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Research Article

EVALUATION OF ANTIBACTERIAL RESISTANCE OF BIOFILM FORMS OF AVIAN *ESCHERICHIA COLI* TO FLUOROQUINOLONES

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ABSTRACT

Bacterial biofilms are the common cause of antibiotic resistance. Several studies have been carried out to determine the most effective antibacterial agent in treating biofilm associated infections. The present study focused on measuring the antibiotic sensitivity of avian Escherichia coli biofilms to fluoroquinolone drugs, ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin. The parameters like 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 E. coli by macrobroth 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 E. coli were found to be non-significant. BEC values determined against the biofilm forms of E. coli during the study period were found to be non-significant among the tested fluoroquinolones. The results of the present study demonstrated that fluoroquinolone drugs were effective in vitro against both the planktonic and biofilm forms of avian E. coli.

Keywords: antibiotic resistance, biofilms, biofilm elimination concentration (BEC), Escherichia coli, fluoroquinolones, minimum bactericidal concentration (MBC), minimum inhibitory concentration (MIC)

INTRODUCTION

Antibacterial agents are commonly used as growth promoters and as therapeutic and prophylactic agents in poultry. Injudicious usage of antibacterial drugs over a period of time has led to the emergence of antibiotic resistance in pathogenic bacteria. 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)]. Biofilm adheres to a substrate encased within the synthesized extracellular matrix of the polysaccharide glycocalyx moiety. Bacterial colonies in biofilm have nutritional limitations, grow slowly and have restricted mobility compared to the free forms (planktonic) of bacteria. This might contribute significantly to increased resistance to antibacterial agents as well as to combat the natural host defenses [(4, 5, 6)].

Colibacillosis is a common infectious disease in poultry caused by *Escherichia coli*. It produces persistent and recurrent morbidity and mortality in poultry. Antibiotic resistance in *E. coli* strains is a major economic constraint in the poultry industry [7, 8]. Several research studies have recently been conducted to evaluate the antibiotic sensitivity of biofilm forms of *E. coli*. Among the various drugs studied, ciprofloxacin was found to be effective against steady state biofilms of *E. coli* [9, 10]. Further studies revealed that Ciprofloxacin at therapeutic concentrations was effective against both planktonic forms and biofilm forms of *E. coli* [11].

Biofilm infections are of considerable significance in clinical medicine. 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 *E. coli*.

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 'O' 78 avian strain of *E. coli*, obtained from the Institute of Animal Health and Veterinary Biologicals (IAH&VB), Bangalore, India. Standard staining procedures and biochemical tests were carried out for confirmation of the organisms [12].

Antimicrobial drugs

The fluoroquinolone drugs, ciprofloxacin, moxifloxacin, 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

E. coli culture used for this study was tested for antimicrobial susceptibility against the fluoroquinolone drugs, ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin by antimicrobial sensitivity test method [13] using antimicrobial sensitivity test discs (Hi Media laboratories, Mumbai, India).

Preparation of free form of *E. coli*

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

Preparation of biofilm form of *E. coli*

Growth medium for *E. coli* biofilm

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.

Procedure

To the biofilm growth medium, *E. coli* inoculum containing 10^9cells/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 [(14)] and expressed as colony forming unit (CFU/ml). Similarly, viable counts were determined on days 1, 3, 7, 10, 14 and 20 post inoculation [(15)].

Estimation of minimum inhibitory concentration (MIC, «g/ml) by macrobroth dilution method [(16)] for planktonic and biofilm cells of *E. coli*

A two-fold serial dilution of fluoroquinolone antibacterial drug in tryptic soya broth was prepared. One ml of planktonic *E. coli* inoculum at a concentration of 10^6CFU/ml was added to one ml of each dilution of fluoroquinolone drug preparation. Then the tubes were incubated at 37^0C for 18 to 24 hours. Biofilm form of *E. coli* was also processed in the same method. 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 *E. coli*. The MIC values for planktonic and biofilm forms of *E. coli* were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

Estimation of minimum bactericidal concentration (MBC, «g/ml) by macrobroth dilution method [(16)] for planktonic and biofilm cells of *E. coli*

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 *E. coli* at a concentration of 10^6 CFU/ml was added. The test tubes were then incubated at 37^0C 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 *E. coli* were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

Estimation of biofilm elimination concentration (BEC, «g/ml) for biofilm cells of *E. coli*

To one ml of *E. coli* 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 37^0C 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 *E. coli* was found to be sensitive to all the fluoroquinolone drugs tested such as ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin.

Table 1. Antimicrobial sensitivity test of *Escherichia coli*

Sl. No.	Antimicrobial disc	Disc content (µg)	Diameter of zone of inhibition (mm)*
1	Ciprofloxacin	5	32
2	Enrofloxacin	5	26
3	Moxifloxacin	5	27
4	Sparfloxacin	5	26
5	Norfloxacin	10	27
6	Pefloxacin	5	26
7	Ofloxacin	5	24

* 17 mm or more is considered as sensitive

Minimum inhibitory concentration (MIC, «g/ml)

The minimum inhibitory concentrations of ciprofloxacin, enrofloxacin, moxifloxacin sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the planktonic and biofilm forms of *E. coli* determined on days 1, 3, 7, 10, 14 and 20 were compared by paired “t” test. On analysis, the MIC values for planktonic forms of *E. coli* revealed no significant difference (P>0.05) with the MIC values of biofilm forms. Also the MIC values of planktonic and biofilm forms of *E. coli* showed no significant difference among the fluorquinolone drugs tested. The MIC values of planktonic and biofilm forms of *E. coli* against the tested fluoroquinolones during the period of 20 days are collectively presented in Tables 2 and 3 respectively.

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Table 2. Comparison of the minimum inhibitory concentration (MIC, «g/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for planktonic cells of *Escherichia coli*

Day	Minimum inhibitory concentration (μg/ml)						
	Ciprofloxacin	Enrofloxacin	Moxifloxacin	Sparfloxacin	Norfloxacin	Pefloxacin	Ofloxacin
1	0.0267 ± 0.0021	0.0233 ± 0.0021	0.0267 ± 0.0033	0.0367 ± 0.0021	0.0450 ± 0.0042	0.0517 ± 0.0030	0.0483 ± 0.0030
3	0.0233 ± 0.0021	0.0133 ± 0.0021	0.0317 ± 0.0030	0.0300 ± 0.0025	0.0333 ± 0.0021	0.0500 ± 0.0044	0.0450 ± 0.0022
7	0.0267 ± 0.0021	0.0217 ± 0.0016	0.0250 ± 0.0022	0.0250 ± 0.0022	0.0317 ± 0.0040	0.0283 ± 0.0030	0.0317 ± 0.0030
10	0.0250 ± 0.0022	0.0217 ± 0.0030	0.0233 ± 0.0021	0.0217 ± 0.0016	0.0300 ± 0.0025	0.0383 ± 0.0030	0.0300 ± 0.0036
14	0.0150 ± 0.0022	0.0150 ± 0.0022	0.0250 ± 0.0022	0.0300 ± 0.0025	0.0233 ± 0.0021	0.0450 ± 0.0034	0.0350 ± 0.0022
20	0.0217 ± 0.0016	0.0150 ± 0.0022	0.0333 ± 0.0033	0.0283 ± 0.0030	0.0317 ± 0.0040	0.0450 ± 0.0042	0.0483 ± 0.0030

Mean ±SD, n=6

P >0.05

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Table 3. Comparison of the minimum inhibitory concentration (MIC, «g/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cells of *Escherichia coli*

Day	Minimum inhibitory concentration (μg/ml)						
	Ciprofloxacin	Enrofloxacin	Moxifloxacin	Sparfloxacin	Norfloxacin	Pefloxacin	Ofloxacin
1	0.0400 ± 0.0025	0.0400 ± 0.0025	0.0550 ± 0.0034	0.0733 ± 0.0033	0.0867 ± 0.0033	0.0850 ± 0.0042	0.1167 ± 0.0042
3	0.0450 ± 0.0022	0.0350 ± 0.0022	0.0550 ± 0.0022	0.0550 ± 0.0022	0.0917 ± 0.0030	0.0933 ± 0.0049	0.0850 ± 0.0042
7	0.0383 ± 0.0016	0.0383 ± 0.0016	0.0533 ± 0.0021	0.0517 ± 0.0030	0.0717 ± 0.0047	0.0750 ± 0.0022	0.0683 ± 0.0030
10	0.0417 ± 0.0016	0.0450 ± 0.0022	0.0433 ± 0.0021	0.0433 ± 0.0021	0.0783 ± 0.0030	0.0583 ± 0.0030	0.0817 ± 0.0040
14	0.0333 ± 0.0021	0.0317 ± 0.0016	0.0483 ± 0.0030	0.0500 ± 0.0025	0.0617 ± 0.0030	0.0833 ± 0.0042	0.0800 ± 0.0025
20	0.0383 ± 0.0030	0.0350 ± 0.0022	0.0500 ± 0.0036	0.0600 ± 0.0036	0.0867 ± 0.0042	0.0867 ± 0.0033	0.0867 ± 0.0033

Mean ±SD, n=6

P>0.05

Minimum bactericidal concentration (MBC, «g/ml)

The minimum bactericidal concentrations of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the planktonic and biofilm forms of *E. coli* determined respectively on days 1, 3, 7, 10, 14 and 20 were found to be non-significant. The data presented in Tables 4 and 5 depicted the MBC values of each fluoroquinolone drug determined on specific days for planktonic and biofilm forms of *E. coli* 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.

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Table 4. Comparison of the minimum bactericidal concentration (MBC, «g/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for planktonic cells of *Escherichia coli*

Day	Minimum bactericidal concentration (µg/ml)						
	Ciprofloxacin	Enrofloxacin	Moxifloxacin	Sparfloxacin	Norfloxacin	Pefloxacin	Ofloxacin
1	0.0383 ± 0.0030	0.0317 ± 0.0016	0.0350 ± 0.0022	0.0467 ± 0.0042	0.0567 ± 0.0033	0.0517 ± 0.0030	0.0500 ± 0.0036
3	0.0333 ± 0.0021	0.0250 ± 0.0022	0.0433 ± 0.0033	0.0450 ± 0.0022	0.0467 ± 0.0033	0.0667 ± 0.0033	0.0567 ± 0.0042
7	0.0333 ± 0.0021	0.0233 ± 0.0021	0.0383 ± 0.0016	0.0333 ± 0.0021	0.0417 ± 0.0030	0.0400 ± 0.0025	0.0383 ± 0.0030
10	0.0250 ± 0.0022	0.0283 ± 0.0016	0.0250 ± 0.0022	0.0283 ± 0.0030	0.0433 ± 0.0033	0.0483 ± 0.0030	0.0500 ± 0.0036
14	0.0233 ± 0.0021	0.0250 ± 0.0022	0.0333 ± 0.0021	0.0300 ± 0.0025	0.0300 ± 0.0025	0.0500 ± 0.0025	0.0400 ± 0.0036
20	0.0333 ± 0.0021	0.0233 ± 0.0033	0.0433 ± 0.0033	0.0450 ± 0.0022	0.0433 ± 0.0033	0.0450 ± 0.0022	0.0483 ± 0.0040

Mean ±SD, n=6

P>0.05

Table 5. Comparison of the minimum bactericidal concentration (MBC, «g/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cells of *Escherichia coli*

Day	Minimum bactericidal concentration (μg/ml)						
	Ciprofloxacin	Enrofloxacin	Moxifloxacin	Sparfloxacin	Norfloxacin	Pefloxacin	Ofloxacin
1	0.0533 ± 0.0021	0.0567 ± 0.0021	0.0717 ± 0.0047	0.0783 ± 0.0047	0.0867 ± 0.0033	0.0900 ± 0.0036	0.1050 ± 0.0042
3	0.0550 ± 0.0022	0.0550 ± 0.0022	0.0617 ± 0.0030	0.0950 ± 0.0042	0.0917 ± 0.0030	0.1017 ± 0.0047	0.0950 ± 0.0042
7	0.0533 ± 0.0021	0.0517 ± 0.0030	0.0583 ± 0.0030	0.0783 ± 0.0030	0.0717 ± 0.0047	0.0817 ± 0.0030	0.0750 ± 0.0022
10	0.0433 ± 0.0021	0.0467 ± 0.0021	0.0550 ± 0.0022	0.0567 ± 0.0033	0.0783 ± 0.0030	0.0717 ± 0.0030	0.0883 ± 0.0030
14	0.0500 ± 0.0036	0.0533 ± 0.0021	0.0600 ± 0.0025	0.0633 ± 0.0042	0.0617 ± 0.0030	0.0867 ± 0.0033	0.0817 ± 0.0030
20	0.0550 ± 0.0022	0.0467 ± 0.0021	0.0650 ± 0.0042	0.0817 ± 0.0030	0.0867 ± 0.0042	0.0900 ± 0.0036	0.0817 ± 0.0030

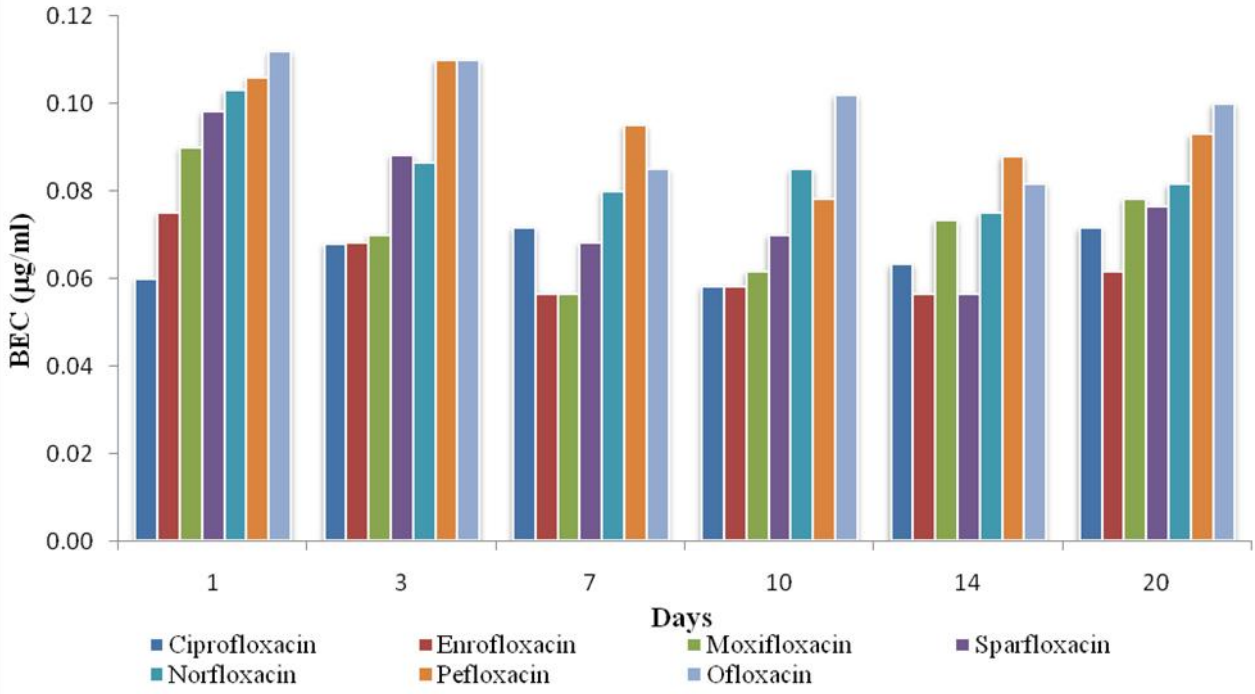
Mean ±SD, n=6

P>0.05

Biofilm elimination concentration (BEC, «g/ml)

The BEC values of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin, and ofloxacin for the biofilm forms of *E. coli* are presented in Fig. 1. 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.

Fig.1. Comparison of biofilm elimination concentration (BEC, mg/ml) of ciprofloxacin, enrofloxacin, moxifloxacin, sparfloxacin, norfloxacin, pefloxacin and ofloxacin for biofilm cell forms of *Escherichia coli*



DISCUSSION

In the present study, avian *E. coli* 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 greater lipophillic nature [17] and tissue penetration abilities of the fluoroquinolones [18]. These results were in accordance with similar research studies [11, 19, 20, 21].

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 are 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 [18, 22]. Confocal scanning laser microscopy studies demonstrated pores/channels permeating through the bacterial biofilms [23]. 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 [22] wherein ciprofloxacin can effectively induced detachment in biofilm cells for drug penetration. The results of the present

study were in accordance with the reports [11, 24] where enrofloxacin and ciprofloxacin were found to be effective against the planktonic and the biofilm cell forms of *E. coli*.

In the present study, it was found that 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 bioflms 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 interfered with the elimination of biofilms at normal MBC [25]; 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].

CONCLUSION:

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 *E. coli*. These research findings should be applied *in vivo* to determine the efficacy of fluoroquinolones in treating chronic/biofilm related infections.

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