure 1: Comparison of the minimum inhibitory concentration (MIC, µg/ml) ofciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for planktoniccells of *S. gallinarum*.
(n=6, p>0.05)

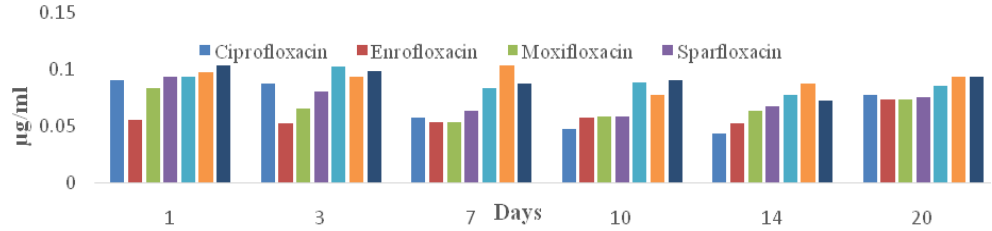

Figure 2: Comparison of the minimum inhibitory concentration (MIC, µg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cells of *S. gallinarum*.
(n=6, p>0.05)

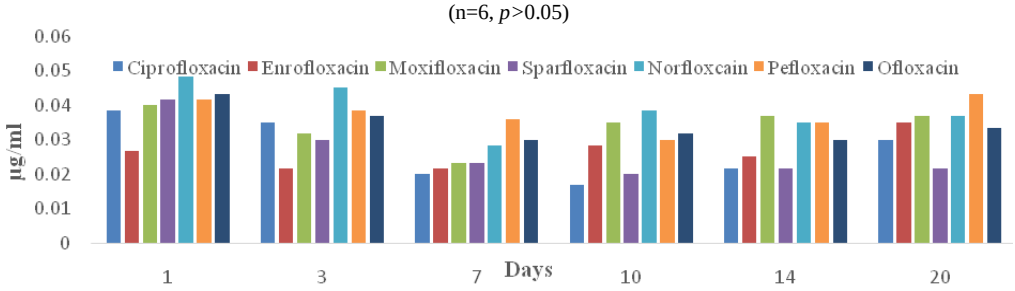

Figure 3: Comparison of the minimum bactericidal concentration (MBC, µg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for planktonic cells of *S. gallinarum*.
(n=6, p>0.05)

On analysis, the MIC values for planktonic forms of *S. gallinarum* revealed no significant difference (p>0.05) with the MIC values of biofilm forms. Also the MIC values of planktonic and biofilm forms of *S. gallinarum* showed no significant difference among the fluorquinolone drugs tested. The MIC values of planktonic and biofilm forms of *S. gallinarum* against the tested fluoroquinolones during the period of 20 days are collectively presented in Figure 1 and Figure 2 respectively.

Minimum bactericidal concentration (MBC, µg/ml)

The minimum bactericidal concentrations of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the planktonic and biofilm forms of *S. gallinarum* determined respectively on days 1, 3, 7, 10, 14 and 20 were found to be non-significant. The data presented in Figure 3 and Figure 4 depicted the MBC values of each fluoroquinolone drug determined on specific days for planktonic and biofilm forms of *S. gallinarum* and did not differ significantly (p>0.05) among the fluoroquinolone drugs. In this study, MBC values of the fluoroquinolone drugs tested were found to be higher than their corresponding MIC values.

Table 1: Antimicrobial sensitivity test of *S. gallinarum*.

Antimicrobial disc	Disc content (µg)	Diameter of zone of inhibition (mm)*
Ciprofloxacin	5	23
Enrofloxacin	5	22
Moxifloxacin	5	21
Sparfloxacin	5	21
Norfloxacin	10	19
Pefloxacin	5	17
Ofloxacin	5	18

*17 mm or more is considered as sensitive

Biofilm elimination concentration (BEC, µg/ml)

The BEC values of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the biofilm forms of *S. gallinarum* are presented in Figure 5. The BEC values determined on days 1, 3, 7, 10, 14 and 20 were found to be non-significant (p>0.05) among the tested fluoroquinolone drugs. Also, BEC values were found to be higher than their respective MBC values.

DISCUSSION

Antimicrobial resistance development in bacterial organisms could be associated mainly with injudicious use of the antibiotics for therapeutic purposes. This would be expressed as poor permeation of antibacterial drugs to the target site or rapid drug inactivation or the modification of target drug site. The antibacterial resistance could be either intrinsic or acquired through plasmids. Additional ways of resisting the actions of antibacterial agents by bacteria is by formation of biofilms. In the present study, avian *S. gallinarum* was found to be sensitive for the fluoroquinolone drugs tested, such as ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin, using the antimicrobial sensitivity test. This could be attributed to the higher lipophilic nature of fluoroquinolones so that the drug can easily enter the bacterial cells and binds with higher affinity to topoisomerase targets[22,23].

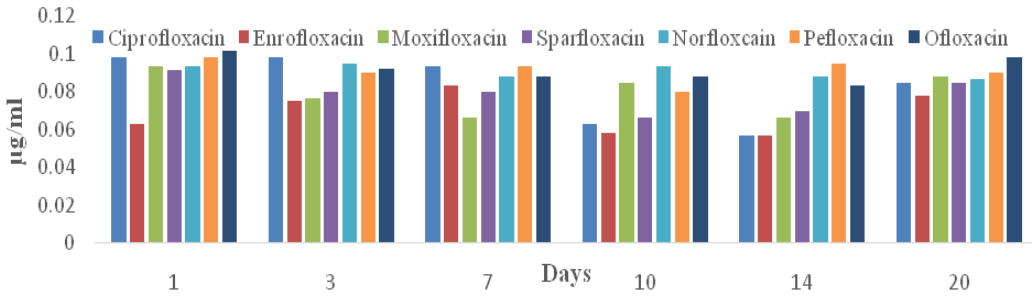

Figure 4: Comparison of the minimum bactericidal concentration (MBC,µg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cells of *S. gallinarum*. (n=6, p>0.05)

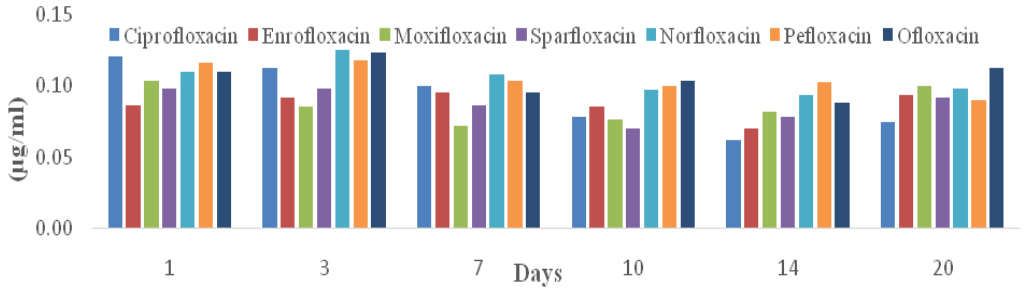

Figure 5: Comparison of the biofilm elimination concentration (BEC, µg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cells of *S. gallinarum*. (n=6, p>0.05)

These findings were in accordance with similar research studies[24,25,26,27]. In this study, the MIC (µg/ml) of the tested fluoroquinolone drugs revealed no difference (p>0.05) for the inhibition of planktonic cell form and biofilm cell forms of *S. gallinarum*, whereas MIC of norfloxacin and pefloxacin on Day 3 and 7, respectively were higher against the biofilm cells as compared to MIC for planktonic cells. This might be due to the complexity of biofilm structure[1] requiring a higher drug concentration of these drugs for the inhibition of bacterial growth. The comparison of MICs for ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin against the planktonic and biofilm cells of *S. gallinarum* revealed that all drugs were effective in inhibiting the planktonic and biofilm forms. This could be attributed to the better penetrating ability of fluoroquinolones through the biofilm via the bacterial pores or channels[23,28]. The MBC (µg/ml) of the tested fluoroquinolone drugs revealed no difference (p>0.05) for the inhibition of planktonic cell form and biofilm cell forms, whereas MBC of pefloxacin and ofloxacin on Day 10 and 20, respectively was higher against the biofilm cells as compared to MIC for planktonic cells. This could be due to the complexity of biofilm structure[1]or any changes in CFU/ ml of the bacterial organisms. The results were in accordance to the reports where enrofloxacin and ciprofloxacin, respectively was found to be effective against the planktonic cell forms and the biofilm cell forms of *S. gallinarum*[27,29]. The biofilms are colonisation of bacterial organisms. The surface pores or channels of bacteria penetrate through the biofilms, so forming the pathway of antibiotic penetration[30]. Since, fluoroquinolones are meant for their good penetrating ability, these drugs can enter through the biofilms and reach the target site of drug action. The biofilm elimination concentration of the tested fluoroquinolone drugs for the biofilm cells of *S. gallinarum* revealed no difference (p>0.05) among each other. The BEC of fluoroquinolone drugs were higher than MBCs observed. The reason might be due to the production of

an exopolysaccharide matrix or glycocalyx by biofilms, which prevents the access of antibiotics to the bacterial cells embedded in biofilm[1,31]. The minimum inhibitory concentration (MIC, µg/ml) and minimum bactericidal concentration (MBC, µg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin revealed no significant difference ($p>0.05$) for the inhibition of planktonic cells and biofilm cells during the study period. This indicates that all the fluoroquinolone drugs tested were effective in inhibiting both the planktonic and biofilm cells. This could be attributed to the ability of fluoroquinolones to penetrate biofilm via the bacterial pores or channels[23,28]. Confocal scanning laser microscopy studies demonstrated pores/channels permeating through the bacterial biofilms[30]. It could be hypothesized that the fluoroquinolones can penetrate through these bacterial pores in the biofilms to reach the target site of action. This could be further correlated to the report wherein ciprofloxacin can effectively induced detachment in biofilm cells for drug penetration[28]. The results of the present study were in accordance with the reports where enrofloxacin and ciprofloxacin were found to be effective against the planktonic and the biofilm cell forms of *E. coli*[27,29]. In the present study, the BEC values obtained were higher than the MBCs observed for the individual drugs. This might be possibly due to the additional factors contributing for the increased resistance of biofilms such as the complex structure of the bacterial biofilms, lower penetration of antibacterial agents into biofilm, growth rate of bacteria in biofilm forms and altered gene expression in biofilms[1]. Bacterial biofilms are composed of several layers and act as a barrier for the antimicrobial penetration. This might have increased the resistance for the elimination of biofilms at normal MBC[32]; hence the BEC values for the fluoroquinolone drugs tested would be higher. Moreover, the extracellular matrix of biofilms is negatively charged, the interaction of drug molecules with such a negatively charged matrix could also be a contributing factor for higher value of BEC[4].

CONCLUSIONS

From this study, it could be concluded that fluoroquinolone antibacterial agents, ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin were effective *in vitro* against the planktonic and biofilm forms of avian *S. gallinarum*. These research findings should be further applied *in vivo* to determine the efficacy of fluoroquinolones in treating chronic/biofilm related infections.

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AUTHOR'S CONTRIBUTION

Kumar K: writing original draft, conceptualization, **Honnegowda:** methodology, investigation. **Mayanna A:** writing, review, and editing, supervision, resources. **Chandrakala:** writing, review, and editing. All authors revised the article and approved the final version.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

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