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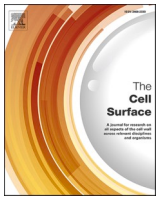

Ultrastructural and morphological studies on variables affecting *Escherichia coli* with selected commercial antibiotics

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ABSTRACT

Background: Many studies reported the effects of antibiotic exposure on *E. coli* bacterial growth and cell modification. However, scarce descriptive information on ultrastructural effects upon exposure of commercial antibiotics.

Methods: This study described the morphological and ultrastructural alterations caused by selected antibiotics (amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin) that targeted cell wall, plasma membrane, and cytoplasmic density, and also proteins synthesis. We determined extracellular morphological changes of exposure through scanning electron microscopy (FESEM) and intracellular activities through transmission electron microscopy (TEM) investigation.

Results: FESEM and TEM micrograph of *E. coli* exposed with selected antibiotics shows ultrastructural changes in beta-lactam class (amoxicillin-clavulanate, ceftriaxone) elongated the cells as the cell wall was altered as it inhibits bacterial cell wall synthesis, polymyxin class (polymyxin B, colistin) had plasmid and curli-fimbriae as it breaking down the plasma/cytoplasmic membrane, and aminoglycoside class (gentamicin, and amikacin) reduced ribosome concentration as it inhibits bacterial protein synthesis by binding to 30 s ribosomes.

Conclusion: Morphological and ultrastructural alterations of *E. coli*'s mechanism of actions were translated and depicted. This study could be reference for characterization studies for morphological and ultrastructural of *E. coli* upon exposure to antimicrobial agents.

1. Introduction

Escherichia coli (*E. coli*) is an encapsulated Gram-negative rod, facultative anaerobe, and a part of our normal gastrointestinal microbiota. Despite it being a catalase positive pathogen, it has several virulence factors; flagella and pili which are contributed to movement and adherence. *E. coli* is very genetically diverse and resistant *E. coli* exhibits resistance to most classes of antibiotics due to the presence of antibiotic resistant genes in their genome or mobile genetics element (Poirel et al., 2018 Jul), an impermeable outer membrane (Lowe, 1982) and also efflux pumps (Amaral et al., 2014). β-lactam, polymyxin, aminoglycoside classes of antibiotics have been used clinically for the treatment of *E. coli* infections due to their unique mechanism of action targeting the cell wall, plasma membrane and cytoplasmic density, and protein synthesis respectively (Hasan et al., 2022).

All antibiotics are classified by their mechanisms of action. Bacterial cells are surrounded by cell walls which are made of peptidoglycan. Antibiotics for example, β-lactam, acts as an inhibitor of cell wall synthesis that affect bacterial cell wall at different stages of peptidoglycan synthesis and cell wall assembly (Liu and Breukink, 2016). While polymyxin acts as disruptors of cell membrane which disrupt the integrity of cell membrane by binding to membrane phospholipids (Trimble et al., 2016). As for aminoglycoside class, it is a protein inhibitor that interfere with bacterial protein synthesis at different steps of the process, including formation of the 30S initiation complex (Rivera et al., 2021).

The unique mechanism of antibiotic actions on *E. coli* in terms of targeting in cell wall, plasma membrane, and cytoplasmic density could

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be studied in depth for revealing cellular structures and functions to observe the high-resolution real-time of cells and cellular processes of *E. coli*. Therefore, with the help of electron microscope (EM) which can easily achieve a nanometer level resolution, its molecular biology techniques and microscopy are used to study the mechanism of action of antimicrobial compounds (Khan et al., 2019). EM is used in biomedical research to study the ultrastructural morphology of bacterial cells. Field emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) aid the visualization of images at high resolution and provide detailed information on normal or abnormal external and internal cellular morphology such as the cell membrane, cytoplasm, nucleus, organelles and cytoskeletal structures (Dholvitayakhun et al., 2017). These FESEM and TEM methods have greater advantages over conventional light microscopy because they can give three-dimensional images and higher image resolutions compared to light microscopy.

Although many studies have reported the effects of antibiotic exposure on bacterial growth (Martínez, 2017) and cell modifications (Ponmalar et al., 2022), however, information describing their ultrastructural effects microscopically upon exposure with commercial antibiotics that are used in clinical setting are scarce. This study described the morphological and ultrastructural alterations caused by selected antibiotics that targets cell wall, plasma membrane, and cytoplasmic density, and also proteins synthesis. The effect on membrane permeability of the bacterial cells upon exposure with the antibiotic was also investigated with FESEM and/or TEM.

During the investigation, an optimized transmission electron microscopy (TEM) method to accurately visualize and elucidate antibiotic interactions with bacteria is conducted. The objective of the present investigation was to determine the susceptibility of selected *E. coli* strains towards selected antibiotics, to study the extracellular of selected *E. coli* strains upon exposure with antibiotics using FESEM, and to determine the membrane permeability and intracellular activities changes in *E. coli* after exposure to selected antibiotics through TEM.

2. Materials and methods

2.1. Kirby-bauer disk diffusion

The bacteria were exposed to antibiotic by adopting the Kirby Bauer assay method (Hudzicki, December 2009). The *Escherichia coli* (Migula) Castellani and Chalmers (ATCC 25922) strain was streaked from the glycerol stock onto a MH-2 agar and incubated for 16 h at 37 °C. The single colony was picked up and inoculated into a MH-2 broth and incubated for 16 h at 37 °C in an orbital shaker. The bacterial suspension's turbidity was adjusted to that of a McFarland standard of 0.5 by adding PBS (1 ×). The MH-2 agar was lawned with the bacterial suspension using a sterile cotton swab. The selected antibiotic impregnated discs were placed on the MH-2 agar plates using a sterile forceps and the plates were incubated for 16 h at 37 °C (facing upwards).

2.2. Chemical fixation

The bacteria were scraped off (just at the edge of clearing zone) (Fig. 1) using modified inoculation loop and were processed for the FESEM and TEM observation. The schematic diagram on sample collection was presented in Fig. 2. The sensitive zone rim edge for each antibiotic exposure was punctured using the modified adjustable wire loop according to the size of the ring as shown in Fig. 1. Then, the punctured ring was transferred into a sterile embryo dish filled with 0.1 M of phosphate buffer solution (PBS) then dislodge the bacteria by scrapping the ring using sterile loop. Followed with transferring the mixture of dislodged bacteria and 0.1 M PBS into sterile 1.5 ml tube then centrifuged at 3000 rpm for 5 mins to obtain the pellet of the bacteria sample. The supernatant was discarded and put in the fixative containing 2.5 % glutaraldehyde in 0.1 M PBS for 1 h. After 1 h in fixative, 4 % of potassium dichromate, $K_2Cr_2O_7$ in 0.1 M PBS for another 1 h. The sample underwent post fixation process with 1 % osmium tetroxide (OsO_4) for 15 mins and en bloc staining with 2 % aqueous uranyl acetate for another 5 mins after fixation.

2.3. FESEM

Prior to proceed with FESEM processing, cover slips were coated with poly-L-lysin and left to air-dry. After post fixation and en bloc, the fixatives were removed completely and rinsed with 0.1 M PBS, then proceed with dehydration phase using graded series of ethanol (30 %, 50 %, 70 %, 90 %, and 100 % for three times) for 5 mins respectively, thus drop 100 µl of the samples on the coated cover slips. Sample was then transferred to a gasket and need to be in 100 % ethanol before putting it into the critical point dryer (Baltec CPD-030, Balzers, Liechtenstein) to remove all the extracellular fluid and at the same time maintaining the intracellular fluid of the sample. For critical point drying with carbon dioxide (CO_2), the gasket was put in the chamber when the chamber was cooled to 10 °C and followed by sequence of medium in and out to remove the ethanol presence in the chamber, this sequence was repeated 6 times. Then, the chamber was heated into 42 °C for 2 h and ensured the pressure was above 85 bar. After drying and gas out the chamber, the sample was put on the stub and sprayed with platinum layers for sample conductivity using Sputter coater (Baltec

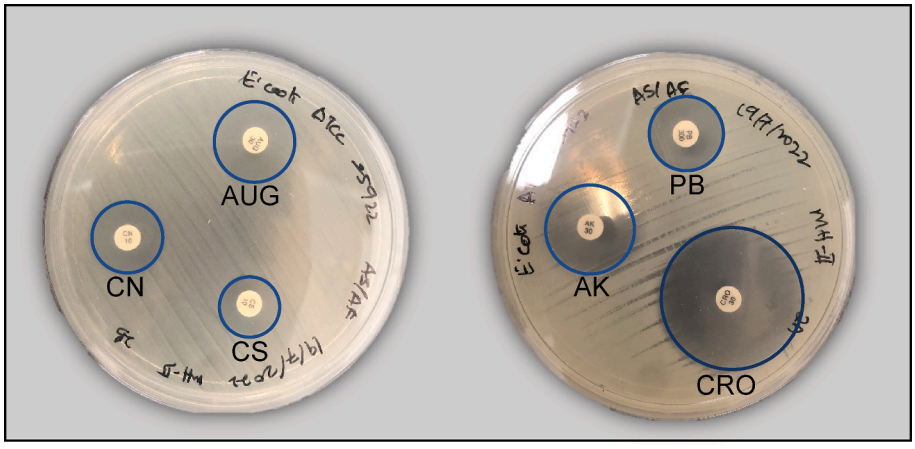

Fig. 1. Kirby-Bauer methods showing the inhibition zone of *E. coli* ATCC25922. Blue circles denote the scrapping area. AUG = Amoxicillin-clavulanate, CRO = Ceftriaxone, PB = Polymyxin B, CS = Colistin, CN = Gentamicin, and AK = Amikacin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

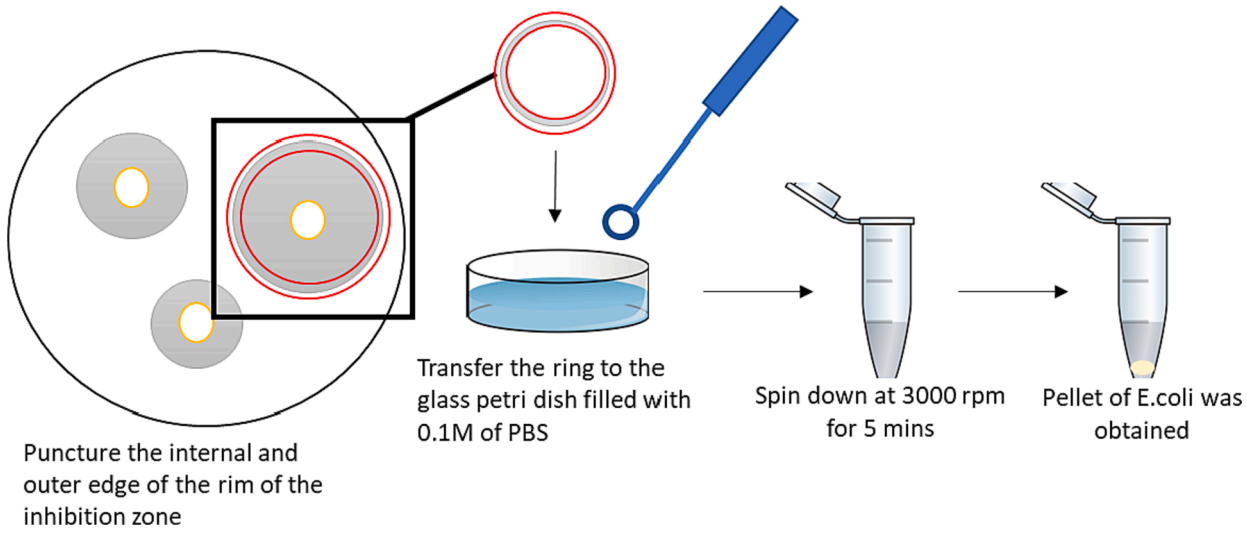

Fig. 2. Schematic diagram of optimized sample collection from the inhibition zone to study the characteristics of antibiotic affects using electron microscopy.

SCD-005, Balzers, Liechtenstein). The sample was then ready to be viewed under FESEM (Teneo FEI, USA).

2.4. TEM

For TEM, the sample was fixed according chemical fixation description similar as method in FESEM which after 10 μL of the sample were taken for FESEM processing, the remaining part of the sample proceeded for TEM processing. Scraped antibiotic-exposed bacteria were then prepared for TEM method. The sample underwent post fixation process with 1 % osmium tetroxide (OsO_4) for 15 mins and en bloc staining with 2 % aqueous uranyl acetate for another 5 mins after fixation. All the fixatives must be removed completely for dehydration phase by using graded series of acetone (30 %, 50 %, 70 %, 90 %, and 100 % for three times) for 5 mins respectively. One part of EPON and one part of 100 % acetone was added for 15 mins before filtering the sample out into the beem capsule. The sample was then degassed in MK II Embedding oven for another 10 mins and left in MK II Embedding oven overnight (24 h). The embedded sample block was cut into 80 nm thin-sectioned using an ultramicrotome (UC7, Leica Microsystems, Vienna, Austria). The sectioned samples were then transferred onto uncoated TEM 200 mesh copper grids (SPI Supplies Brand, 3.05 mm). The grid was subsequently viewed under TEM (FEI Tecnai Spirit G2, USA).

3. Results

3.1. Antibiotic susceptibility test

The inhibition zone diameters for *E. coli* ATCC 25922 upon exposure to amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin antibiotics were successfully determined using the Kirby-Bauer assay (Fig. 1). The diameter of inhibition zones as shown in Table 1 was an average result from three replicates and determined based on the CLSI 2020, 31st edition. The *E. coli* ATCC 25922 was observed to be susceptible for all tested antibiotics based on the CLSI except for the polymyxin B since no range of breakpoint is stated for it in CLSI 2020, 31st edition.

3.2. Analysis of extracellular morphology effects of E. coli exposed to selected antibiotics by FESEM

The processing for FESEM to analyze the extracellular morphology effects of *E. coli* after exposure to the selected antibiotics fulfilled our objective. In total we produced 45 micrographs of E coli after treatment with the different antibiotics. The images were taken post exposure to each antibiotic, respectively as results obtained showing the formation of distortion on the bacterial surface. In addition, the shape of the bacteria also corresponded towards the action of amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin (Fig. 4). In the control sample (the unexposed bacteria), the cells looked perfect rod-shaped and undamaged (Fig. 3). However, it was found that the bacteria became elongated and enlarged before undergoing a rupturing process after they had been exposed to the antibiotics. The ruptured areas can be seen as labelled whereas the cells looked flatten and torn. Furthermore, fimbriae was presented prominently post polymyxin B and colistin exposure. These changes of bacteria extracellular structures were significant as we compared to the control bacteria in Fig. 3 where the structures of control *E. coli* were observed to be consistent and remained unaltered.

Table 1
Results of susceptibility test *Escherichia coli* ATCC 25922.

Antibiotic	Disc content	Diameter of inhibition zone (mm)			
		Resistant	Intermediate	Sensitive	Average results (n = 3)
Amoxicillin-clavulanate	**30 μg**	**≤ 13**	**14–17**	**≥18**	**20**
Ceftriaxone	**30 μg**	**≤ 19**	**20–22**	**≥ 23**	**33**
Polymyxin B*	**300 IU**				**18**
Colistin (9)	**10 μg**	**≤11 mm**	**12–13**	**≥14 mm**	**15**
Gentamicin	**10 μg**	**≤ 12**	**13–14**	**≥ 15**	**25**
Amikacin	**30 μg**	**≤ 14**	**15–16**	**≥ 17**	**24**

*There are no interpretive categories mentioned in CLSI 2020 for polymyxin B since broth microdilution is the only approved method for diagnostic.

3.3. Analysis of membrane permeability and intracellular activities changes in E. coli exposed to selected antibiotics by TEM

A total of 45 TEM electron micrographs were taken to see the ultrastructural changes of *E. coli* ATCC 25922 by the action of selected antibiotics. The electron micrograph of the TEM shows that there are changes in intracellular activities of the bacteria when exposed to antibiotics. The unexposed control bacteria group (Fig. 5) clearly shows a smooth undamaged surface, uniform overall size, and rod-shaped shape. In the antibiotic exposed bacteria group micrograph of amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin (Fig. 6), the shape of bacteria becomes irregular and there was

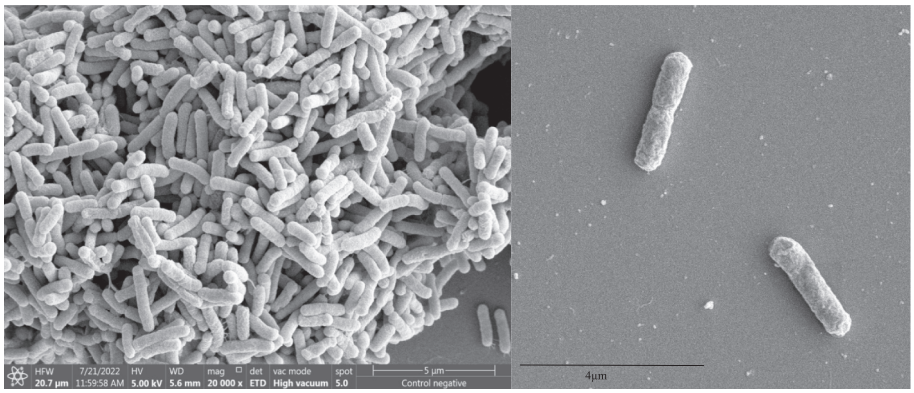

Fig. 3. FESEM micrograph showing consistent shape of *E. coli* ATCC 25922 as the control whereby it was not treated with any antibiotics, magnification in 20,000x and 30,000x under 5.0 kV.

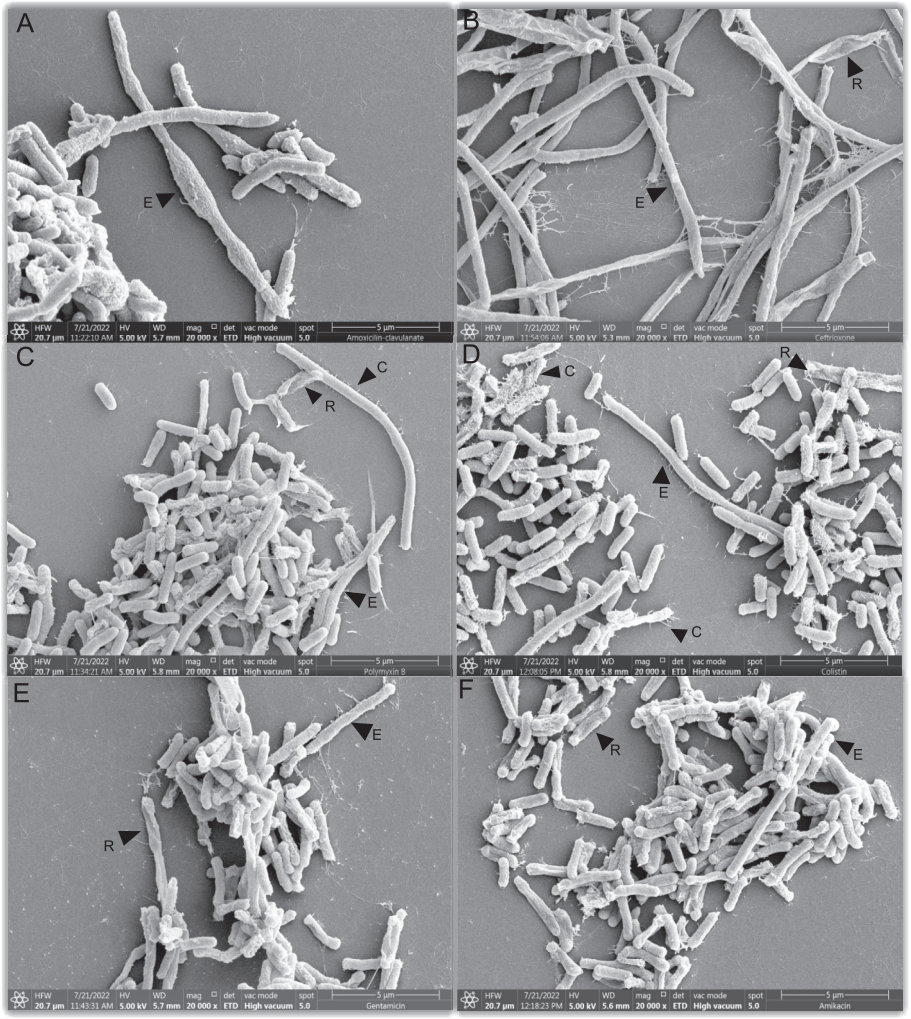

Fig. 4. FESEM micrograph of *E. coli* exposed with A) Amoxicillin-clavulanate, B) Ceftriaxone, C) Polymyxin B, D) Colistin, E) Gentamicin, and F) Amikacin; (black arrow) shows extracellular structural changes in the presence of E = elongation, R = rupturing, and C = curli fimbriae, magnification in 20,000x under 5.0 kV.

formation of additional structures in the cell polarity in the curved regions. Cell polarity localized proteins that are regulated temporally and spatially involved essentially in all aspects of cell function such as in growth, division, cell cycle regulation, differentiation, motility, signal transduction, and multi-enzyme complexes (Treuner-Lange and Søgaard-Andersen, 2014). Furthermore, the shape of the bacteria upon exposure to amoxicillin-clavulanate, ceftriaxone, gentamicin and amikacin was found to be increased in size on average than the control group caused by the elongation occurrence (Fig. 6; A, B, E, and F). It was also noticeable on the appearance of plasmids and vacuoles in the cross-section of bacterial surface. Furthermore, curli fimbriae was clearly seen on the bacterial surfaces upon exposure to polymyxin B and colistin (Fig. 6; C and D) which corresponded and correlated with the results of the SEM micrographs.

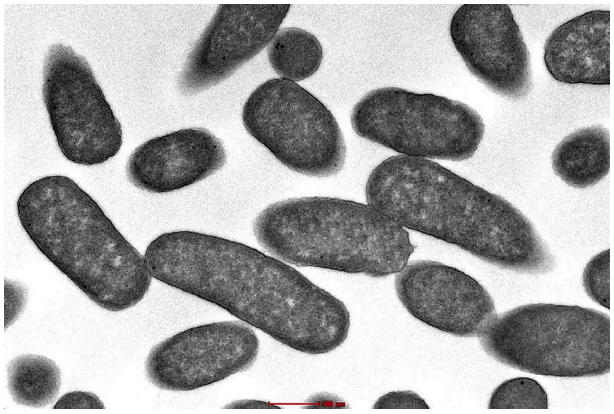

Fig. 5. TEM micrograph showing normal *E. coli* ATCC 25922 as the control not treated with any antibiotics. Magnification 4400x.

4. Discussion

The Kirby-Bauer test is a widely used and effective way to test if a bacterium is resistant or sensitive to several antimicrobial compounds simultaneously. We measure the diameter of inhibition zone by determining a greater sensitivity forming larger zones and lower sensitivity forming smaller zones. In summary, the Kirby-Bauer assay is a fast and effective way to test potential antimicrobial properties of compounds against a single bacterium. The diameter of the zone of inhibition, relates to the sensitivity of bacterium to a compound and the assay works by inhibiting growth but not directly killing bacteria. Therefore, with this approach, we proceeded the use of antibiotic exposed *E. coli* in studying the extracellular morphology effects through SEM and determining the membrane permeability and intracellular activities changes in *E. coli* using TEM.

Here we report the observation of *E. coli* ultrastructure upon exposure to commercial antibiotics disc which are amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin. These antibiotics are chosen based on their mechanism of action; amoxicillin-clavulanate and ceftriaxone (interrupting bacterial cell wall development by binding covalently to penicillin-binding-proteins in the peptidoglycan) (Iyer and Kenakin, 2022); polymyxin B and colistin (disrupting bacterial cell membrane) (Cai et al., 2015), and gentamicin and amikacin (inhibiting bacterial protein synthesis by binding to 30S ribosomes (Krause et al., 2016 Jun).

Evaluation by SEM and TEM were used to determine the ultrastructural morphological alterations caused by the antibiotic exposure. Results obtained from SEM and TEM displayed strong evidence that targeting bacterial cell wall is the main antibacterial mechanism used by amoxicillin-clavulanate and ceftriaxone. Besides, unlike the unexposed cells, the *E. coli* exposed to selected antibiotics faced dramatic morphological changes. The elongation of bacterial cell wall is probably

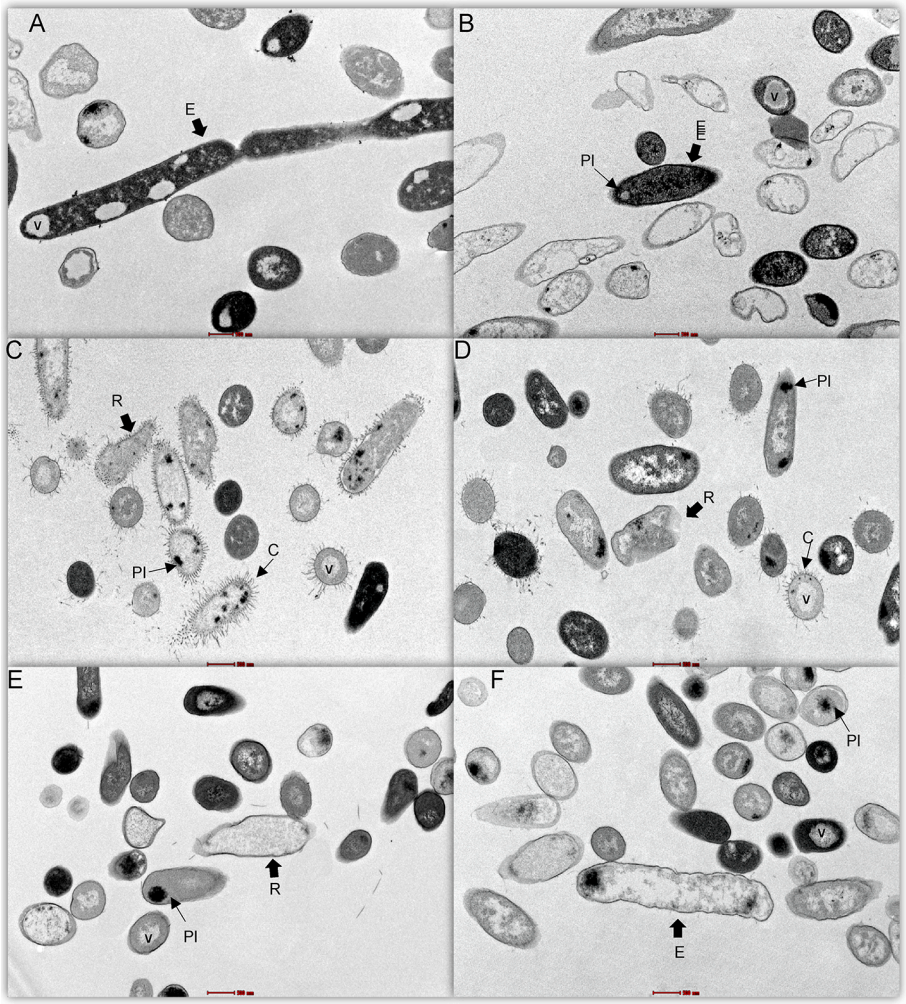

Fig. 6. TEM micrograph of *E. coli* ATCC 25922 exposed to A) Amoxicillin-clavulanate, B) Ceftriaxone, C) Polymyxin B, D) Colistin, E) Gentamicin, and F) Amikacin. The black arrow shows intracellular structural changes in presence of E = elongation, R = rupturing, V = vacuole, C = curli, and Pl = Plasmid. Magnification 4400x.

a result of the strong interaction between the negatively charged membrane and the positively charge beta-lactam antibiotics with high hydrophobic content (Malanovic and Lohner, 2016 Sep). Unlike the normal mammalian cell membranes which consist primarily of phospholipids and cholesterol (Luchini and Vitiello, 2021), bacterial membranes are richer in highly electronegative lipids because of the presence of peptidoglycan which is rich in carboxyl and amino groups (Vollmer et al., 2008). These peptidoglycan layer tend to cause the bacterial membrane which is highly negative in charge and thus attract the positively charged antimicrobial peptides to attach to the bacterial membranes (Malanovic and Lohner, 2016 Sep).

In addition to their polarly negative charge of bacterial cell attracted to positively charge antibiotic, elongation occurrence could possibly be due to the peptidoglycan synthesis. Upon beta-lactam exposure (amoxicillin-clavulanate and ceftriaxone), peptidoglycan of the *E. coli* was observed to be inhibited at the septal wall but not at the lateral wall which led the cells to elongate without division. The elongation in beta-lactam exposed-bacterial cells were found to be drastically long, however, in polymyxin class (polymyxin B and colistin) and aminoglycosides class (gentamicin, and amikacin) exposed bacteria, the cells were considerably long but not as disruptive as beta-lactam exposed bacteria and they are comparatively shorter in length. The cationic amphiphilic antibiotics are known to have electrostatic affinity against negatively charged bacterial cell membrane and integrate via hydrophobic interaction resulting in membrane destruction and disintegration (Lohner and Prossnigg, 2009).

In this study, we determined that exposure to antibiotic (amoxicillin-clavulanate) caused *E. coli* to have increased number and size of holes and development of vacuole-like structures on the cells. While for the intracellular observation, the vacuoles frequency was increased during wide range of antibacterial exposure which was proven in all selected antibiotics applied in this study. This formation of vacuole-like or holes could be caused by reaction oxygen species (ROS). ROS are necessary for various physiological functions but an imbalance in favor of reactive oxygen species results in oxidative stress (OS) (Fasnacht and Polacek, 2021). Antibiotic activities contribute to increment of OS in bacterial cells, which leads to vacuole formation (a marker of effective antibacterial activity) (Zhao and Drlica, 2014). Signification of cytoplasmic vacuolization has in the vast discussion yet it always accompanies programmed death of organisms and manifestation of conservative mechanism of cell death, that appeared in unicellular and preserved in multicellular organisms (Shubin et al., 2016).

Following exposure of polymyxin class antibiotics to *E. coli*, images in FESEM and TEM showed prominent structure of curli fimbriae development. Colistin were established in interacting with lipopolysaccharide on the surface of bacterial cell wall and then across the outer membrane via the self-promoted uptake pathway, resulting in disruption of the normal barrier property of the outer membrane (Sabnis et al., 2021). Subsequently, the outer membrane was transiently open thereby allowing passaging of antibacterial agents into the cell to the drug target site(s), likely to be located on the plasma membrane (Ahmed et al., 2020). Besides that, compared to unexposed cells, most of the tubular and curli fimbria-like radiant appendages were broken and had disappeared upon exposure to polymyxin B, which have similar mechanism to colistin.

Moreover, in polymyxin exposed bacterial cells, increased plasmid frequency was detected compared to other antibiotics exposed cells. Plasmid could function variously; in this situation, plasmid could possibly aid *E. coli* to survive external stress from the antibiotic's activities. However, antibiotics could also interfere with the evolution of plasmid stability as reported by Tanita Wein (Wein et al., 2020). The study discovered that antibiotics led to plasmid amplification, resulting in plasmid instability. These reflected in our study too that under specific regime of polymyxin B exposure, the circumstantial condition affected *E. coli* to amplify plasmids which was considerably more compared to other antibiotics.

As for the aminoglycoside class which inhibits bacterial protein synthesis, exposure to amikacin and gentamycin resulted in reduction of ribosomes concentration in *E. coli* that can be seen in images of TEM. In terms of quantifying the number of ribosomes reduction was integrally tough, as the proportion of nucleoid that was visible varies from one cross section to other cells, respectively. Other studies proposed this incident happened due to defect in cell envelope, which allows influx of liquid and efflux of ribosome during the interaction between the antibiotics and the ribosomes (Kapoor et al., 2017).

5. Conclusion

This study highlighted the potential of microscopy in helping to achieve more thorough understanding of the modification of bacterial cell structural upon exposure to antibiotics. In summary, our studies described the morphological and ultrastructural alterations caused by exposure of *E. coli* to several selected antibiotics that targets cell wall, plasma membrane, and cytoplasmic density and protein synthesis through investigation of FESEM and TEM. The results of electron microscopy clearly presented the selected antibiotics; amoxicillin-clavulanate, ceftriaxone, polymyxin B, colistin, gentamicin, and amikacin have their own mechanism of actions which inhibit the bacteria cells of *E. coli* from multiplying by disrupting the protein synthesis. From this study, the mechanism of actions was translated and depicted in terms of morphological and ultrastructural alterations; beta-lactam class (amoxicillin-clavulanate, ceftriaxone) elongated the cells as the cell wall was altered as it inhibits bacterial cell wall synthesis, polymyxin class (polymyxin B, colistin) had plasmid and curli-fimbriae as it broke down the plasma/cytoplasmic membrane, and aminoglycoside class (gentamicin, and amikacin) reduced ribosome concentration as it inhibits bacterial protein synthesis by binding to 30 s ribosomes. This study also gave opportunities for us to develop better techniques in improving experimental design to be definitively identified and more accurately measured. Finally, this study could be reference for characterization studies for morphology and ultrastructure of *E. coli* upon exposure to antimicrobial agents.

Ethics statement

This research project involved the isolation and culturing of Escherichia coli bacteria isolates from bacteriology laboratory, IMR and no human biospecimens will be obtained. Approval granted via Expedited Review by Medical Research and Ethics (MREC) Malaysia Chairperson/ Deputy Chairperson. (Research ID: RSCH ID-22-00989-SXQ, NMRR ID: NMRR ID-22-00695-LPT).

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CRediT authorship contribution statement

Nur Afrina Muhamad Hendri: . **Nur Asyura binti Nor Amdan:** . **Shelly Olevia Dounis:** . **Norzarila Sulaiman Najib:** Resources, Project administration, Funding acquisition. **Santhana Raj Louis:** Visualization, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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