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Evaluation of Enrofloxacin Sensitivity on Free and Biofilm Form of *Salmonella* Pullorum

G. Kavitha, G.K. Chandrakala, and S.M. Byregowda

Institute of Animal Health and Veterinary Biologicals,

Hebbal, Bangalore – 560 024.

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Corresponding author:

drkavitha85@gmail.com

Abstract

In the present study, Enrofloxacin sensitivity of free/planktonic and biofilm forms of *Salmonella* Pullorum was assessed for a period of 20 days. The parameters like Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Biofilm Elimination Concentration (BEC) were evaluated.

The investigation revealed that the MIC and MBC values for biofilm form were found to be slightly higher than planktonic form. The values of MIC and MBC of enarofloxacin against planktonic as well as biofilm forms of *S. Pullorum* were found non significant (P>0.05) during the study period. The BEC values obtained were found higher than the MBC values. Hence, the findings suggested that the fluroquinolone antibacterial drug, enrofloxacin was found effective against the planktonic and biofilm forms of *S. Pullorum* and can be used for its management.

Introduction

More than half of the infectious diseases that affect mildly compromised individuals involve bacterial species that are commensals and very common in our environments. For example, *E. coli*, *Salmonella*, *Streptococcus suis* and *S. agalactiae*, which colonize the mucosal membranes. It is difficult to eradicate such infections from a farm, and in many cases they may even cause chronic infections in livestock, which may show the presence of biofilm bacteria surrounded by an exopolysaccharide matrix. Rediscovery of a microbiological phenomenon, the 'biofilms' exhibited a distinct phenotype with respect to gene transcription and growth rate, where bacteria undergo transition from a planktonic (loner) existence to a community-based existence in which they must interact with many neighbours of various species in close proximity (Prakash *et al.*, 2003).

Pullorum disease is global in nature i.e. it has been found substantially in all poultry producing area of the world. This disease causes severe economic loss with persistent and recurrent morbidity and mortality. The disease is characterized by bacillary white diarrhea. Chicken are the natural host and turkeys are also affected with this infection. *Salmonella* spp. and *Pseudomonas aeruginosa* isolates growing as planktonic populations were sensitive to

enrofloxacin, gentamicin, ampicillin, oxytetracycline, and trimethoprim/sulfadoxine, but as a biofilm, these bacteria were only sensitive to enrofloxacin (Olson *et al.*, 2002).

Several research studies have recently been conducted to evaluate the antibiotic sensitivity of biofilm forms of *E. coli*. The antibiotic sensitivity of bacterial biofilms to ciprofloxacin was reported by Ashby *et al.* (1994). Studies revealed that Ciprofloxacin at therapeutic concentrations was effective against both planktonic forms and biofilm forms of *E. coli* (Ramesh, 2003). Fluroquinolones are synthetic antibacterial agents used extensively in veterinary and human medicine because of their high potency and rapid bactericidal action (Brown, 1996). Fluoroquinolone drugs were effective in vitro against both the planktonic and biofilm forms of avian E. coli (Kumar *et al.*, 2014). Thus, the present study was undertaken to find out the sensitivity of the planktonic and biofilm forms of *S.*Pullorum to enrofloxacin antibiotic.

Materials and Methods

The present study was carried out using *S. Pullorum* (SP17) maintained at IAH & VB, Bangalore. Enrofloxacin was obtained from Vetcare India Pvt. Ltd.

Preparation of free and biofilm forms of *S.Pullorum*

The free form of *S. Pullorum* culture was grown in 3.0 per cent TSB (Tryptic Soya Broth) at 37 °C. The culture grown in tryptic soya broth was harvested on days 1, 3, 7, 10, 14 and 20 after inoculation. Free forms were then quantified and expressed as colony-forming units per milliliter (CFU/ml).

The Bentonite clay used for growing biofilm was procured from Loba chemie Pvt. Ltd., Mumbai. The bentonite clay (0.3%) was suspended in 0.16 per cent tryptic soya broth and incubated at 37 °C for the production of *S.* Pullorum biofilms (Prakash *et al.,* 2002). Standard staining procedures and biochemical tests were carried out for confirmation of the organisms

Salmonella Pullorum 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 harvested 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 and expressed as colony forming unit (CFU/ml). Similarly, viable counts were determined on days 1, 3, 7, 10, 14 and 20 post inoculation.

Minimum Inhibitory Concentration (MIC, mg/ml) and Minimum Bactericidal Concentration (MBC, mg/ml)

MIC and MBC were determined by macrobroth dilution method on days 1, 3, 7, 10, 14 and 20 of post inoculation. A two-fold serial dilution of enrofloxacin in tryptic soya broth was prepared (from 1.024 mg/ml to 0.002 mg/ml). One ml of inoculum at a concentration of 10^6 CFU/ml was added to one ml of each dilution of enrofloxacin 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 enrofloxacin that resulted in complete inhibition of growth of planktonic/biofilm cells.

For MBC determination after the inhibitory phase of the test , 0.1 ml from each tube was subcultured on to a nutrient agar plate. The plates were then incubated overnight and the MBC was determined as the lowest concentration of enrofloxacin, subculture of which was lethal to 99.9 per cent of the original inoculum.

Biofilm Elimination Concentration (BEC, mg/ml)

The BEC was determined for biofilm forms of *S.Pullorum* on days 1, 3, 7, 10, 14 and 20 of post inoculation. One ml of each dilution of enrofloxacin (from 1.024 mg/ml to 0.002 mg/ml) prepared in tryptic soy broth (TSB) was added to one ml of *S.Pullorum* biofilm culture containing 10^6CFU/ml. 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 5 minutes and 0.1 ml from each tube was dropped on to the surface of nutrient agar plate. The BEC was the minimum amount of enrofloxacin required to eliminate 99.9 per cent cells in the biofilms.

Statistical analysis

The paired't' test was used to assess the significance of the differences of two means. The computer software GraphPad Prism version 4 was used to analyze the data.

Results and Discussion

The MIC values for biofilm form were slightly higher than for planktonic form (Table 1). This could be because of the formation of thick and stable exopolysaccharide (EPS) matrix. The EPS secreted by biofilm bacteria, acts as a physical/ chemical barrier, thus preventing penetration by antibodies or many antibiotics (Prakash *et al.,* 2003). Moreover, EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells. The paired 't' test was carried out to compare MIC values of enrofloxacin for the planktonic and biofilm forms of *S.Pullorum.* There is no significant difference (P>0.05) between the planktonic and biofilm forms with respect to MIC values of enrofloxacin.

There was an increase in the trend of MBC values for the biofilm forms (Table 1). This trend is correlated with the process involved in the biofilm formation. Biofilm-forming microorganisms have been shown to elicit specific mechanisms for initial attachment to a surface, micorcolony formation, development of a stable and thick three-dimensional community structure (microcolony formation with EPS matrix) and maturation (Prakash *et al*., 2003).

After day 10 and 14 of inoculation, the MBC values for biofilm were reduced to 0.0582 $\pm$ 0.003072 mg/ml and 0.0566 $\pm$ 0.003332 mg/ml respectively (Table 1). This could be attributed to the detachment of cells from biofilm matrix. Further, these cells act as planktonic cells. Occasionally, for mechanical reasons, some bacteria are shed from the colony or (more frequently) some bacteria stop producing EPS and are thus released into the surrounding environment (Prakash *et al.,* 2003). Biofilm cells may be dispersed either by shedding of daughter cells from actively growing cells, or detachment as a result of nutrient levels or quorum sensing, or shearing of biofilm aggregates (continuous removal of small portions of the biofilm) because of flow effects (Baselga *et al*., 1994).

Table 1. The MIC and MBC of enrofloxacin for planktonic and biofilm cells of *Salmonella Pullorum*.

Day	Minimum Inhibitory Concentration (mg/ml)		Minimum Bactericidal Concentration (mg/ml)	
	Planktonic cells	Biofilm cells	Planktonic cells	Biofilm cells
1	0.0254 $\pm$ 0.002101	0.0545 $\pm$ 0.002216	0.0317 $\pm$ 0.003072	0.062 $\pm$ 0.002106
3	0.0217$\pm$ 0.001656	0.0517+ 0.003063	0.030 $\pm$ 0.002583	0.0750 + 0.004280
7	0.0218 $\pm$ 0.001666	0.0522 $\pm$ 0.003332	0.0318 $\pm$ 0.003072	0.0822 $\pm$ 0.003312
10	0.02883 $\pm$ 0.003063	0.0567 $\pm$ 0.003321	0.0380 $\pm$ 0.00234	0.0582 $\pm$ 0.003072
14	0.0252 $\pm$ 0.002236	0.0515 $\pm$ 0.001664	0.0366 $\pm$ 0.002108	0.0566 $\pm$ 0.003332
20	0.0350 $\pm$ 0.002235	0.0732 $\pm$ 0.003332	0.0462 $\pm$ 0.003072	0.0782 $\pm$ 0.004012

The Paired 't' test revealed no significant difference (p>0.05) between the planktonic and biofilm forms with respect to MBC values of enrofloxacin.

This could be attributed to the better penetrating ability of enrofloxacin (Muller *et al.,* 1999) to reach the target site of action through the bacterial pores or channels in the biofilm formed by *S.Pullorum* (De beer *et al.,* 1993). The results were in accordance with the reports of Olson *et al*. (2002) and Ramesh (2003).

The BEC values obtained were higher than the MBC values (Table 2). This could be attributed to the complex structure of biofilm which acts as a barrier. This might have interfered with the elimination of biofilms at normal MBC. The

Table 3 The Biofilm Elimination Concentration (mg/ml) of enroflozacin for the biofilm cells of *Salmonella Pullorum*

Day	Biofilm Elimination Concentration (mg/ml)
1	0.08666 ± 0.003332
3	0.09166 ± 0.003072
7	0.0950 ± 0.003414
10	0.0850 ± 0.003415
14	0.0700 ± 0.005164
20	0.09332 ± 0.002107

Production of an exopolysaccharide matrix or glycocalyx by biofilms prevents the access of antibodies to the bacterial cells embedded in biofilm (Thien–Fah and O' Toole, 2002). Moreover, the extracellular matrix of biofilms is negatively charged, the interaction of drug molecules with such a negatively charged matrix (Wilson, 2001) could also be a contributing factor for higher value of BEC.

The present study indicated that the differences of MIC, MBC and BEC values of enrofloxacin against planktonic as well as biofilm forms of *S. Pullorum* were non significant. Hence, it can be concluded that the planktonic & biofilm forms of *S. Pullorum* were sensitive *in vitro* to enrofloxacin. In future perspectives, these research findings could be applied *in vivo* to determine the efficacy of enrofloxacin in treating *Salmonella* infections in poultry.

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