Ultrastructural Study On The Effects Of Antibiotics On *Staphylococcus aureus* ATCC 25923

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ABSTRACT

The purpose of this study is to give better understanding in the morphological changes in the *Staphylococcus aureus* ATCC 25923 from the perspectives of electron microscope. The morphological changes seen in bacteria treated by amikacin, gentamicin and ciprofloxacin were same, such as the thicker outer membrane, condensing of ribonucleic, indentation and hole formation appearances and presence of mesosomes. The extra features seen in the ciprofloxacin treated bacteria compared to the amikacin and gentamicin was the coagulated DNA. However, the oxacillin and vancomycin treated bacteria were bigger in size and varies in shapes compare to other antibiotics treated bacteria. The other features that are more distinct in the oxacillin and vancomycin treated bacteria were the number of mesosomes and detachment of outer membrane. The above mentioned defects were not seen in the control group. Furthermore, the Transmission Electron Microscope Rapid Method that was used in the study gave us the accurate understanding on the ultra structural changes that occurs in the antibiotics treated bacteria that is coherent to the literature review of the how the antibiotics works.

INTRODUCTION

Staphylococcus aureus can produce a wide variety of disease from relatively benign skin infection such as folliculitis and furunculosis, to deep seated and life threatening conditions including erysipelas, deep abscesses, osteomyelitis, pneumonia, sepsis and endocarditis. The heterogeneity of these diseases and the unique ability of *Staphylococcus aureus* to develop resistance to antibiotic agents reflect its extraordinary capacity to adapt to a variety of environments (Guignard *et al.,* 2005).

In view of this, we like to demonstrate the effects of antibiotics on *Staphylococcus aureus* ATCC 25923 thus a providing the exact mechanisms of action of antibiotics to the bacteria from the perspectives of electron microscopy. In this study, the Transmission Electron Microscope (TEM) Rapid Method was applied to get to nearest first generation of the bacteria reaction to the antibiotics. This was done by fixing the bacteria for 20 minutes because the generation time *Staphylococcus aureus* are 20 minutes.

MATERIAL AND METHODS

Bacteria Culture

Staphylococcus aureus ATCC 25923 was obtained from Bacteriology Unit, Institute for Medical Research, Kuala Lumpur. The concentration of the bacteria that was used in this study is 1.4 X 107 bacteria per test and the ratio between the bacteria and the fixative solution was at

1:15.

Disc Diffusion Test

A suspension of *Staphylococcus aureus* ATCC 25923 at 1.0 x 108 CFU/ml was made and were streaked in at least three directions over the surface of the Mueller-Hinton agar to obtain a uniform growth. After the plates were dried for five minutes, antibiotic disks (ciprfloxacin, amikacin, oxacillin, gentamycin, and vancomycin disks) were placed on top the swabbed agar. The plates were incubated at 37°C/overnight. Following the overnight incubation, the bacteria around the rim of inhibition growth zone around each disk were used for electron microscope analysis.

TEM Rapid Method

The bacteria that were fixed for 20 minutes in the 2% glutaraldehyde in 0.1M PBS were washed with distilled water (X6). Then the bacteria were washed with distilled water (X3), after it were stained with 2% uranyl acetate for 5 minutes. The excess osmium tetraoxide was discarded after the stained bacteria were exposed to osmium tetraoxide for 5 minutes. The dehydration of the expose bacteria to osmium tetraoxide was conducted to the series concentrations of acetone (50%, 70%, 90% and 100%X2), respectively 5 minutes. Polymerization was done with pure epoxy resin in the embedding oven at 75°C/2 hours and 90°C/2 hours, after the bacteria infiltrated by mixture of acetone and epoxy resin (1: 1) for 15 minutes. The blocks were trimmed and cut to 90 nm ultra thin sections and mounted on 200 mesh thin bar copper grids (Agar). The specimens then were stained with Reynold's stain for 1 minute. Each specimen was examined at 30 000 magnification by using Technai G2 TEM at an accelerating voltage of 90 KV.

RESULTS

A total of 45 TEM electron micrographs were taken to see the changes of the inner ultra structures of *Staphylococcus aureus* ATCC 25923. These images showed that there were changes to the outer membranes, plasma membrane, ribonuclues and nucleotides of the bacteria upon exposure to antibiotics. In Figure A, the control group where TEM electron micrograph of the bacteria clearly shows a defined outer membrane (OM), plasma membrane (PM), ribonucleic (R), and nucleotides (N). In contrast with Figure B, which shows the effects of amikacin, the thickening of the outer membrane (TW), condensing of the ribonucleus (CR), indentation and hole formation appearances (PA) and presence of mesosomes (M). Overall, the size of the amikacin treated bacteria is larger than the control group. The same appearances were seen in the gentamicin and ciprofloxacin treated bacteria (Figure C and Figure F). The main feature present in the ciprofloxacin treated bacteria compared to amikacin and gentamicin treated bacteria was the coagulated DNA (CD). The oxacillin (Figure D) and vancomycin (Figure E) treated bacteria showed irregular shape and the sizes are much bigger compare to the control and other antibiotics group. However, the TW and CR were not seen in the oxacillin and vancomycin treated bacteria. Detachments of the outer membranes (DOM) were more evident on the oxacillin and vancomycin treated bacteria that were not seen in the other antibiotics treated bacteria. Besides that, the M were also more evident in the oxacillin and vancomycin treated bacteria compared to the other antibiotics treated bacteria. However, all the defects in the inner and outer core of the antibiotics treated bacteria were not seen in the control group.

DISCUSSION

In this study we used the Kirby Bauer Method as the sensitivity method for antibiotics reaction in the bacteria because of its nearest outcome on the bacteria treated by the antibiotics to

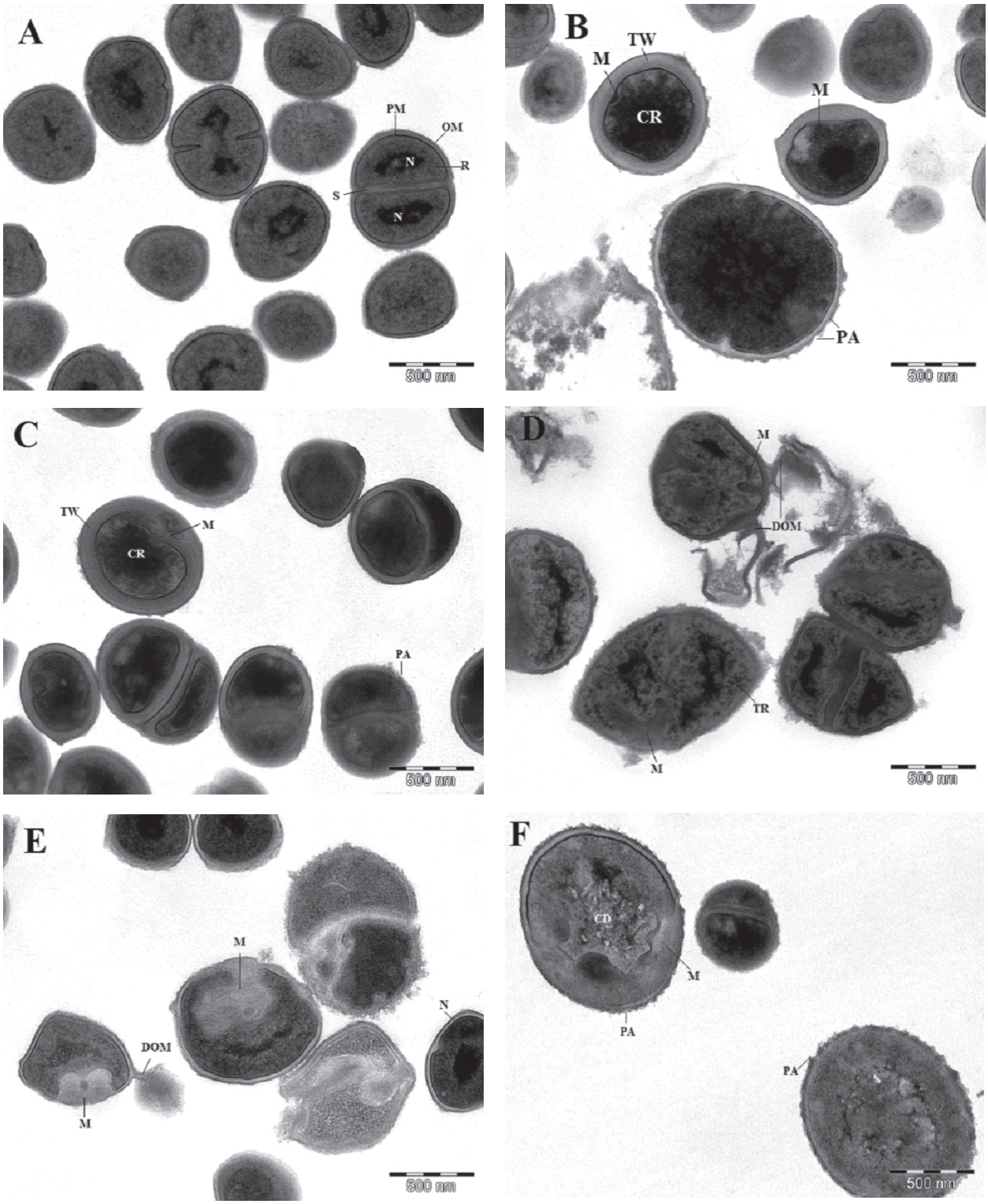

Figure 1. The electron micrograph of control group (A) shows very distinct OM=Outer Membrane, PM=Plasma Membrane, R=Ribonucleus, N=Nucleotide, and S=Septa of the bacteria. Antibiotic treated bacteria such as amikacin (B), gentamicin (C) and ciprofloxacin (F) clearly shows thickening of the outer membrane (TW), condensing of the ribonucleus (CR), indentation and hole formation appearances (PA) and presence of mesosomes (M). Oxacillin (D) and vancomycin (E) presented with thicker mesosomes and detachment of outer membrane (DOM). But the ciprofloxacin treated bacteria show the main feature of coagulated DNA (CD). Magnification for all the electron micrographs are at 30 000X.

the vivo conditions. This is base on scientific evidences that the hard surface would give similar morphological changes to the bacteria, rate of growth of the bacteria and employ a good condition for a serious infection as compared to the liquid medium (Schito, 2006).

The first group of antibiotics that was used is the Aminoglycosides (Amikacin and Gentamicin). In scientific reports indicates that aminoglycosides bind irreversibly to the 30S subunit of bacterial ribosomes and interfere with the proofreading process that helps assure the accuracy of translation. The antibiotics reduce the rejection rate for tRNAs that are near matches for the codon. This leads to misreading of the codons or premature termination of protein synthesis. There is also evidence scientific that the aminoglycosides prevent the transfer of the peptidyl tRNA from the A-site to the P-site, thus preventing the elongation of the polypeptide chain (Schito, 2006).

The role aminoglycosides reacts on the bacteria were clearly seen in the Figure B and C. Appearance of TW and CR of the bacteria indicates that the some of the bacteria were going on the defensive mode. We believe that the CR really is the mRNA of the bacteria. The mentioned mRNA was indicated by the dark area of the inner structure of the bacteria but they were more diffuse compare than control batch (Figure A). In the control batch, the mRNA was still the double helix form and the DNA was more centralized and compact. The more the mRNA was released means that the rate of protein synthesis was increased. These increases were interrelated to the TW of the bacteria. The main reason of the TW by the bacteria was to avoid anymore insurgent of the aminoglycosides and to maintain the osmotic pressure of initial attack of the aminoglycosides. Besides producing more peptidoglycan to protect themselves, the bacteria were developing M. M are the invagination of the PM that helps the chromosomes replicate and distribute the electron transfer systems for respiration of the bacteria. Nevertheless, the electron micrograph (Figure B and C) also indicates that some of bacteria were affected by the osmotic pressure and clearly shown by the larger size and thinning of the OM. The appearance of PA were due to the bacterial enzymes called autolysins put breaks in the peptidoglycan in order to allow for insertion of peptidoglycan building blocks (monomers of NAG-NAM-peptide). For the aminoglycosides treated bacteria, the insertion of the peptidoglycan building block does not happen. So, the monomers were not attached to the growing end of the bacterial cell wall by the transglycosidase enzymes and the transpeptidase enzymes. The failure to insert the peptidoglycan would cause the outer membrane to have a PA appearances. The PA appearances were caused by the movement of the water molecule from the environment to the intracellular of the bacteria because of the difference osmotic pressure between the outer and inner surrounding of the bacteria. The movement of the water molecule would expand the outer membrane of the bacteria to its limit and this would present the PA appearances at the surface of the bacteria. If this event were to prolong, it would cause the lyses of the bacteria.

The second group of antibiotics that were used in this study was from the Glycopeptides (i.e. vancomycin) and Penicillins (i.e. oxacillin) groups. Mode of action of glycopeptides were to bind to the peptides of the peptidoglycan monomers and block the formation of glycosidic bonds between the sugars by the transglycosidase enzymes, as well as the formation of the peptide cross-links by the transpeptidase enzymes. The penicillin's mode of action is to bind to the transpeptidase enzymes (also called penicillin-binding proteins) responsible for resealing the cell wall as new peptidoglycan monomers are added during bacterial cell growth and blocks the transpeptidase enzymes from cross-linking the sugar chain. This would make the cell wall of the bacteria to be weaker and subsequently osmotic pressure would lyses the bacteria (Schito, 2006) .

The effects of the antibiotic that were mentioned above correlates to the electron micrographs in Figures D and E. The severity damages to the outer membrane of the bacteria are more evident to the oxacillin group compare to the vancomycin group. The DOM (Figure D) were clearly seen in the oxacillin treated bacteria than the vancomycin treated bacteria. The damages were more prominent in the oxacillin treated bacteria (Figure D) compare to the vancomycin treated bacteria (Figure E); this was base on the number of bacteria that were treated by the antibiotics. In theory, the

vancomycin treated bacteria should show more damages that the oxacillin treated bacteria because its reaction on the both transglycosidase and transpeptidase enzymes in the building block of the peptidoglycan compared to oxacillin only affecting on the transpeptidase enzymes. However, the severity of the damage for oxacillin treated bacteria is higher compared to the vancomycin treated bacteria because dysfunctional of one enzyme by oxacillin could not discharge or balanced the osmotic pressure as easily as the two enzymes that were effected by vancomycin. Beside that, the M that were present in the treated bacteria for oxacillin and vancomycin are different structurally compared to the aminoglycosides treated bacteria. The difference was the M are thicker in size due to the effects of the osmotic preassure that occurred during dysfunction of the peptidoglycan building block. Beside that, oxacillin and vancomycin treated bacteria do not show any defensive mode like the aminoglycosides antibiotics such as the TW and CR of the treated bacteria. The thickening of the M also indicates that the treated bacteria by oxacillin and vancomycin failed in its defense to maintain the inner core replication of the bacteria.

The third group of antibiotics that was used in this study is fluoroquinolones (i.e., ciprofloxacin). The fluoroquinolones work by inhibiting one or more of a group of enzymes called topoisomerase, DNA topoisomerase enzymes, including DNA gyrase, are essential in the unwinding, replication, and unlinking of bacterial DNA during DNA replication. These enzymes also enable bacterial DNA to become circular and supercoil. In *Staphylococcus aureus* ATCC 25923, the main target for ciprofloxacin is the DNA gyrase (topoisomerase II), an enzyme responsible for supercoiling of bacterial DNA during DNA replication. The effects of ciprofloxacin on the bacteria could easily see in the electron micrograph Figure 1F where the center region of the treated bacteria by ciprofloxacin presents a DNA coagulated effect. The term of DNA coagulated effects means that the function of DNA gyrase was stopped from fulfilling its main core in supercoiling the DNA during DNA replication. In other words, the mRNA that was freed from the supercoil DNA by topoisomerase IV could not be coiled back because the DNA gyrase was immobilsed by the ciprofloxacin. Continuous replication of the bacteria will be stopped eventually because the bacteria are running out of mRNA that was supposed to be recycled for its multiplication. The TW effects that were seen in the aminoglycosides, were also present in this of fluoroquinolones group. In short, the TW effects are only present only when the bacteria are threaten within in its replication or during making the essential proteins (like the peptidoglycan) in growing phase of the bacteria. Besides that, the presences of mesosomes are to safeguard the inner core of the bacteria for continual survival of the species in hostile environments, such as environment presented by the fluoroquinolones and aminoglycosides.

CONCLUSION

The TEM Rapid Method that was used in this study has significantly given us the true event in how the *Staphylococcus aureus* ATCC 25923 reacts to the antibiotics. This method could have a better insight of the effects of the other antibiotics on different kinds of bacteria. The only recommendation is that the fixation time should follow the generation time of the bacteria.

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