RESEARCH ARTICLE

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

Kumar Kamashi¹, Honnegowda², Mayanna Asha³, Chandrakala³

¹Anatomy, Physiology and Pharmacology Academic Program, School of Veterinary Medicine, St. George’s University, Grenada, West Indies.
²Department of Pharmacology and Toxicology, Veterinary College, University of Agricultural Sciences, Hebbal, Bangalore, India.
³Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore, India.

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 and biofilm forms of avian S. gallinarum were found to be non-significant.

Results: The MIC 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. Under electron microscopy, biofilm revealed a pattern of colonization of bacterial cells in multiple layers. The bacterial cells bind firmly to the surface by producing exopolysaccharide glyocalyx polymers, forming a matrix inside which microcolonies develop. As the size and number of the adherent microcolonies increases, they coalesce to form biofilms. Bacterial biofilms are bacterial colonies adhering to a substrate, encased within the synthesized extracellular matrix of carbohydrate polysaccharide glyocalyx moiety and thus protected from various antagonistic agents including antibiotics. 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. and contributed for the development of multiple drug resistance. The virulence – associated plasmid of strains of S. gallinarum contributes toward virulence in fowl typhoid. Fluoroquinolones are synthetic antibacterial agents used in veterinary/human medicine because of their high potency and rapid bactericidal action. 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\textsuperscript{12,13}. For \textit{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\textsuperscript{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 \textit{S. gallinarum}.

\section*{Materials and Methods}

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

\textbf{Culture}

The present study was conducted using Type I culture of \textit{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\textsuperscript{17}.

\textbf{Antimicrobial drugs}

The fluoroquinolone drugs, ciprofloxacin, moxifloxacin, sparflloxacin, norfloxacin, pefloxacin and ofloxacin were procured from Astrazeneca Pharmaceuticals Pvt. Ltd., Bangalore, India and enrofloxacin was obtained from Vetcare, Bangalore, India.

\textbf{Antimicrobial sensitivity test}

Antimicrobial susceptibility of \textit{S. gallinarum} was determined for the fluoroquinolone drugs, ciprofloxacin, enrofloxacin, moxifloxacin, sparflloxacin, norfloxacin, pefloxacin and ofloxacin by antimicrobial sensitivity test method\textsuperscript{18} using antimicrobial sensitivity test discs (Hi Media laboratories, Mumbai, India).

\textbf{Preparation of free form of \textit{S. gallinarum}}

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

\textbf{Preparation of biofilm form of \textit{S. gallinarum}}

\textbf{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.

\textbf{Procedure}

To the biofilm growth medium, \textit{S. gallinarum inoculum} containing 10\textsuperscript{5} 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\textsuperscript{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\textsuperscript{20}.

\textbf{Estimation of minimum inhibitory concentration (MIC, \(\mu g/ml\)) by macro broth dilution method for planktonic and biofilm forms of \textit{S. gallinarum}}

A two-fold serial dilution of fluoroquinolone antibacterial drug in tryptic soya broth was prepared. One ml of planktonic \textit{S. gallinarum inoculum} at a concentration of 10\textsuperscript{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 \textit{S. gallinarum}. The MIC values for planktonic and biofilm forms of \textit{S. gallinarum} were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

\textbf{Estimation of minimum bactericidal concentration (MBC, \(\mu g/ml\)) by macro broth dilution method\textsuperscript{21} for planktonic and biofilm cells of \textit{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 \textit{S. gallinarum} at a concentration of 10\textsuperscript{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 \textit{S. gallinarum} were determined on days 1, 3, 7, 10, 14 and 20 of post inoculation.

\textbf{Estimation of biofilm elimination concentration (BEC, \(\mu g/ml\)) for biofilm cells of \textit{S. gallinarum}}

To one ml of \textit{S. gallinarum} biofilm inoculum containing 10\textsuperscript{6}CFU/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 plates were then inoculated overnight and 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.

\textbf{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.

**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.

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. 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 fluoroquinolone 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.

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. These findings were in accordance with similar research studies.

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 bacterial cells as compared to MIC for planktonic cells. This might be due to the complexity of biofilm structure 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. 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 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. 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. 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. 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. Confocal scanning laser microscopy studies demonstrated pores/channels...
permeating through the bacterial biofilms. 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. 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. 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. 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, 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.

CONCLUSION
From this study, it could be concluded that fluoroquinolone antibacterial agents, ciprofloxacin, enrofloxacin, moxifloxacin, sparflloxacin, 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.

ACKNOWLEDGEMENTS
The authors thank the Dean, Veterinary College, University of Agricultural Sciences, Hebbal, Bangalore, India for providing assistance during the study period. Special memorable thanks to the former Director, Late Dr. G. Krishnappa, Institute of Animal Health and Sciences (IAH and VB), Hebbal, Bangalore, India for his encouragement, guidance and for providing the necessary facilities for carrying out this research. A special thanks to the Scientists and technical staffs of IAH and VB for offering technical services to carry out the research in IAH and VB. Complimentary samples of drugs were provided by Astrazeneca Pharmaceuticals, Bangalore and Vetcare, Bangalore, India.

AUTHOR’S CONTRIBUTION
The manuscript was carried out, written, and approved in collaboration with all authors.

CONFLICT OF INTEREST
No conflict of interest associated with this work.

REFERENCES
1. Thien-Fah C, O’Toole GA. Mechanism of biofilm resistance to antimicrobial agents. Trends Microbiol 2001; 9:34-39. https://doi.org/10.1016/S0966-842X(00)01913-2
2. Nickel JC, Heaton J, Morales A, Costerton JW. Bacterial biofilm in persistent penile prosthesis- associated infection. J Urol 1986; 135:586-588. https://doi.org/10.1016/S0022-5347(17)45747-8
3. Costerton JW. Structure and plasticity at various organization levels in the bacterial cell. Can J Microbiol 1988; 34:513-521. https://doi.org/10.1139/m88-088
4. Loosdrecht MCMV, Lyklem J, Norde W, Zhender. Influence of interfaces on microbial activity. Microbiol Rev 1990; 54:75-87. PMID: 2181260
5. Wilson M. Bacterial biofilms and human disease. Sci Progress 2001; 84:235-254. https://doi.org/10.1016/j.scip.2017.07.012
6. Wimpenny JWT, Kinniment SL. Measurement of the contribution of adenylate concentrations and adenylate charge across Pseudomonas aeruginosa biofilms. Appl Environ Microbiol 1992; 58: 1629-1635. PMID: 1352444
7. Goyal SM, Hoodley AW. Salmonellae and their associated R-plasmids in poultry processing wastes. Revista-de-Microbiologia-Brazil. 1979; 10: 50-58. https://doi.org/10.1006/rmbi.1998.3022-8
8. Anjanappa M, Harbola PC, Verma JC. Plasma profile analysis of field strains of Salmonella gallinarum. Indian Vet J 1994; 71: 417-421. PMID: 12146886
9. Barrow PA, Simpson JM, Lovell MA, Binns MM. Contribution of Salmonella gallinarum large plasmid toward virulence in fowl typhoid. Int Immunol 1987; 55:388-392. PMID: 3804442
10. Brown SA. Fluoroquinolones in animal health. J Vet Pharmacol Therapy 1996; 19: 1-14. https://doi.org/10.1111/j.1365-2855.1996.tb00001.x
11. Sanders CC. Ciprofloxacin: in vitro action, mechanism of action and resistance. Rev Infect Dis 1988; 10:516-527. https://doi.org/10.1093/infdis/10.3.516
12. Mizuuchi K, Fisher LM, O’Dea MH, Gellert M. DNA gyrase action involves the introduction of transient double standard breaks into DNA, Proc. Natl. Acad. Sci., USA, 1980; 77:1847-1851. https://doi.org/10.1073/pnas.77.4.1847
13. Kato J, Nishimura Y, Iinamura R, Niki H, Higara S, Suzuki H. New topoisomerase essential for chromosome segregation in E. coli. Cell 1990; 63: 393-404. https://doi.org/10.1016/0092-8674(90)90172-b
14. Domagala JM, Hanna LD, Heifitz CL, Hutt MP, Sanchez JP, Solomon M. New structure activity relationships of the quinolone antibacterials using the target enzyme. The development and application of a DNA gyrase assay. J Med Chem 1986; 29: 394-404. https://doi.org/10.1021/jm00153a015
15. Chow RT, Dougherty TJ, Fraimow HS, Bellin EY, Miller MH. Association between early inhibition of DNA synthesis and the MICs and MBCs of carboxyquinoiline antimicrobial agents for wild-type and mutant [gyrA{ompF} acrA] Escherichia coli K-12. Antimicrob Agents Chemother 1988; 32: 1113-1118. https://doi.org/10.1128/JAC.32.8.1113
16. Piddock LJV, Walters RN, Diver JM. Correlation of quinolone MIC and inhibition of DNA, RNA and protein synthesis and induction of the SOS response in Escherichia coli. Antimicrob Agents Chemother 1990; 34: 2331-2336. https://doi.org/10.1128/JAC.34.12.2331
17. Cruickshank R, Duguid JP, Marimion BP, Swain RHA. In: Medical Microbiology, 12th Edn. 1975, Churchill Livingstone, Edinburgh, London.
18. Bauer AW, Kirby WMM, Sherris JS, Turkem M. Antibiotic susceptibility testing by a standardised method. Am J Clin Pathol 1966; 45: 493-496. https://doi.org/10.1093/647952
19. Miles AA, Misra SS. The bactericidal power of blood. J Hyg 1938; 38: 732. 
https://doi.org/10.1017/s002217240001158x
20. Shivarahaj D. Biofilm production of Escherichia coli and Salmonella gallinarum and evaluation of oral Escherichia coli vaccines in chicks. M.V.Sc. Thesis; Univ Agri Sci., Bangalore, India, 1998. 
https://doi.org/10.5812/jjm.18971v2
21. Matsen JM. Bacterial susceptibility testing and assays. In: Clinical Diagnosis and Management by Laboratory Methods. Ed. Henry JC, 17th Edn. W.B. Saunders Co., Philadelphia. 1989; 1322-1352. 
https://doi.org/10.1128/CMR.00095-17
22. Hooper DC. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin Infect Dis 2000; 31: S24-S28. 
https://doi.org/10.1086/314056
23. Muller M, Stab H, Brunner M, Moller JG, Lackner E, Eichler HG. Penetration of moxifloxacin into peripheral compartiments in humans. Antimicrob. Agents Chemother 1999; 43: 2345-2349. PMID: 10508004
24. Tang HJ, Chang MC, Ko WC, Huang KY, Lee CL, Chuang YC. In vitro and in vivo activities of newer fluoroquinolones against Vibrio vulnificus. Antimicrob Agents Chemother 2002; 46:3580-3584. 
https://doi.org/10.1128/AAC.46.11.3580-3584.2002
25. Wright DH, Gunderson B, Hovde LB, Ross GH, Ibrahim KH, Rotschafer JC. Comparative pharmacodynamics of three newer fluoroquinolones versus six strains of Staphylococci in an in vitro model under aerobic and anaerobic conditions. Antimicrob. Agents Chemother 2002; 46:1561-1563. 
https://doi.org/10.1128/AAC.46.5.1561-1563.2002
26. Kaji C, Watanabe K, Apicella MA, Watanabe H. Antimicrobial effect of fluoroquinolones for the eradication of nontypeable Haemophilus influenzae isolates within biofilms. Tohoku J Exp Med 2008; 214 (2):121-8. 
https://doi.org/10.1620/tjem.214.121
27. Ramesh N. Studies on resistance to antibacterial agents with reference to the plasmid profile and biofilm formation in certain poultry pathogens. Ph.D. Thesis, 2003; Univ. Agri Sci, Bangalore, India. 
https://doi.org/10.1128/cmr.1.2.228
28. Suci PA, Mittelman MW, Yu FP, Geesey GG. Investigation of ciprofloxacin penetration into Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 1994; 38: 2125-2133. 
https://doi.org/10.1128/aac.38.9.2125
29. Olson ME, Ceri H, Morck DW, Buret AG, Read PR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res 2002; 66:86-92. 
PMID: 11989739
30. De Beer D, Stoodley P, Roe F, Lewandowski Z. Oxygen distribution and mass transport in biofilms. Biotechnol Bioeng 1993; 43:1131-1138. 
https://doi.org/10.1002/bit.260431118
31. Stewart PS. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob. Agents Chemother 1996; 40: 2517-2522. PMID: 8913456
32. Christensen BB, Sternberg C, Andersen JB, Molin OS. In situ detection of gene transfer in a model biofilm engaged in degradation of benzyl alcohol. APMIS (Suppl.) 1998; 84: 25-28. 
https://doi.org/10.1111/j.1600-0463.1998.tb05644.x