Morphological and Ultrastructural Changes of Escherichia Coli and Klebsiella Pneumoniae Carriers of β-lactamase when subject to β-lactam Antibiotic

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Abstract

In our manuscript, we examine the structure and morphology, we take an imperative look at this pathway. We collected clinical isolates from patients at the Oncology Center of Mansoura University from urine samples. The isolates were then screened for antibiotic resistance and beta-lactamase enzyme production, with a focus on the most prevalent bacterial isolates. To further investigate the impact of one particular beta-lactam antibiotic on Escherichia coli and Klebsiella pneumoniae, both known producers of the beta-lactamase enzyme, we utilized Transmission Electron and Scanning Electron Microscopes. Our study aims to discuss the various changes induced by cefoxitin, including its effects on the characteristics of clinical isolates such as the cell wall structure of E. coli and K. pneumoniae.

The aim of this manuscript is to discuss the occurrence of alterations caused by beta-lactam antibiotics. Photomicrographs will be used to describe these alterations and provide information about the agents responsible for the changes. We will focus on the structural and morphological changes that are consistently observed.

**Keywords:** Beta lactam antibiotic, Mechanism of action, Scanning Electron Microscopy, Transmission Electron Microscopy, Bacterial morphological changes.

Introduction

*Escherichia coli* and *Klebsiella pneumoniae* are well known as the most antibiotic-resistant bacteria widespread, and it is the reason for more mortality and morbidity in hospitals (Mammina et al 2012). Beta-lactam drugs are the most used antibacterial due to its efficiency and low toxicity (Haeggman et al 2004). Beta-lactam bacterial targets is the Penicillin binding protein and the inhibition of this protein destroy the cell wall of bacteria (Stefanova et al 2003). There are many studies were reported on the changes in morphological and structure of bacteria introduce by cephalosporins in many species (Buijs et al 2008).
The production of beta-lactamase is an important resistance mechanism in pathogenic bacteria as a response to beta-lactam (Haeggman et al. 2004). Beta-lactamases have the ability to inactivate cephalosporins and penicillins (Robin et al. 2005). The majority of Escherichia coli and Klebsiella pneumoniae possess genes for beta lactamase resistance (Nelson et al. 2003). Clinical practice of using cephalosporins for treating the pathogenic bacteria was followed by the discovery of beta-lactamases, which conferred its resistance to cephalosporins (Haeggman et al. 2004). Many of the sulphydryl variable active site (SHV) gene (170 variants) and Transmission electron microscopy (TEM) (200 variants) have been discussed (Bush and Jacoby 2012). Another group of extended spectra of beta-lactamases is the CTX gene which is widely found in many species of the Enterobacteriaceae family specially K. pneumoniae (Saladin et al. 2002). Infections treatment of beta-lactamases (Escherichia coli and Klebsiella pneumoniae) is limiting to the use of Cephalosporins which found to be a more effective treatment against these infections (Lascols et al. 2013). The increasing of multidrug resistance in many countries is leading to the less effectiveness of antibiotics for treating bacterial infection (Cress et al. 2014). Thus, we need to develop new studies, in which old antibiotic should be reused. In order to obtain a new option for treating bacterial infections, many studies have used drugs in combination with beta-lactam and others (Hirsch et al. 2013).

Elucidation of the antibacterial mechanism in manipulated drugs is a key in discovering a new antibacterial therapy, this information permits solving the problem related to the resistance of bacteria (Silver 2011). Disrupting of the cytoplasmic membrane, might cause many problems more than those which target the cell wall. Information about antibiotic action mechanisms gives understanding of drug interaction (Auerbach et al. 2010) and optimizing drug activity and structure (Gwynn et al. 2010). Lastly, drug information of action mechanism enables scientists to select the combination of drugs for reducing resistance (Oldfield and Feng 2014).

**Material and Methods**

**Bacterial Isolates**

Urine samples were collected by the medical center staff nurses then transferred to the laboratory. The samples were incontinently reprocessed for urine culture. Samples received were streaked on agar of MacConkey type. The plates were incubated for 24h at 37°C. The Bacterial growth appearance of all bacterial isolates was included in our study. Strain identification was performed using the Vitek 2 system( bioMérieux,) at Mansoura University Oncology Center.

**Screening for Antibiotic resistance.**

All the isolates were tested for antibiotic resistance (Oxoid) using the disk diffusion method. Freshly grown colonies were suspended in ordinary saline, McFarland's standard, and then inoculated onto Mueller Hinton agar with a sterile cotton swab. Afterward, all antibiotic discs were placed with a gap of 20 mm and incubated at 35 ± 2°C for 16-18 hours. The isolates with less susceptibility to antibiotics (zone width of ≤22 mm) around the disks were suspected to be antibiotic resistant. Results were noted according to the Institute of Clinical and Laboratory Standards (CLSI, 2014).

**Beta lactamase Production**

The isolates were tested for the production of beta-lactamase enzyme using synergy test. Synergy test used for isolation of E. coli and K pneumoniae beta-lactam resistance. Using cefepime (30 μg), cefotaxime (30 μg) and amoxicillin with clavulanic acid (20/10 μg) as a β-lactamase inhibitor on Mueller Hinton agar, were tried and the plates bacteria were incubated at 37°C for 24 hours, if the zone of inhibition around the amoxicillin + clavulanic acid and single disc was ≥ 5 mm, beta lactamase enzyme was considered as a positive for this strain (Paterson and Bonomo 2005).

**Scanning Electron Microscopy (SEM)**

Escherichia coli and Klebsiella pneumoniae isolates were subjected to cefoxitin antibiotics sensitivity test. Bacterial cells were centrifuged
and Perfusion or immersion fixation of the tissue using a modified Karnovsky (1965).
Leave tissue overnight at 4°C in 2.5 % buffered glutaraldehyde + 2 % paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 then wash 3 times for 15 minutes in 0.1 M sodium phosphate buffer + 0.1M Sucrose. Postfixation for 90 minutes in 2 % sodium phosphate buffered osmium tetroxide pH 7.4. Washing 3 times for 15 minutes in 0.1 M sodium phosphate.
Dehydrate with ethanol 30%, 50%, 80%, 90%, 96%, and 100% 2 times for 15 minutes. The samples were dried, then coated with gold palladium membranes and observed in a Jeol JSM-6510 L.V SEM, The microscope was operated at 30 KV at Faculty of Agriculture, Mansoura University, Egypt.

Transmission Electron Microscopy (TEM)
Escherichia coli and Klebsiella pneumoniae isolates were subjected to cefoxitin antibiotics at 37°C for 6 hours. Under the same condition the control for each isolate was included, without antibiotic. Perfusion or immersion fixation of the tissue using a modified Karnovsky (1965).
Leave tissue overnight at 4°C in 2.5 % buffered glutaraldehyde + 2 % paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 then wash 3 times for 15 minutes in 0.1 M sodium phosphate buffer + 0.1M Sucrose. Postfixation for 90 minutes in 2 % sodium phosphate buffered osmium tetroxide pH 7.4. Washing 3 times for 15 minutes in 0.1 M sodium phosphate.
Dehydrate with ethanol 30%, 50%, 80%, 90%, 96%, and 100% 2 times for 15 minutes. The samples were dried, then coated with gold palladium membranes and observed in a Jeol JSM-6510 L.V SEM ,The microscope was operated at 30 KV at Faculty of Agriculture, Mansoura University, Egypt.

Results
Gram-negative bacilli were the predominant bacteria found in the cultured samples. Among the isolated bacteria, Escherichia coli accounted for 42.0%, Klebsiella pneumoniae for 35.0%, Enterococci for 9%, Pseudomonas for 5%, Proteus mirabilis for 4%, Citrobacter for 2%, Breaundima for 1%, and Kluyvera cryorescens for 1% (Figure 1).

![Figure 1](image1.png)

Figure 1. Bacterial distribution among studied cases.

Susceptibility to antibiotics
It was discovered that K. pneumoniae exhibited a high resistance to amoxicillin-clavulanic acid, followed by E. coli and Enterococci. However, azlocillin demonstrated a positive effect on the majority of bacterial isolates (Figure 2).

![Figure 2](image2.png)

Figure (2): Antibiotic sensitivity among isolated bacterial species, AMC(Amoxicillin-clavulanic)-AMG(Amoxicillin)-ATM(Azetronam)-AK(Amikacin)-AZT(Azlocillin)- and AZM (Azithromycin).

Also, K. pneumoniae and E. coli exhibited resistance to cefuzidime, cepodoxime, and ciprofloxacin. Conversely, the cefuzidime antibiotic demonstrated greater efficacy against Breaundima, whereas cepodoxime was
effective against *Pseudomonas* and *Breundimona* (Figure 3).

*K. pneumoniae* and *E. coli* exhibited resistance to cefoxitin but were sensitive to gentamycin. Conversely, *Proteus* demonstrated greater sensitivity to cefoxitin, while *Enterococci* and *Pseudomonas* were more effectively treated with gentamycin (Figure 4).

*E. coli* and *K. pneumoniae* displayed resistance to piperacillin-tazobactam, levofloxacin, and trimethoprim-sulpham. *Breundimona*, on the other hand, showed sensitivity to piperacillin-tazobactam, while *Pseudomonas* exhibited sensitivity to levofloxacin, vancomycin, ampicillin-sulbactam, and vancomycin (Figure 5).

**Figure (4)** Antibiotic sensitivity among isolated bacterial species, CAZ (Ceftazidime), CEP (Cefpodoxime), CIP (Ciprofloxacin), CES (Cefoprazon), CRO (Cefotaxim), CTX (Cefotaxime), CN (Cephalexin), CTF (Cephalothrin), CIR (Cefuroxime), CEF (Clarithromycin), CT (Cefitubutens), CT (Ceftriaxone), C (Chloramphenicol), CTC (Chlortetracycline), and CFM (Cefixime).

**Figure (5)** Antibiotic sensitivity among isolated bacterial species, PRL (Piperacillin), RA (Rifampin), S (Streptomycin), SXT (Trimethoprim-sulpham), SCF (Cefoprazon-sulbactam), SAM (Ampicillin-sulbactam), VA (Vancomycin), TPZ (Piperacillin-tazobactam), and LEV (Levofloxacin).

**Prevalence of Beta-lactamase in E. coli and K. pneumoniae (According to Synergy Test)**

When the zone of inhibition around the single disc and combination was equal to or greater than 5 mm, the strain was considered positive for Beta-lactamase in *E. coli* and *K. pneumoniae* isolates (Figure 6).

**Figure (6)**: Double disk synergy test for *E. coli* and *K. pneumoniae*. AMC (Amoxicillin + Clavulanate), CTX (Cefotaxime), FEP (Cefepime) show clear extension of edge of the Cefepime inhibition zone towards the clavulanate).

**Ultrastructural and Morphological Analyses**

**Scanning Electron Microscopy (SEM)**

The effect of cefoxitin on the bacterial cell structures of beta-lactamase developing *Klebsiella pneumoniae* and *Escherichia coli* was examined using Scanning Electron Microscopy (SEM) images. The SEM images of the control and treated isolates are shown in Figures 7 and 8, respectively. As depicted in Figures 7A and 7B for *E. coli* and *K. pneumoniae*, respectively, the control isolate exhibited compact bacterial cell structures with a particulate surface. However, the cefoxitin-treated isolate displayed abnormal morphology in both *Escherichia coli* and *Klebsiella*...
pneumoniae (Figures 8A and 8B, respectively). The SEM images revealed a reduction in cell adherence, unclear bacterial cell edges, loss of turgidity, collapse, and fewer particles on the surface after treatment with cefoxitin. Additionally, there was a decrease in mass, and the bacterial cells appeared looser and thinner under the pressure of cefoxitin compared to the control. Furthermore, the ability of E. coli and K. pneumoniae to produce beta-lactamase was reduced with cefoxitin treatment. Bacterial isolates treated with the antibiotic cefoxitin exhibited apparent particulate near the interface, and the walls of these isolates were observed to be thickened (Felice et al., 1986). There was a significant reduction in the consistency and frequency of cell division at the air interface in the cefoxitin-treated isolates compared to the control group. These findings suggest that cefoxitin was able to penetrate the entire colony and act on bacteria at the edges. The action of cefoxitin was observed along the entire length of the colony-air interface, indicating that it entered the system to some extent, rather than being limited to isolated directions.

Figure (7): SEM images of the control isolate beta-lactamase producing E. coli (A) and (B) K. pneumoniae using cefoxitin.

Figure 8. SEM images of the cefoxitin-treated isolates beta-lactamase-producing E. coli (A) and (B) K. pneumoniae using cefoxitin.

Transmission Electron Microscopy (TEM)

The effect of cefoxitin on the bacterial cell structures of beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae was visualized using TEM. Cefoxitin-treated bacterial cells exhibited noticeable action, as indicated by lysed cells, bloated cells, and cell debris, particularly near the air interface and membrane (Figure 9A and B) for E. coli. However, the untreated bacterial cells (Figure 9C and D).

The effect of cefoxitin on the bacterial cell structures of beta-lactamase-producing K. pneumoniae was also observed using TEM. It had a significant impact on the cells, as evidenced by lysed cells (Figure 10A and B). However, the untreated bacterial cells (Figure 9C and D).

Figure (9): Transmission electron micrographs of isolate E. coli. (A and B) Cell subjected to Cefoxitine presence of large electron lucent space due to increased periplasmic space (s) and reduced cytoplasmic material (c) and ruptured cell wall(w). (C and D) Untreated bacterial cell preserved morphology, cytoplasmic membrane, cell wall, and cytoplasm intact.

Figure (10): (A–D) Transmission electron micrographs of isolate K. pneumoniae. (A and B) Cell subjected to Cefoxitine presence of large electron lucent space due to increased periplasmic space (s) and reduced cytoplasmic material (c) and ruptured cell wall(w). (C and D) Untreated bacterial cell preserved morphology, cytoplasmic membrane, cell wall, and cytoplasm intact.
Discussion

Beta-lactamase mediated bacterial resistance is a crucial mechanism for drug resistance in Enterobacteriaceae. Beta-lactamases are frequently found in isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Infections caused by these isolates, which produce beta-lactamases, can guide the selection of the most appropriate antibiotic. The purpose of this study is to investigate the impact of cefoxitin on the development of beta-lactamase resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from patients with urinary tract infections under various clinical conditions.

In this study, the presence of different bacterial cell morphological disorders caused by K. pneumoniae and *E. coli* isolates after subject to cefoxitin. Subjection of the K. pneumoniae and *E. coli* isolates to cefoxitin, caused changes in cell morphology in all isolates, with consecutive septa formation and cell membrane and cell wall disorganization in many cells. Bacteria undergo a variety of changes in cell morphology when treated with antibiotics (Nikola et al., 2022). These morphological changes often involve alterations in cell size. For example, *Escherichia coli* decreases its cell size when exposed to cell wall-targeting antibiotics (Harris and Theriot, 2016). The effects of changes in cell size and shape on bacterial growth and motility have been well-characterized (Ojicic et al., 2019). Several studies have reported on the morphology of Gram-negative bacteria in response to cephalosporins (Nishino and Nakazawa, 1972; Lorian and Atkinson, 1975). Additionally, different genes encoding beta-lactamases in *K. pneumoniae* isolates have been shown to cause changes in cell morphology when exposed to beta-lactams (Dyana et al., 2015). There are differences in the structural changes induced by cephalosporins. At low concentrations, stretched cells are observed, while at high concentrations, spheroplasts are common. Changes in the concentration at which cell growth is affected have been studied with various cephalosporins, including cephalaxin, cefamandole, cefazolin, cephalothin, cefmetazole, cefoxitin, and ceftadine, which fall into the intermediate range. Cephaloridine and cephalosporin, on the other hand, show almost no growth (Ubbukata et al., 1979). In a study by Spratt (1975), the morphological changes in *E. coli* caused by different beta-lactams were described, and the relationship between filminization in the test bacteria was discussed. The concentration range of cefmenoxime, where *E. coli* cell extension was wider compared to cefazolin, and cell lysis occurred at low concentrations, was also mentioned.

This changes in morphology of cell was reported previously in other studies using resistant and sensitive isolates from different species, such as *K. pneumoniae*, *P. aeruginosa*, and *Serratia marcescens* treated with carbapenems, monobactams, and cephalosporins. The studies showed that the penicillin binding proteins inactivation associated with inability of cell division (Buijs et al., 2008, Rajeshwari et al., 2009). The changes in cell morphology were previously reported in other studies using resistant and sensitive isolates from different species, such as *K. pneumoniae*, *P. aeruginosa*, and *Serratia marcescens*, that were treated with carbapenems, monobactams, and cephalosporins. These studies showed that the inactivation of penicillin binding proteins was associated with the inability of cell division (Buijs et al., 2008, Rajeshwari et al., 2009). The results demonstrate that *E. coli* and *K. pneumoniae*, which have beta-lactamases, can induce a change in cell ultrastructure and morphology when exposed to beta-lactams. Therefore, these antibiotics still have residual activity, despite the phenotypic resistance of the isolates.

References


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الملخص العربي

عنوان البحث: التغييرات الخارجية والمورفولوجية في العزلات السريرية إشريشيا كولاي وكلبسيلا نيومانيا الحاملة لإنزيم البيتاالاكتاماز عند تعرضها للمضاد الحيوي البيتاالاكتام

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الملخص:

اهتمام هذه الدراسة بفحص البكتيريا المعالجة المضاد الحيوي ببيتاالاكتام و ذلك بحثًا عن التغييرات التي تطرأ عليها مورفولوجيتها وكذلك بنيتها التحتية الدقيقة، حيث اشتملت هذه الدراسة على خصائص العزلات إكليمية إشريشيا كولاي وكلبسيلا نيومانيا والتي تم جمعها من عينات البول وفحص مقاومتها للمضادات الحيوية لمرضى مركز الأورام بجامعة المنصورة، إضافة إلى فحص إنتاجها لإنزيم بيتا لاكتاماز من بين جميع العزلات وقد كانت أكثر العزلات البشرية انتشارا هي الإشريشيا كولاي والكلبسيلا نيومانيا، كما استخدمت المجهر الإلكتروني الماسح والناقل لتوضيح تأثير أحد المضادات الحيوي للبيتا لأكتام على بكتريا إشريشيا كولاي والكلبسيلا نيومانيا بعد أن تم التعرف عليها كمنتجة لإنزيم البيتا لاكتاماز، تناولت الدراسة أيضا تعرف على المتغيرات التي تحدث للخلايا البكتيرية عند استخدام البيتا لاكتاماز و قد أظهرت النتائج أن عزلات إشريشيا كولاي والكلبسيلا نيومانيا التي تحتوي على البيتا لاكتاماز يمكن أن تخضع لتفصيات مورفولوجية وبنى تحتية للخلايا عند تعرضها للمضاد الحيوي البيتا لكتام، مما يشير إلى أن هذا المضاد الحيوي له نشاط منتفي على الرغم من حدوث المقاومة المظهرية في هذه العزلات.