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INHIBITION OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* BIOFILM BY DNA SYNTHESIS AND PROTEIN SYNTHESIS INHIBITORS

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Abstract: *Corynebacterium pseudotuberculosis* represents the causative factor of caseous lymphadenitis, a ruminant disease that contributes to major economic loss in most sheep farming countries. The present study was performed to determine the efficacy of DNA synthesis (nalidixic acid) and protein synthesis inhibitors (streptomycin and tetracycline) on *C. pseudotuberculosis* biofilm. Biofilm of *C. pseudotuberculosis* clinical isolate was developed in the flat-bottom microplate. Field-emission scanning electron microscope and resazurin assay were used to investigate the structure and antimicrobial susceptibility of *C. pseudotuberculosis* biofilm, respectively. All test inhibitors were evaluated in the range between 3.125 µg/ml and 100 µg/ml. Results demonstrated that *C. pseudotuberculosis* biofilm formed a three dimensional and heterogeneous structure on the surface. The highest biofilm percentage inhibition shown by nalidixic acid, streptomycin and tetracycline were 71.57%, 87.44% and 74.73%, respectively. Significant ($p<0.05$) correlation of antibiofilm susceptibility between these inhibitors and other antimicrobials were also demonstrated herein. The findings of the present study suggest the potential use of streptomycin for the control of caseous lymphadenitis.

Keywords: *Corynebacterium pseudotuberculosis*, biofilm, caseous lymphadenitis, antibiofilm susceptibility.

Introduction

Corynebacterium pseudotuberculosis is a Gram-positive bacterium and one of the significant veterinary pathogens. The bacterium not only causes caseous lymphadenitis (CLA) in small ruminants but also causes other chronic diseases such as ulcerative lymphangitis in horses and bovine mastitis. CLA is well known to affect sheep and goats leading to the decrease in wool production, weight loss and carcass condemnation thus causing great economic loss to livestock industry (Dorellaa *et al.,* 2006; Shi *et al.,* 2019). *C. pseudotuberculosis* can survive several weeks in environment and spread within the flock or herd. The contamination of superficial wounds which may be caused by several procedures such as shearing, ear tagging and castration often increases the probability of the transmission among the animals. The formation of the abscess in superficial lymph nodes and subcutaneous tissue is the common external form of CLA. Meanwhile, the visceral form of CLA is shown by the abscess formation in internal organ such as kidney, spleen, liver and lungs. The uses of antibiotic in controlling the CLA infection is a hard task as the bacteria stay protected inside the abscess which encapsulated it (Ilhan, 2020).

Biofilm represents a major bacterial growth mode. Extracellular matrix which contains a wide variety of proteins, glycoproteins and glycolipids that surrounds the biofilm cells plays role in cellular attachment to surface, stabilization of the overall biofilm structure and protection against the antimicrobial regime. Development of biofilm begins with attachment of bacteria to the surfaces, followed by the formation of microcolony, biofilm maturity and finally biofilm dispersion (Johari *et al.,* 2020). It has been established that difficulty in

disease control is due to the biofilm formation. In general, the biofilms comprise multiple cell types such as dormant cells, dead cells, aerobic cells and fermentative cells (Rani *et al.,* 2007). The variety of physiological states represented in a biofilm such as DNA synthesis, protein synthesis, intercellular signalling and nutrient flux may contribute to the physiological heterogeneity and antimicrobial resistance (Machineni, 2020).

Nalidixic acid (DNA synthesis inhibitor), streptomycin (protein synthesis inhibitor) and tetracycline (protein synthesis inhibitor) are often used to contain a wide spectrum of bacterial infections in humans (Walia *et al.,* 2004). Their inhibitory effects on *C. pseudotuberculosis* biofilm have previously been demonstrated (Yaacob *et al.,* 2021). Yaacob *et al.* (2021) demonstrated that streptomycin (3.12 µg/ml, 12.5 µg/ml, 25 µg/ml and 100 µg/ml), tetracycline (3 µg/ml, 12.5 µg/ml and 50 µg/ml) and nalidixic acid (25 µg/ml and 50 µg/ml) significantly ($p < 0.05$) attenuated *C. pseudotuberculosis* biofilm. To date, there is still no information on the correlation between their antibiofilm activities. While many works have highlighted epidemiology and pathophysiology of CLA (Shi *et al.,* 2019; Selim *et al*., 2021), the information related to high-resolution observation of *C. pseudotuberculosis* biofilm and inhibition of *C. pseudotuberculosis* biofilm is still limited. Therefore, the present study was carried out to investigate the morphology of *C. pseudotuberculosis* biofilm and susceptibility of *C. pseudotuberculosis* biofilm towards DNA synthesis and protein synthesis inhibitors.

Materials and Methods

Test Microorganism

A clinical isolate of *C. pseudotuberculosis* was obtained from Veterinary Laboratory Service Unit (VLSU), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). It was cultivated in nutrient broth (Difco Laboratories, USA) at 37°C for 48 hours and adjusted to optical density (OD) of 0.7 at 600 nm before the biofilm assay.

Inhibitors and Chemicals

Commercial inhibitors and chemicals used in this study were nalidixic acid (Sigma, USA), tetracycline (Sigma, USA), streptomycin (Sigma, USA), dimethyl sulfoxide (Merck, Germany), ethylenediaminetetraacetic acid (Sigma, USA), sodium chloride (Sigma, USA), ethanol (Merck, Germany), formaldehyde (Merck, Germany), resazurin (Sigma, USA) and crystal violet (Sigma, USA).

Imaging of C. Pseudotuberculosis Biofilm

Biofilm imaging was performed according to the method suggested by Yaacob *et al.* (2021). *C. pseudotuberculosis* biofilm was developed in a 6-well microplate. Overnight inoculum (4 ml) and fresh nutrient medium (1 ml) were loaded into the microplate wells. The glass cover slips were placed in the microplate wells with the surface of interest facing upward. After overnight incubation at 37°C, the glass cover slips were pulled out from the microplate wells and washed with saline buffer twice. The biofilm on the glass cover slip was fixed by immersing it into formaldehyde (4%) at 4°C for 3 hours, washed with sterile distilled water thrice and serially dehydrated using ethanol (25% once for 10 minutes, 50% once for 10 minutes, 75% once for 10 minutes and 100% twice for 10 minutes). The slide was air dried overnight before observation using field-emission scanning electron microscope (FESEM) (Hitachi, Japan) at 5000 x magnification.

Antibiofilm Microplate Assay

The antibiofilm activities of DNA synthesis and protein synthesis inhibitors and common chemicals against *C. pseudotuberculosis* were evaluated according to Yaacob *et al.* (2021) method. Resazurin solution (0.02%) was prepared and stored at 4°C in the dark until further use. Inhibitors were prepared in the range between 3.12 ug/ml and 100 ug/ml. Solutions of ethylenediaminetetraacetic acid (EDTA) were prepared in the range between 15.6 mM and 500 mM. Ethanol and dimethyl sulfoxide (DMSO) solutions were prepared in the range between

3.12% and 100%. Overnight inocula (200 µl) and test solutions (50 µl) were added into the microplate wells. Equal volume of intellectual property (IP)-protected antibiofilm cocktail and fresh broth were also added as positive and negative controls, respectively. After overnight incubation at 37°C, the medium was discarded and the microplates were washed with distilled water twice and heat-fixed at 60°C for 30 minutes. To suspend the biofilm fractions, 220 µl of PBS and 30 µl of 0.02% resazurin were loaded into the microplate wells. The microplate was incubated at 37°C for at least 3 hours and microplate reader (ThermoFisher Scientific, USA) was used to measure absorbance values at 570 nm. The mean absorbance values from the triplicate microplate assay were used to calculate the percentage inhibition of biofilm according to the following equation:

$$\text{Percentage (\%) inhibition} = \frac{(OD_{(\text{negative control})} - OD_{\text{experimental}}) \times 100}{OD_{(\text{negative control})}} \quad (1)$$

Statistical Analysis

To investigate the relationship between the selected inhibitors and other chemicals, the absorbance data obtained from the antibiofilm microplate assay was used. To determine the relationship between all antibiofilm susceptibility profiles, the Pearson correlation coefficient test was carried out (Oggioni *et al.,* 2015).

Results and Discussion

Biofilm Morphology

Figure 1 shows the FESEM image of *C. pseudotuberculosis* biofilm. *C. pseudotuberculosis* formed a heterogeneous biofilm structure. Biofilm cells were encapsulated by self-produced matrix while several pores were detected throughout the biofilm structure.

Biofilm Inhibitory Actions

Figure 2 displays the antibiofilm activities of DNA synthesis inhibitor (nalidixic acid), protein synthesis inhibitors (streptomycin and tetracycline) and other chemicals. All compounds tested herein inhibited the biofilm viability of *C. pseudotuberculosis*. The percentage of biofilm inhibition was found to be in the range between 12.92% and 87.44%. The highest biofilm percentage inhibition shown by nalidixic acid, streptomycin and tetracycline were 71.57%, 87.44% and 74.73%, respectively.

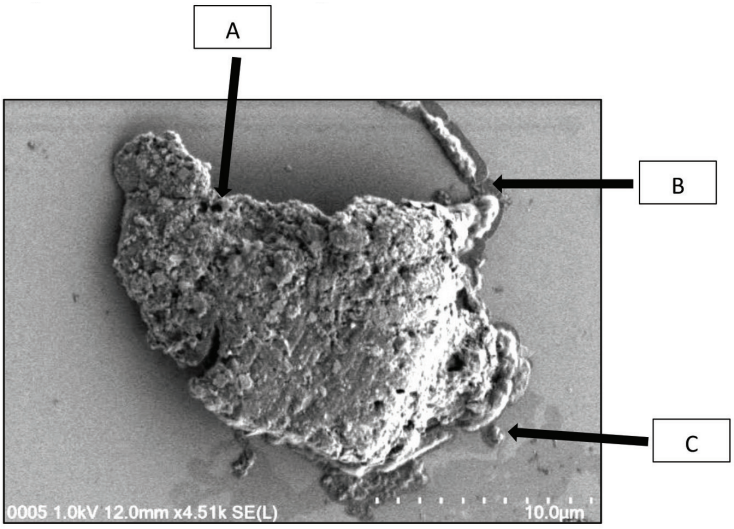

Figure 1: Biofilm morphology of *C. pseudotuberculosis* at 5000X magnification. (A) Pore formation; (B) Self-produced matrix; (C) *C. pseudotuberculosis* cells

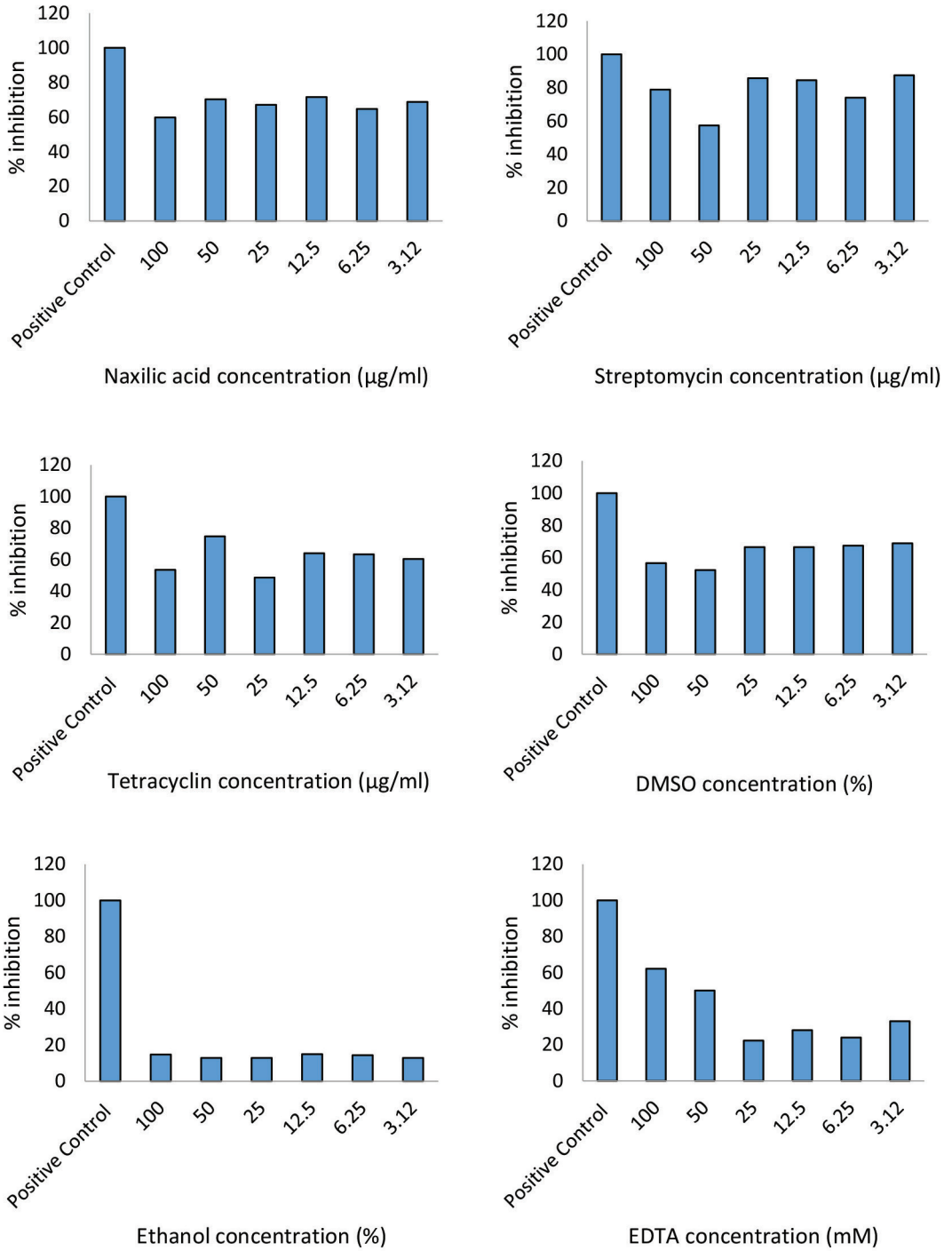

Figure 2: Antibiofilm activities of selected compounds against *C. pseudotuberculosis*. IP-protected antibiofilm cocktail was used as positive control

Correlation Coefficients

Table 1 shows the correlation between all antibiofilm susceptibility profiles of *C. pseudotuberculosis* biofilm. The nalidixic acid showed strong correlation (correlation coefficient > 0.7) with streptomycin and tetracycline and moderate correlation (0.5 < correlation coefficient < 0.7) with DMSO. The streptomycin showed moderate correlation (0.5 < correlation coefficient < 0.7) with tetracycline, DMSO and ethanol. The tetracycline did not show any strong or moderate correlation with other chemicals.

The present study used FESEM to investigate the morphology of *C. pseudotuberculosis* biofilm. Scanning electron microscopy employs an electron beam that allows the high magnification observations of various biofilm components including extracellular matrix and cell-to-cell interactions (Habimana *et al.,* 2009). FESEM is able to capture images with low electrostatic distortion and a higher spatial resolution than conventional SEM by using a field emitter instead of a thermionic emitter.

Nutrient transport is crucial for the biofilm development and is dependent on the structure of biofilm. Effective diffusion coefficient of nutrients is known to vary at different locations within the biofilm. In the present study, FESEM image revealed the pore formation in the heterogeneous *C. pseudotuberculosis* biofilm. The pores in heterogeneous biofilms are exposed to the nutrient liquid and allow the nutrient liquid to intrude and exit freely (Ning *et al.,* 2014). The heterogeneity and porosity of biofilm structure have also been demonstrated by other works (Mahat *et al.,* 2012; Wang *et al.,* 2012; Yahya *et al.,* 2017). Our work provides the first evidence for the heterogenous three-dimensional structure and pore formation of *C. pseudotuberculosis* biofilm, allowing us to understand how the biofilm develops.

Streptomycin is a common antibiotic used to fight against a wide range of bacterial infections including tuberculosis, brucellosis, endocarditis, plague and Burkholderia infection. It is known to eliminate the bacterial infection by attenuating the binding between formyl-methionyl-tRNA and 30S sub unit during translation. The present study demonstrated the efficacy of streptomycin against *C. pseudotuberculosis* biofilm. This finding does not corroborate with that of Olson *et al.* (2002) that revealed the low efficacy of streptomycin against *C. pseudotuberculosis* biofilm (MBEC values > 256 µg/ml). This discrepancy may be due to different culture medium used in the individual work in which the nutrient broth was used herein while tryptic soy broth containing fetal bovine serum was used by Olson *et al.* (2002). The mode of action of streptomycin against *Pseudomonas aeruginosa* biofilm is known to be mediated by its binding to a cell signalling receptor, LasR protein (Khan *et al.,* 2020). On the other hand, treatment with streptomycin has also caused differential expression of transporters, virulence factors, signalling proteins, multidrug efflux pumps and regulatory proteins in *Staphylococcus aureus* biofilm (Kumar & Ting, 2016).

Table 1: Correlation between antimicrobial susceptibility profiles of *C. pseudotuberculosis* biofilm. All shaded cells show statistically significant correlations. NA: Nalidixic acid; ST: Streptomycin; TC: Tetracycline; DMSO: Dimethyl sulfoxide; EDTA: Ethylenediaminetetraacetic acid; ETH: Ethanol

	NA	ST	TC	DMSO	EDTA	ETH
NA	1					
ST	0.81	1				
TC	0.71	0.67	1			
DMSO	0.54	0.69	0.46	1		
EDTA	0.35	0.27	0.43	0.33	1	
ETH	0.49	0.62	0.49	0.34	0.12	1

Nalidixic acid is a heterocyclic carbonic acid derivative that is usually used for treating urinary tract infections. In bacteria, it effectively interferes topoisomerase IV and DNA gyrase and initiates cleavage complexes. The present study showed the efficacy of nalidixic acid against *C. pseudotuberculosis* biofilm. Information on the impact of nalidixic acid on the biofilm formation by Gram-positive pathogen is still limited. However, the present result contradicts with Irwin *et al.* (2013) that demonstrated no bactericidal activity of nalidixic acid (<2000 mg/l) against the biofilm formation by *Proteus mirabilis,* a Gram-negative pathogen. This may be due to the outer membrane which is present in Gram-negative pathogens but not Gram-positive pathogens.

Tetracycline is a broad-spectrum antibiotic commonly used to treat mild-to-moderate microbial infections. It specifically binds to the 30S bacterial ribosomal subunit and attenuates translation mechanism by hindering the interaction between aminoacyl tRNA and acceptor site on the mRNA ribosome complex. The present work demonstrated the efficacy of tetracycline towards *C. pseudotuberculosis* biofilm. Treatment with tetracycline has previously been shown to substantially decrease the biofilms of *Achromobacter sp.*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus pumilus* (Liaqat *et al.,* 2009).

DNA synthesis and protein synthesis represent the major biological processes taking place in the microbial biofilm although its growth rate is very low. These processes keep changing with time as the biofilm grows and are sensitive to extreme intracellular changes. The diversity in DNA synthesis and protein synthesis contributes to the special ecology and resistance to antibiofilm agents. In the present study, none of the DNA synthesis and protein synthesis inhibitors eliminated *C. pseudotuberculosis* biofilm. This is probably due to the existence of the cells that grow slowly and dormant cells in *C. pseudotuberculosis* biofilm. Non-growing cells are normally less susceptible to antimicrobials targeting the macromolecule synthesis processes (Rani *et al*., 2007; Fleischmann *et al.,* 2021).

DMSO is an organosulfur compound with a trigonal pyramidal molecular geometry. It is frequently used as a solvent to dissolve a wide range of pharmaceutical drugs. For many decades, it is also known to be effective against pathogenic microorganisms. The present study demonstrated inhibition of *C. pseudotuberculosis* biofilm by DMSO. The antibiofilm activities of DMSO against *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhimurium* biofilms have previously been demonstrated (Yahya *et al.,* 2017; Yahya *et al.*, 2018).

EDTA is a metal-chelating agent commonly used to treat mercury and lead poisoning. It also often used to treat biofilm colonization in dentistry, veterinary medicine and medical devices. Structurally, it has a protonated conjugate base as a ligand and a precursor namely H4EDTA. Antibacterial potential of EDTA is due to its ability to complex with and remove Mg^{2+} and Ca^{2+} ions from bacterial cell membrane. Herein, EDTA was effective against *C. pseudotuberculosis* biofilm. The biofilm inhibitory effect of EDTA against other Gram-positive pathogens has also been shown (Juda *et al.,* 2008; Chang *et al*., 2012).

Correlation coefficient measures the degree of statistical relationship between two variables. Herein, the correlations between the antibiofilm susceptibility profiles were demonstrated. Oggioni *et al.* (2015) has reported the correlations between biocides and antibiotics susceptibility profiles in *Staphylococcus aureus*. The correlation coefficient values were found to be in the range between 0.32 and 0.86. The correlations between antibiotics and DMSO demonstrated in the present study also corroborate the fact that DMSO enhances the effects of antibiotics in a wide range of inflammatory diseases (Patil, 2013). On the other hand, the combination between tetracycline and EDTA in topical applications may also be possible although their correlation is low (Lambert *et al.,* 2004).

Conclusion

This study demonstrated the heterogeneous biofilm formed by *C. pseudotuberculosis* and the antibiofilm actions of DNA synthesis and protein synthesis inhibitors against *C. pseudotuberculosis*. Streptomycin that showed the highest percent inhibition against *C. pseudotuberculosis* biofilm and strong correlation with other antimicrobials may be useful for the control of CLA disease. The molecular mechanism underlying the antibiofilm action of streptomycin needs further investigation.

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