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In-Vitro Antimicrobial Activity of Membrane-Acting Antibiotics Action Against *Streptococci*

Zidan A. Bashir* [1, 2]
[1] Microbiology Program, School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia, UKM Bangi, Selangor Darul Eshan, Malaysia.
[2] Department of Zoology, Faculty of Science, Sebha University, Libya.

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ABSTRACT

Streptococcus equi sub*S. equi* a group C *streptococcus* is a gram-positive bacterium and is the causative agent of one of the most prevalent diseases of horses known as strangles, although, a live attenuated *S. equi* vaccine for prevention of the strangles has been introduced, there are presently still no proven vaccine against *S. equi*. In the effort to develop an effective vaccine against *S. equi*, the minimum inhibitory concentrations of antimicrobials acting bacterial membrane - triclosan and 4-Methylumbelliferone (4-MU) on *S. equi* have been investigated. These were determined by serial dilution technique on three strains of the equine *S. equi*: 4047, K3, and NCTC 9682. In addition, sensitive disk test on solid media were also carried out. The result of this study demonstrated that the three strains of *S. equi* are very sensitive to triclosan as they inhibit certain membrane functions. However, these strains are very resistant to 4-MU. Further research to investigate the mechanism of interaction between these antimicrobial agents and the bacterial membrane components is required.

INTRODUCTION

Streptococcus equi sub*S. equi* a group C streptococcus is a gram-positive bacterium and is the causative agent of one of the most prevalent diseases of horses known as strangles (Todd, 1910). Strangles has been estimated to be responsible for nearly 30 % of equine infections reported worldwide (Chanter, 1997). Infection with *S. equi* rapidly spreads to the lymph nodes of the head where bacterial multiplication proceeds unhindered by a massive infiltration. In one or more lymph node infection progress and a sinus tract forms to allow pus to drain through the nearest site of egress, either through upper respiratory tract mucosa or through the skin. Symptoms associated with this disease are mucopurulent nasal discharge, inspiratory dyspnoea, loss of appetite and pyrexia. Some complications are often associated with strangles i.e. rupture of abscesses which can be formed anywhere within the body organs and which leads to death in about 10 % of the cases, oedema of the limbs, eye lids and gums, circulatory failure also leading to death due to entrapped of antibody and *S. equi* M-like protein in capillary beds (Sweeney *et al.,* 1987; Galan & Timoney, 1985). As a result of this disease, in addition to the animal suffering, economic factors are also a burden. Although, a live attenuated *S. equi* vaccine for the prevention of strangles has recently been introduced, there are presently still no proven effective vaccines against *S. equi* (Harrington *et al*, 2002). Thus, effective treatment is needed and more attention has been focused on vaccine development. The membrane of *S. equi*, like other micro-organisms contains lipids and proteins which are the major components of all membranes (Kakizaki *et al.,* 2002). Below is an overview of some of the major components of the *S. equi* membrane. Membranes of group A and C Streptococci have been reported to contain numerous phospholipids mainly cardiolipin and phosphatidylglycerol (Tlapak-Simmons *et al.,* 1999). In recent years, advances have been made in the development of anti-microbial compounds in an effort to check the harmful effects of microorganisms. Many anti-microbial compounds do not need to penetrate a microorganism in order to exert their anti-microbial action. Many anti-microbial compounds act not on the cell wall or

* *Corresponding Author*
E mail: 4zidanukm@gmail.com

external layers, which are comparatively inert metabolically, but on the plasma membrane (Rose, 1976). Some anti-microbial compounds that may affect bacteria such as *S. equi* include pantoyl lactone, allyl mercaptan and 4- methyl-umbelliferone (4-MU). These compounds act on the plasma membrane by combining with proteins, lipids or other compounds in the membrane that are relevant to one or more vital physiological roles thereby disrupting the integrity of the membrane. Phospholipids dispersions can adopt different structures such as micelles, bilayers, lipidic and Hexagonal-H_{11} phase which can greatly affected the functional behaviour of membranes (de Kruijff *et al.,* 1985). Thus, several antibacterial agents such as triclosan and 4-MU inhibit the biochemical activities of microorganisms by distorting the structural arrangement of these phospholipids.

In this study the antimicrobial activities of various membrane active agents against *Streptococci* was determined. The study focuses on the effects of sub-MIC concentrations of selected agents penicillin, triclosan, 4-methylumbelliferone and on lipoprotein and polar lipids metabolism. Initial experimentation will focus on the equine pathogen *S. equi* strains 4047, K3 and NCTC 9682, assessed by using serial dilution technique. Triclosan, 4-methylumbelliferone sensitivity Disk test on solid media to determine the diameter of the clear zone were also investigated.

MATERIALS AND METHODS

Bacteria strains

The following three strains of *S. equi were* used for this study : 4047, K3 and NCTC 9682 strains. The strains of bacteria were supplied by (Dr. I. Sutcliffe Northumbria University) and were cultured in Brain Heart Infusion (BHI) broth at 37 ^{o}C overnight. The bacteria were grown in the media until they became turbid. A Gram stain test was carried out to confirm the bacteria as gram-positive cocci in chains. Each strain was then cultured on Brain Heart Infusion (BHI) agar - a solid medium by streaking method and incubated at 37 ^{o}C overnight. Colonies were observed on the agar plate, which were clearly labelled according to the strain of the bacteria and kept in the refrigerator for subsequent experiments.

Preparation of the media

Preparation of Brain Heart Infusion (BHI) broth

7.4 g of BHI (37 g/L) was weighed and added to 200 mL of distilled water, and then thoroughly mixed and autoclaved, the broth medium was then disposed into 10 pieces of 20 ml McCartney tubes and autoclaved.

Preparation of brain heart agar (BHI agar)

9.4 g of BHI agar (47g/L) was weighed in a red-capped flask; 200 mL of distilled water was added to it and then thoroughly mixed before autoclaving. However, after slight cooling, approximately 20 mL of the mixture were poured in each petri dish. Plates were allowed to solidify before being taken to the oven for about 10 minutes to dry.

Preparation of BHI supplemented with 50 mg/ L calcium

50 mg/mL of CaCl solution was prepared and autoclaved. Also the BHI medium was prepared as above and autoclaved. Then 100 µL of CaCl stock solution was added into 100 mL of BHI broth and labelled as BHI + Ca to be used in the preparation of daptomycin serial dilutions. The final BHI broth was supplemented with a final concentration of 50 mg/L calcium and used for the serial dilution.

Preparation of agar plates containing different 4 - MU concentrations:

Four batches of 100 µL of BHI agar were prepared as previously described. After autoclaving, it was incubated for 40 minutes in a water bath that had been set at 50 ^{o}C. Quickly after the incubation, in a fume cupboard the following additions of 4-MU in DMSO were added to each batch and labelled respectively; 500 µg/mL, 250 µg/mL and 125 µg/mL. After each addition the agar was gently swirled to ensure that 4-MU and DMSO were dissolved thoroughly throughout the agar, then the contents of each batch was poured into four sterile petri dishes well labelled with the final concentration of 4 - MU. Plates were kept in a fume cupboard overnight to allow DMSO to evaporate.

Antimicrobial compounds

These agents were prepared as stocks as follows:

Preparation of penicillin stock

200 mg/mL penicillin stock was prepared by the weighing out of penicillin in a sterile Eppendorf tube. 0.36 mg of penicillin was weighed and dissolved in 180 mL of sterile distilled water and kept in the freezer as a stock.

Preparation of triclosan stock.

200 mg/mL of triclosan stock was prepared by weighing the triclosan in a sterile eppendorf and dissolving it in 200 mg/mL of ethanol.

Briefly, 0.056 µg of triclosan was weighed and dissolved in 280 µL of ethanol, labelled and kept in the freezer as a stock. Preparation of 4-Metylumbelliferone (4 - MU) stock. 200 mg/mL of 4 - MU stock was prepared by weighing out 4 - MU in a sterile bijou and dissolved in 200 mg/ml of DMSO (dimethylsulfoxide). 177 mg of 4-MU was dissolved in 885µL of DMSO, labelled and kept in the freezer as a stock.

Preparation of antibiotic disks for triclosan and 4-MU.

Twenty millipore filter disks were sterilised in a beaker, then dried in a drying oven. For each antibiotic, 6 millipore disks were arranged in a sterile petri dish marked with the name of the antibiotic on the lid. 50 µL of 200 mg/mL antibiotic stock was added to each disk and kept in a fume hood overnight to dry.

Broth (tube) assay for Minimum Inhibitory Concentrations (MIC) of antibiotics

Preparation of the media and starter culture

BHI broth and BHI agar were prepared as previously described. Starter culture was prepared by inoculating two McCartney tubes containing 20 mL of BHI broth with single colony of *S. equi* 4047 and then kept in the incubator at 37 ^{o}C till the next day. Two tubes of 20 mL broth were necessary to overcome the possibility of failure of growth in one of the McCartney tubes.

Broth (tube) assay for MIC of penicillin

The bacterial growth in the starter culture was used for the experimental work on the second day. The MIC measurement of the antibiotics was by broth dilution assay. The antibiotics serial dilution was done as follows: The previously prepared 200 mg/mL penicillin stock was diluted to 2 mg/mL by adding 10 µL of the stock into 990 µL of BHI broth in a sterile test tube. Then 12 sterilized test-tubes, each containing 1 mL BHI broth were prepared. From the tube containing 2 mg/mL penicillin stock, 1 mL was transferred into the first test-tube that contained 1 mL BHI broth and labelled as tube No 1 with a penicillin concentration of 1mg/ml. Then from tube No 1, two fold serial dilutions were carried out up to the tube No. 11, from which 1mL was discarded. Tube No. 12 was kept as a control. The final concentrations of penicillin in the various tubes are given in the table below.

Preparation of inocula

Inocula was prepared by dilution of starter culture, 1:100 by transferring 200 µL of the overnight starter culture into 20 mL BHI broth in a McCartney tube. The suspension was thoroughly mixed and 25 µL were used to inoculate each of the 12 tubes of the prepared serial dilution.

Total viable count determination

From the *S. equi* inocula, 100 µL was transferred into a sterilized test tube containing 900 µL of BHI broth to give 10^{-1} dilution. Three further 10 – fold serial dilutions were made i.e. to a 10^{-4} dilution. Then 100 µL each from the *S. equi* dilutions 10^{-3} and 10^{-4} were used to inoculate two agar plates respectively. A sterilized glass spreader was used to spread the inocula on the agar plates. Each plate was labelled with the name of the organism inoculated and the dilution factor. The tubes and plates were then placed in a closed jar containing a lit candle to create an anaerobic condition then incubated at 37 ^{o}C overnight.

Broth assay for other antibiotics

The broth assay was preformed using alternative antibiotics. A method similar to the penicillin mentioned in the previous section was used except in the following cases. In the case of the revised method for pantoyl lactone from the stock previously prepared (the second method). 1mL was transferred into the first test-tube that contained 1mL BHI broth and labelled as tube No 1 with a pantoyl lactone concentration of 100 mg/L. Then from tube No 1, two fold serial dilutions were carried out up to tube No 11 from which 1ml was discarded. Tube No 12 was kept as a control. The final concentrations of pantoyl lactone in the various tubes are given. In the case of 4-MU, from the previously prepared stock section. 20µl was added into a test tube containing 4 mL of BHI broth, immediately mixed and the final concentration was 1mg/mL. Two fold serial dilutions were carried out up to tube No 11, from which 1ml was discarded. Tube No 12 was kept as a control. Also, for each test series a 13th tube was set up containing 1mL BHI broth and 5 µL of DMSO and was inoculated and used as a control for the level of DMSO (0.25 %) in tube 1.

Disc diffusion assay of triclosan and 4-MU

A method similar to the daptomycin mentioned in the previous section was used, except the six plates containing agar media were prepared and were inoculated with 50 µL of inocula that had been prepared previously. Total viable count determination was carried out above while all the plates were incubated under anaerobic conditions at 37 ^{o}C overnight.

Plate assay for 4-MU

Agar media, starter culture for the three strains of *S. equi* and inocula were prepared as above. Each strain was inoculated onto each of the plates in the dilution series by adding 25 µL inocula onto four plates with (500, 250, 125, 0) concentrations of 4-MU and spread with a glass spreader. Total viable count determination was carried. Total viable count determination was carried out above while all the plates were incubated under anaerobic conditions at 37 ^{o}C overnight.

RESULTS AND DISCUSSION

Penicillin (Reference antibiotic)

Penicillin, which is a reference antibiotic, was used for *S. equi* 4047 to ascertain the effectiveness of the serial dilution technique in determining the minimum inhibitory concentration of antibiotics. Seven experiments were conducted using penicillin on *S. equi* 4047 and the results were as shown in Table 7. No growth was observed in any tubes containing penicillin within the concentration range of 1mg/mL - 1µg/mL. However, there was growth in tube No. 12, the control tube that did not contain penicillin. Hence, *S. equi* 4047 is sensitive to penicillin with minimum inhibitory concentration < 1µg/mL. For the fact that the technique works perfectly well with penicillin (reference antibiotic), it was employed in determining the MICs of other antimicrobial agents such as pantoyl lactone, triclosan, 4-MU and daptomycin. Pilot experiments were performed on *S. equi* 4047 using triclosan and the results were as displayed in Table 11. Experiment 1 & 7 shows the same pattern with MIC ≥ 31 µg/mL, while experiments 3, 4, 5 and 6 also give similar growth patterns with MIC ≥ 15.6 µg/mL. However, experiment No. 2 provides an exceptional result indicating that the MIC is at least ≥ 62.5 µg/mL.

Considering all the seven experiments performed it appears that the MIC lies between 15.6 µg/mL – 31 µg/mL. In all the experiments, the MIC was ≥ 15.6 µg/mL. Triclosan assay was preformed on the three strains of *S. equi;* 4047, K3 and NCTC 9682. For isolate 4047, no growth was observed in tubes 1-7, indicating that MIC ≥ 15,6 µg/mL, while for strains K3 and 9682 no growth was observed in tubes 1-6, indicating that the MIC ≥ 31µg/ml, as shown in Table 12. However, the assay was repeated to confirm the MIC of triclosan on the three strains of *S. equi*, as shown in Table . The results confirmed the MIC of all the three strains to be ≥ 31 µg/mL. The discrepancy of the MIC in the case of *Strep. equi* 4047, as observed in the first assay, may be due to experimental error. The three strains are sensitive to triclosan at a minimum inhibitory concentration (MIC) of 31µg/mL. Similarly, triclosan inhibits membrane function such as bacterial growth of *Streptococcus sobrinus* at a MIC of 41µM which is equal to (11.9µg/mL) (Villalain *et al.*, 2001). The antibacterial effects of triclosan are mediated, at least at the membrane level, by its impact on the membrane structure without cell lysis. That is, growth inhibition of *Strep. sobrinus* by triclosan does not result from the loss of intracellular materials but instead may be dependent on the inhibition of fatty acid biosynthesis (Villalain *et al.*, 2001). Two experiments were conducted to assess the action of triclosan discs on the three strains of *S. equi*. A clear zone was observed for the three strains of *S. equi* as shown in Figure 2.

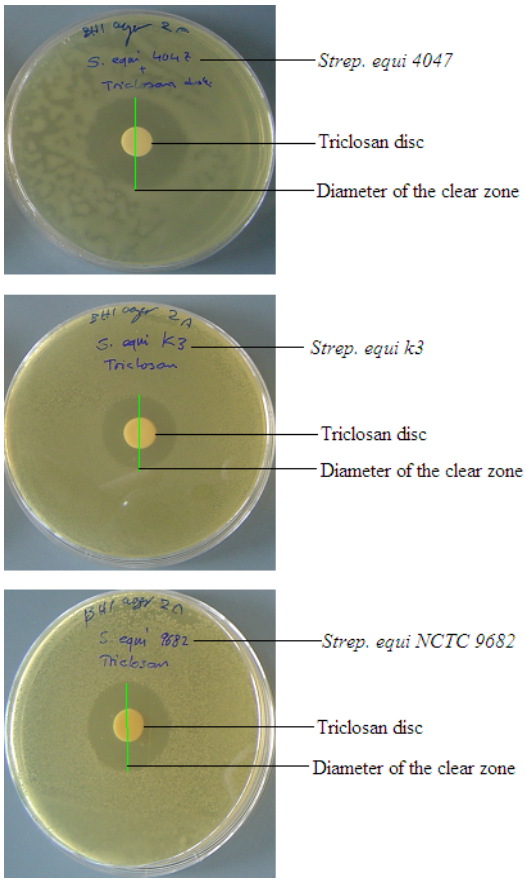

Fig. 1: Triclosan disk assay for *Strep. equi* 4047, K3 and NCTC 9682.

Thus the three strains were considered to be sensitive to triclosan. The action of 4-MU was investigated on the three strains of *S. equi* and the results are as shown in Table 5. For the strains 4047 and 9682 the MIC was observed to be 125 µg/mL while that of strain k3 appears to be > 1 mg/mL. Although, in the case of K3 in tube No 2 there was no growth observed, which could be due to experimental error. The assay was repeated twice to confirm the MICs of 4-MU on these strains as, shown in Tables 5 and 6. These results indicate that the MIC for K3 is probably > 1mg/mL while the MICs for strains 4047 and NCTC 9682 was shown to be at least 500µg/mL. The results of 4-MU disc diffusion assay are shown in Figure 3.

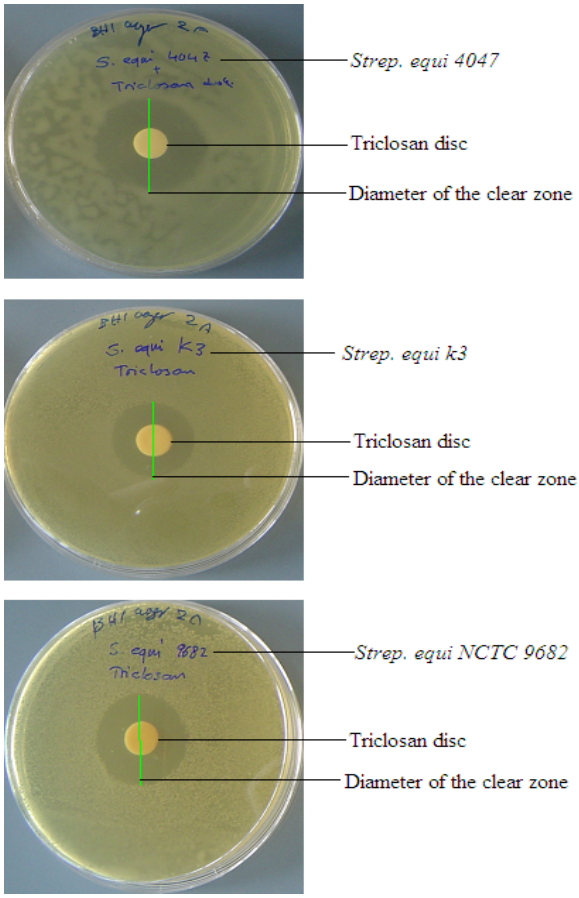

Fig. 2: 4-MU disk diffusion assay for *Strep. equi* 4047, K3 and NCTC 9682.

For strain 4047, the glossy capsule was switched off up to a diameter of about 35 mm and re-appeared at further distances at which the concentration of 4-MU is less. In the case of 4-MU versus strain K3, the results are as shown in Figure 3 were not as obvious as that of strain 4047. This is probably due to the fact that strain K3 is less capsulated (glossy) compared to strain 4047, hence less effect was seen. The result of strain NCTC 9682 was similar to that of K3 with the addition of a very narrow clear zone around the antibiotic disk. The experimental results showed that the three strains of *Strep. equi* are not sensitive to the antibacterial action of 4-MU, although 500µg/mL of 4-MU was able to switch off the glossy capsule of strain 4047. Similar results were obtained

by Kakizaki *et al.,* (2002). That is, 4-MU decreases the production of hylaluronan (HA) without inhibition of bacteria growth (Kakizaki *et al.,* 2001). Comparing plates with 500 μg/ml 4-MU and those containing no antibiotic but only BHI agar, it was observed that the glossy capsules of strain 4047 was switched off in the plate containing 500 μg/mL 4-MU, while glossy capsules could be clearly seen on the plate without 4-MU. On comparing these two plates for strain NCTC 9682, it was observed that there was reduced bacterial growth in the plate containing 500μg/mL 4-MU, while there was heavy bacterial growth in the plate without 4-MU, as seen in Figure 2.

This indicates that antibiotic 4-MU has an effect on strain NCTC 9682 by suppressing its growth. However, no effect on capsule production was evident with this strain.

Table. 1: Shows action of Penicillin for *S. equi* strain 4047 .

Expt NO.	Inoc c.f.u/ml	Tube number, Antibiotic final concentration (μg/mL) & Growth state +/-											
		1	2	3	4	5	6	7	8	9	10	11	12
1	$1_X 10^7$	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
2	4.2 x10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
3	5.5 x10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
4	4. 5 x10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
5	2.5 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
6	7.5 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +
7	3.4 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 –	3.9 –	2.0 –	1.0 –	Zero +

Table. 2: Shows action of Triclosan for *S. equi* strain 4047.

No	Inoc c.f.u/ml	Tube number & antibiotic final concentration (μg/mL) & Growth state +/-											
		1	2	3	4	5	6	7	8	9	10	11	12
1	5.5 $\times 10^6$	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 +	7.8 +	3.9 +	2.0 +	1.0 +	zero +
2	4.5 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 +	15.6 +	7.8 +	3.9 +	2.0 +	1.0 +	zero +
3	2.5 x10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 +	3.9 +	2.0 +	1.0 +	zero +
4	7.5 x10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 +	3.9 +	2.0 +	1.0 +	zero +
5	3.4 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 +	3.9 +	2.0 +	1.0 +	zero +
6	3.6 x 10 ^{6}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 –	7.8 +	3.9 +	2.0 +	1.0 +	0 +
7	4.40 x 10 ^{7}	1 –	500 –	250 –	125 –	62.5 –	31 –	15.6 +	7.8 +	3.9 +	2.0 +	1.0 +	0 +

Table. 3: Shows action of Triclosan for *Strep. equi* strains 4047, K3, NCTC 9682

Tube NO.	1	2	3	4	5	6	7	8	9	10	11	12
Triclosan conc (μg/mL)	1	500	250	125	62.5	31	15.6	7.8	3.9	2.0	1.0	0
S. equi 4047[1] Growth	-	-	-	-	-	-	-	+	+	+	+	+
S. equi K3[2] Growth	-	-	-	-	-	-	+	+	+	+	+	+
S. equi NCTC[3] 9682 Growth	-	-	-	-	-	-	+	+	+	+	+	+

1. Strain 4047 Number of cells was = 3.6×10^5 c.f.u /mL
2. Strain K3 Number of cells was = 3.2×10^5 c.f.u /mL
3. Strain NCTC 9682 Number of cells was = 1.2×10^{-5} c.f.u /mL

Table. 4: Shows action of Triclosan for *Strep. equi* strains 4047, K3, NCTC 9682.

Tube NO.	1	2	3	4	5	6	7	8	9	10	11	12
Triclosan concentration(μg/ml)	1	500	250	125	62.5	31	15.6	7.8	3.9	2.0	1.0	0
S. equi 4047[1] Growth	-	-	-	-	-	-	+	+	+	+	+	+
S. equi K3[2] Growth	-	-	-	-	-	-	+	+	+	+	+	+
S. equi NCTC[3] 9682 Growth	-	-	-	-	-	-	+	+	+	+	+	+

1. Strain 4047 Number of cells was = 4.40×10^6 c.f.u /mL
2. Strain K3 Number of cells was = 2.328×10^7 c.f.u /mL
3. Strain NCTC 9682 Number of cells was = 1.856×10^7 c.f.u /mL

Table. 5: Shows action of 4-MU for *Strep. equi* strains 4047, K3 and NTCL 9682.

Tube NO.	1	2	3	4	5	6	7	8	9	10	11	12	13
4 MU concentration(µg/ml)	1	500	250	125	62.5	31	15.6	7.8	3.9	2.0	1.0	0	
S. equi 4047[1] Growth	_	_	_	_	+	+	+	+	+	+	+	+	_
S. equi K3[2] Growth	+	_	+	+	+	+	+	+	+	+	+	+	+
S. equi NCTL 9682[3] Growth	_	_	_	_	+	+	+	+	+	+	+	+	+

1- Strain 4047 Number of cells was $= 2.5 \times 10^5$ c.f.u /mL
2- Strain K3 Number of cells was $= 1.75 \times 10^6$ c.f.u /mL
3- Strain 9682 Number of cells was $= 1.4 \times 10^5$ c.f.u /mL

Table. 6: Shows action of 4-MU for *Strep. equi* strains 4047, K3 and NTCL 9682.

Tube NO.	1	2	3	4	5	6	7	8	9	10	11	12	13
4 MU conc(µg/mL)	1	500	250	125	62.5	31	15.6	7.8	3.9	2.0	1.0	0	
S. equi 4047[1] Growth	_	_	_	+	+	+	+	+	+	+	+	+	+
S. equi K3[2] Growth	+	+	+	+	+	+	+	+	+	+	+	+	+
S. equi NCTL 9682[3] Growth	_	_	+	+	+	+	+	+	+	+	+	+	+

1- Strain 4047 Number of cells was $= 1.48 \times 10^6$ c.f.u /mL
2- Strain K3 Number of cells was $= 1.25 \times 10^6$ c.f.u /mL
3- Strain 9682 Number of cells was $= 4.8 \times 10^5$ c.f.u /mL

CONCLUSION

Antibiotics such as triclosan and 4-MU have been shown to have a wide range of antibacterial action on micro-organisms. Triclosan has been shown to inhibit membrane functions such as transport activities of some biochemical materials in *Streptococcus sobrinus* (Villalain *et al.,* 2001). However, the extent and manner to which micro-organisms respond to a given antibiotic varies. Some bacteria are sensitive to certain antibiotics while others are insensitive. This work has been able to demonstrate that the three strains of *Strep. equi* are very sensitive to triclosan as they inhibit certain membrane functions. However, these strains are very resistant to 4-MU. With these findings, it is imperative to conduct further research to investigate the mechanism of interaction between these antimicrobial agents and the bacterial membrane components.

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