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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 sulfhydryl 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 problems 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, 2 times for 15 min. with acetone, 30 min. 2 : 1 acetone : Epon mixture, 30 min. 1:1 acetone: Epon mixture , 30 min. 1 : 2 acetone : Epon mixture, put in Epon pure solution overnight at 4° C put in new fresh Epon solution, put in incubator for ~248 hours at 70° C
For polymerization, cut with an ultramicrotome set to 50 - 100 nm section thickness rinse sections to grids made of copper, post contrast of sections according to Reynolds (1963) for 10 min. using 8 % uranylacetate, and for 5 min . using1 % lead citrate, after drying for 15 min sections may be investigated in a transmission electron microscope . Ultrathin sections were observed at 160 kV using a JEOL JEM-2100 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%, *Breundimona* for 1%, *Raoultella* for 1%, and *Kluyvera cryocrescens* for 1% (Figure 1).

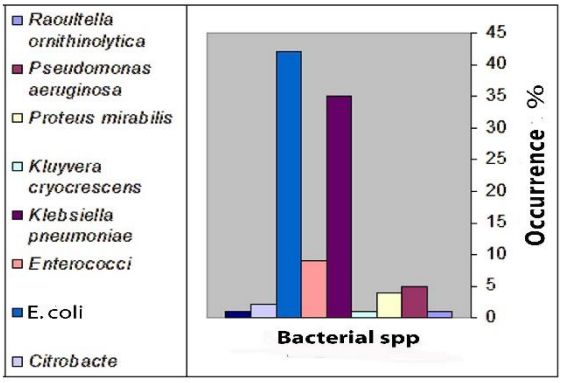

Figure 1. Bacterial distribution among studied cases.

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**).

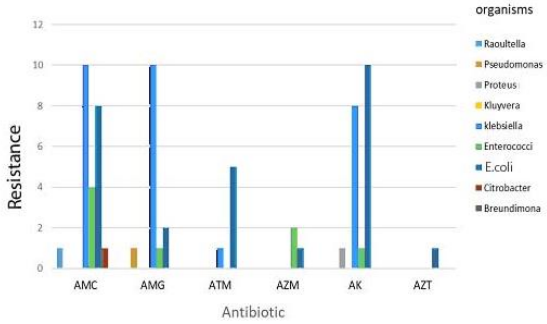

Figure (2): Antibiotic sensitivity among isolated bacterial species, AMC(Amoxicillin- clavulanic)-AMG(Amoxicillin)-ATM(Azetronam)-AK(Amikacin)-AZT(Azlocillin)- and AZM (Azithromycin**)**.

Also, *K. pneumoniae* and *E. coli* exhibited resistance to ceftazidime, cefpodoxime, and ciprofloxacin. Conversely, the ceftazidime antibiotic demonstrated greater efficacy against *Breundimona*, whereas cefpodoxime was

effective against *Pseudomonas* and *Breundimona* (**Figure 3**).

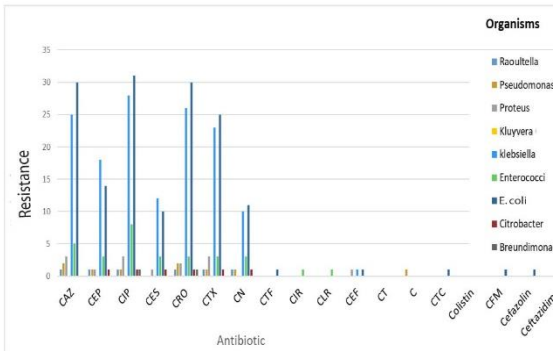

Figure (3). Antibiotic sensitivity among isolated bacterial species,CAZ (Ceftazidime),CEP (Cefpodoxime), CIP(Ciprofloxacin), CES (Cefoprazon), CRO(Cefotriaxon), CTX(Cefotaxime), CN(Cephaloxin), CTF (Cephalothin), CIR(Cefuroxime), CEF (Clarithromycin), CT(Ceftibuten), CT (Ceftolozan/tazobactam), C(Chloramphenicol), CTC(Chlortetracycline), and CFM(Cefixime).

K. pneumoniae and *E. coli* exhibited resistance to cefoxitin but were sensitive to gentamycin. Conversely, *Proteus* demonstrated greater sensitivity to cefoxitin, while *Enterococci* and *Pseudomona*s 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**).

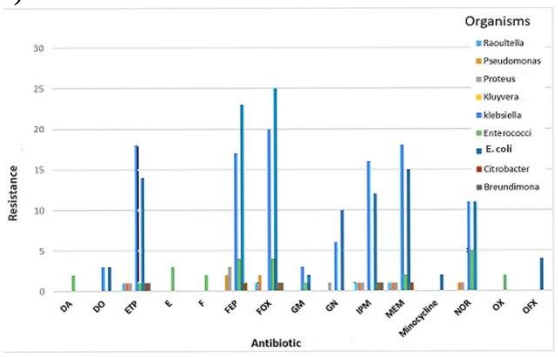

Figure (4) Antibiotic sensitivity among isolated bacterial species,DA (Clindamycin), DO (Doxacycline), ETP (Ertapenem), E (Erythromycin), F (Nitrofurantoin), FEP (Cefepime), FOX (Cefoxitin), GM (Gemifloxacin), GN (Gentamycin), IMP (Impeneme), MEM (Meropem), NOR (Norfloxacin), OX (Ofloxacin), and OFX (Ofloxacin).

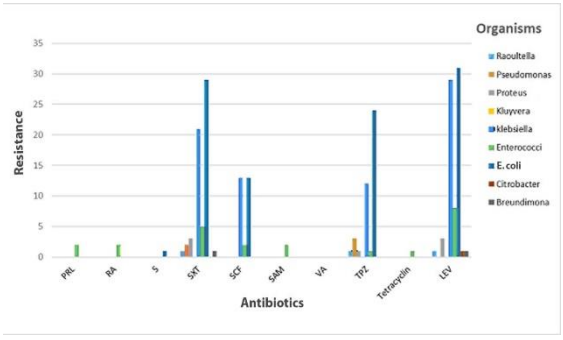

Figure (5) Antibiotic sensitivity among isolated bacterial species, PRL (Piperacillin), RA (Rifampin), S (Streptomycin), SXT (Trimethoprim-sulpham), SCF (Cefoprazon-sulbactam),SAM (Ampicillin-sulbactam),VA (Vancomycin), TPZ (Piperacillin-tazobactam), and LEV (Levofloxacin).

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**).

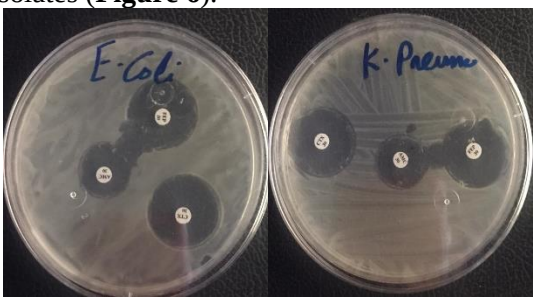

Figure (6): Double disk synergy test for E. coli and K. pneumoniae . AMC (Amoxycillin + Clavulanate), CTX (Cefotaxime), FEP(Cefepime) show clear extension of edge of the Cefepime inhibition zone towards the clavulanate).

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.

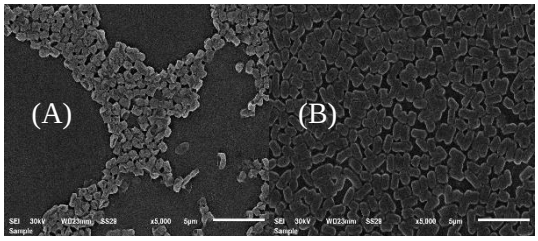

Figure (7): SEM images of the control isolate beta-lactamase producing *E. coli* (A) and (B) K.pneumoniae using cefoxitin.

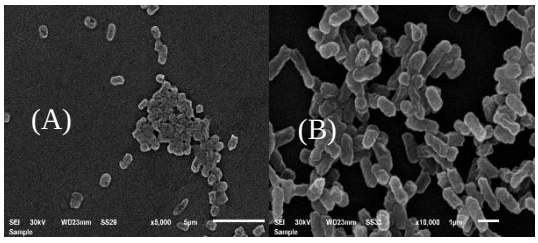

Figure 8. SEM images of the cefoxitin -treated isolates beta-lactamase -producing E. coli (A) and (B) K. pneumoniae using cefoxitin.

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**)

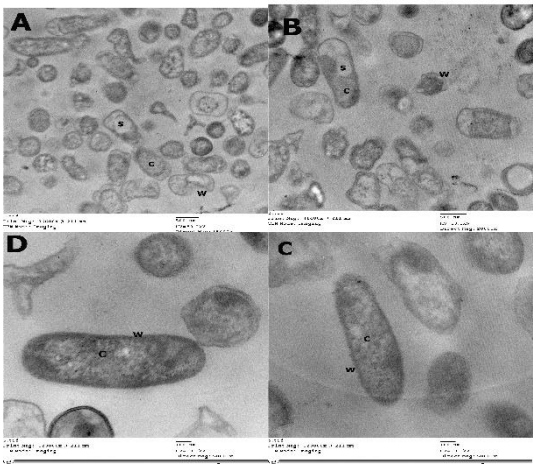

Figure (9): Transmission electron micrographs of isolate *E.coli*. (A and B) Cell subjected to Cefoxitine presence of large electron lucent space due to increased periplasmic space (s) and reduced cytoplasmic material (c) and ruptured cell wall(w).(C and D) Untreated bacterial cell preserved morphology,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

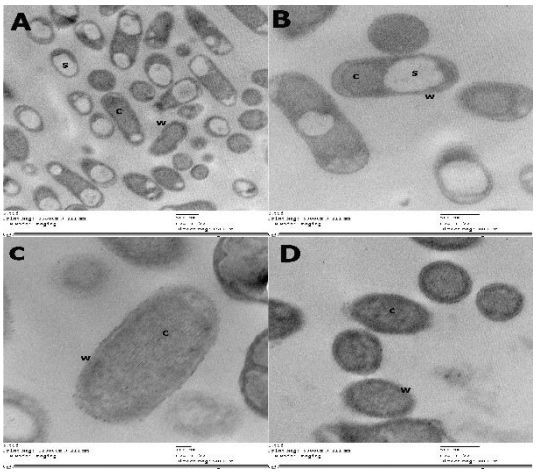

Figure (10): (A–D) Transmission electron micrographs of isolate *K. pneumoniae*. (A and B) Cell subjected to Cefoxitine presence of large electron lucent space due to increased periplasmic space (s) and reduced cytoplasmic material (c) and ruptured cell wall(w).(C and D) Untreated bacterial cell preserved 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 (**Ojkic *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 cephalexin, cefamandole, cefazolin, cephalothin, cefmetazole, cefoxitin, and cefradine, 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 filmization 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, 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.

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الملخص العربي

عنوان البحث: التغيرات الخارجية والمورفولوجية في العزلات السريرية إشريشيا كولاي وكلبسيلا نيومنيا الحاملة لإنزيم البيتالاكتاميز عند تعرضها للمضاد الحيوي البيتالاكتام

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اهتمت هذه الدراسة بفحص البكتيريا المعالجة المضاد الحيوي ببيتالاكتام و ذلك بحثًا عن التغييرات التي تطرأ علىها مورفولوجيتها وكذلك بنيتها التحتية الدقيقة، حيث اشتملت هذه الدراسة علي خصائص العزلات إكلينيكية والسريرية إشريشيا كولاي وكلبسيلا نيومنيا والتي تم جمعها من عينات البول وفحص مقاومتها للمضادات الحيوية لمرضى مركز الأورام بجامعة المنصورة ، وايضا فحص إنتاجها لإنزيم بيتا لاكتاميز من بين جميع العزلات ولقد كانت أكثر العزلات البكتيرية انتشارا هي الإشرشيا كولاى والكلبسيلا نيومنيا ، كما تم استخدام المجهر الإلكتروني الماسح والناقل لتوضيح تأثير أحد المضادات الحيوية للبيتا لاكتام على بكتريا إشرشيا كولاى والكلبسيلا نيومنيا بعد أن تم التعرف عليها كمنتجة لإنزيم البيتا لاكتاماز، تناولت الدراسة ايضا التعرف على المتغيرات التي تحدث للخلايا البكتيرية عند استخدام السيفوكسيتين و قد اظهرت النتائج أن عزلات إشرشيا كولاى والكلبسيل نيومنيا التى تحتوي على البيتالاكتاميز يمكن أن تخضع لتغييرات مورفولوجية وبنية تحتية للخلايا عند تعريضها للمضاد الحيوي بيتا لكتام ، مما يشير إلى أن هذا المضاد الحيوي له نشاط متبقي على الرغم من حدوث المقاومة المظهرية في هذه العزلات.