

Phenotypic detection of some virulence factors and antibiotics susceptibility of *Enterobacter cloacae* isolated from urinary tract infection

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Abstract : In our previous study, we detect (8) isolates of *Enterobacter cloacae* from UTI deepened on phenotypic and under molecular level. Investigation of some the virulence factors were also performed of all clinical isolates and the results showed all bacterial isolates 8(100%) produced adherence while the ability of isolates to produce capsule 6(75%), Bacteriocin 1(12.5%). In addition to that, the results of siderophore, hemolysin and protease tests were showed that 7(87.5%) of isolates were able to produce siderophores, and all isolates under study unable to produce hemolysin and protease Furthermore, the susceptibility of isolates to a variety of antibiotics has been investigated. It has been found that all isolates were sensitive to Ceftriaxon. The isolates were fully resist (100%) to Ampicillin, Cephalothin, Carbinicillin, Tetracyclin and Azethromycin, Cefotaxime (62.5%), Gentamycin (50%), Amikacin (25%). Low level of resistance (12.5%) was exhibited to Nitrofurantion, Ciprofloxacin, and Norfloxacin.

Keywords : *Enterobactercloacae*, hemolysin, antibiotics, sidrophore.

Introduction:

E. cloacae is also an important nosocomial pathogen responsible for bacteremia and lower respiratory tract, urinary tract and intra-abdominal infections, as well as endocarditis, septic arthritis, osteomyelitis and skin and soft tissue infections. The skin and the gastrointestinal tract are the most common sites through which *E. cloacae* can be contracted (1).*Enterobacter* spp.Can cause UTI after catheterization procedures because the catheter may carry microorganisms from the extremities of genitourinary tract causing a contamination of the inner tissues and resulting in cystitis or urethritis (2).*Enterobacter* infections can be acquired from either endogenous or exogenous sources. This is not surprising, given the ubiquitous nature of the organisms. Single source outbreaks have been traced, to contaminated intravenous solutions, blood products, endoscopes, hydrotherapy, distilled water, cotton swabs, stethoscopes, and devices used for monitoring intra-arterial pressure (3).Some strains of bacteria may have a capsule around the bacterial wall It is a polysaccharide layer that lies outside the cell envelope of bacteria, and is thus deemed part of the outer envelope of a bacterial cell. It is a well-organized layer, not easily washed off, and it can be the cause of various diseases. The capsule is considered a virulence factor because it enhances the ability of bacteria to cause disease (e.g. prevents phagocytosis). The capsule can protect cells from engulfment by eukaryotic cells, such as macrophages. A capsule-specific antibody may be required for phagocytosis to occur (4).Siderophores are small organic molecules produced by microorganisms under iron-limiting conditions which enhance the uptake of iron to the microorganisms. In environment, the ferric form of iron is insoluble and inaccessible at physiological pH (7.35-7.40). Under this condition, microorganisms synthesize siderophores which have high affinity for ferric iron.

These ferric iron-siderophore complexes are then transported to cytosol. In cytosol, the ferric iron gets reduced into ferrous iron and becomes accessible to microorganism. In recent times, siderophores have drawn much attention due to its potential roles in different fields. Siderophores have application in microbial ecology to enhance the growth of several uncultivable microorganisms and can alter the microbial communities (5). Clinical strains of *E. cloacae* produce one or more types of siderophores, certain strains of *E. cloacae* can produce enterochelin and hydroxamate compounds which defined as aerobactin and these two compounds represent siderophores (6). Hemolysin is extracellular toxic proteins, they cause destruction of red blood cells, with subsequent release of hemoglobin that can occur by any one of the numerous substance such as bacterial hemolysin that appear to aid the invasive power of bacteria (7). Hemolysin are active on erythrocytes from numerous mammalian species including humans. Binding of α -hly and activation of target cells was found to be independent of the cellular receptor that indicates how the toxin may lyse a variety of nucleated cells different animal species (8). Bacteriocin is a small molecule produced by bacteria, that inhibited closely related strains. It is usually a peptide or protein and is considered to be a narrow spectrum antibiotics, which are phenomenically analogous to yeast and paramecium killing factors, and are structurally, functionally and ecologically divers (9). Proteases refers to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or the properties of the active site (10). It is a well-known fact that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions (11). It has been hypothesized that flagella, organelles required for motility, facilitate the establishment and spread of infection by microbial pathogens within the host. Studies suggest that up to (95%) of urinary tract infections (UTIs) develop in an ascending manner beginning with periurethral colonization by bacteria such as uropathogenic bacteria, followed by migration to the bladder to establish infection, and if left untreated, ascension to the upper urinary tract or ureters and kidneys. Once in the kidneys, bacteria can gain access to the bloodstream, causing bacteremia and sometimes death (12). Antimicrobial agents effective against planktonic bacteria frequently fail to eradicate bacterial biofilms. The problem is that choosing of antibiotics is based on bacterial cultures derived from planktonic bacteria which differ in behavior and in phenotypic form from bacteria in biofilm. (13, 14).

Aims of the Study:

Our study aimed to investigated the phenotypic some virulence factors of *E. cloacae* and study of antibiotics profile for our isolates.

Material and methods:

Isolation of bacteria:

According to previous study of (15).

Hemolysin production:

Hemolysin production was carried out by Inoculating bacterial isolate on blood agar medium at (37°C) for (24-48) hours, An appearance of clear zone around the colonies referred to complete hemolysis (β -hemolysis), greenish zone around the colonies referred to partial hemolysis (α - hemolysis), while no clear or greenish zone referred to non-hemolysis (γ - hemolysis) (16).

Siderophores production test:

M₉ media was prepared and then supplement with (2%) agar. Then sterilization it by autoclave and cooled it to (50°C), Adding (0.25) mg/L glucose (sterilizing by filtration) and (200 μ /L) of dipyriddy. This media inoculating with the organisms and incubating it for (24) hrs. at (37°C). The result is reading as presence of organism's growth or not (17).

Extracellular protease production test:

M₉ media was used for the detection of protease enzyme. After sterilization in autoclave and cooling at (50°C), and (0.25) gm/L glucose (sterilized by filtration) was added, the medium was then supplemented by (1%) casein. Pores was made in agar medium and inoculation of this media with (20 μ l) from bacterial broth in

each pores and incubation at (37°C) for (24) hours; (3ml) of (3%) trichloroacetic acid was added to precipitate the protein. The formation of transparent area around the colony indicated a positive result (18).

Capsule production:

The solutions (India ink) were prepared according to the required microbiological methods: A smear slide is prepared from bacterial suspension on glass slide without fixing and is left to dry. Flood gently with (1%) of india ink and left for one minutes, allowed to dry in air, and examined under the microscope. The organism should be deep purple, and the capsule a faint blue against a light purple background (19).

Bacteriocin production:

The method of (20) had been used: A medial streak of the test strain by vertical line on tryptic soy agar, then incubated at (37°C) for (48) hours to allow bacteriocin to spread around growth line. At the second day, inoculating the indicator or sensitive strain on nutrient agar and incubated at (37°C) to the next day. On the third day, the petri-dish (plate) cover of the streaked plate covering by filter paper impregnated with chloroform in an upright position for (1/2) hour. Scraped the culture by sterilized glass slide into disinfecting vessel, then exposing the plate culture to chloroform vapors and then left the plate open for (1) hour to remove the chloroform. Inoculating the indicator or sensitive strain (grown on nutrient agar) by streaking it crossing the original streak line on TSA plate (which scraped), incubating overnight at (37°C). indicate the bacteriocin production as growth inhibition at the medial streak line.

Adherence Activity:

The ability of *E. cloacae* to adhere to epithelial cells is one of important virulence properties of these bacteria and detected as following steps; Prepare the brain heart infusion broth and incubated under anaerobic condition for (72) hrs. Prepare dilution of bacterial broth by use phosphate buffer (PBS) then take (1.5×10⁸) CFU/ ml. Take the urine epithelial cells by collect a sample of urine and centrifuge, the sediment then transferred to the sterile tube contain PBS (pH 7) after that wash the epithelial cells by PBS by using centrifuge (5000 rpm for 10 minutes) for three times. Filtered the PBS contain epithelial cells by filter paper, then place the epithelial cells on cover slide by press the cover on surface of filter paper then lifted to be dry. Place the cover slides on sterile glass plate then add (5ml) of previously prepared bacterial broth, then place the plate contains the epithelial cells and bacterial broth on incubator for 1hr at (37°C). Wash the cover slides by PBS to remove un adherent bacteria. Fixed the epithelial cells by ethanol for (15) minutes. Stain with Giemsa stain (30%) for (20) minutes then wash the cover slides by D.W and lifted to dry by air. Place the cover slides on glass slides by inverted position, and then tested under light microscope (21, 22, 23).

Antibiotic sensitivity test:

This test was performed by using a pure culture of previously identified bacterial isolates. the most effective antibiotic for each bacterial isolate was determined as recommended by (24).

Results and Discussion:

Investigation of some virulence factors associated with the pathogenicity of *E. cloacae*:

Hemolysin and Siderophore production:

In present study *E. cloacae* isolates were investigated for their ability to produce siderophores. The results show that 7/8(87%) isolates were able to produce siderophores. All isolates detected previously of their ability to produce hemolysin in blood agar and the results showed no hemolysis suggested that *E. cloacae* possess another way for iron acquisition. The results were showed in Table (1). These results are in agreement with (25) who found none of (57) *Enterobacter* spp. tested were hemolytic. (26) mentioned that *Enterobacter* spp. did not produce hemolysin on blood agar, but in another study done by (27) had pointed that clinical isolates of *E. cloacae* can produce low molecular weight hemolysin and this may be putative virulence factor in *E. cloacae* infections. In addition the first report of α -hemolysin production by *E. cloacae* by (28) showed that the hemolysin can tested by specific monoclonal antibody and by DNA-hybridization with an α -hemolysin specific gene prob. The ability of pathogenic microorganisms of obtaining iron from host is essential for its



Figure (1) Capsule production from *E. cloacae*



Figure (2) Ability of *E. cloacae* for adherence to the epithelial cells

Table (2) Bacteriocin production from bacterial isolates

Bacterial test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8
<i>E. coli</i>	-	-	+	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	+	-	-	-	-	-
<i>S. aureus</i>	-	-	+	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	+	-	-	-	-	-

Antibiotics Susceptibility test to *E. cloacae*:

These isolates showed different susceptibility towards antibiotics used in this study, as shown in Figure (3). It has been observed that most isolates are multi-resistant, the highest rate of resistance was seen with Tetracyclin, Ampicillin, Cephalothin, and Carbincillin (fully resist 100%). Whereas some isolates showed resistance in lesser degrees to Azathromycin (62.5%), Cefotaxim (62.5%), Gentamycin (50%), Amikacin (25%). Most of these isolates were sensitive to Ciprofloxacin, Norfloxacin and Nitrofurantoin at the ratio of (12.5%). The results in this study were in accordance with (35) who found that resistance to ampicillin and Cephalothin were (100%). Also the result are identical with those obtained by (36) who have pointed that *E. cloacae* produced chromosomally encoded beta-lactamases that mediated resistance to ampicillin and Cephalosporins. These results are in accordance with (37) who found that *Enterobacter* isolates were highly resist to Ampicillin (98.1%). (38) found the susceptibility of *Enterobacter* spp. To any beta-lactam was poor. (39) found that among *Enterobacter* strain isolated from acute uncomplicated cystitis the resistance to Ampicillin were (92.5%). All isolates in this study were resist to Cephalothin which is first generation Cephalosporins, this result were in agreement with (40) and (39) who have pointed that *E. cloacae* have resistance to Cephalothin. Regarding to Cefotaxime, the results of this study were in agreement with (41) who found that (63%) of *Enterobacter* isolates resist to Cefotaxime. (39) found that the resistance to Cefotaxime among *Enterobacter* reach to (70.8%). Generally, *E. cloacae* resistance to beta-lactam antibiotics is mediated by chromosomally beta-lactamase and also by reduced permeability of these antibiotics inside the cell by alteration in the outer membrane proteins (Porins) (42). The results show that all isolates were fully sensitive to

Ceftriaxone, for this reason Ceftriaxone considered drug of choice in UTIs now days. Antibiotic exposure remains one of the most important risk factors for the acquisition of antibiotic-resistant, Gram- negative microorganisms by hospitalized patients (43). Widespread use of antimicrobial therapy has often been held responsible for co- resistance to four or more unrelated families of antibiotics and the occurrence of multiply resistant strains in hospitals (44).Aminoglycosides have also been used which were including Gentamycin, Amikacin, the resistance to Gentamycin was (50%) while its resistance rate of *E. cloacae* to Amikacin was (25%) , These results are correlated with those of (45) who have showed the *E. cloacae* strains had partial resistance to gentamycin (58%) while its completely sensitive to Amikacin (100%), While (46, 47) had stated that the resistance rate of *E. cloacae* to Gentamycin was (33.3%) while to Amikacin it was (54%). The mechanism of *E. cloacae* resistance to aminoglycosides is mediated by the production of more than one type of aminoglycosidases located on the R plasmid.The results of this study was highest for Gentamycin when compared with (48) who found that, only (1.8%) of isolates were resist to Gentamycin. In contrast the result of this study were low when compared with result reported by (49) for Gentamycin (100%). On the other hand, The resistance rate to Ciprofloxacin and Norfloxacin were (12.5%), this results are identical to (50), who mentioned that the resistance rate of *E. cloacae* to Ciprofloxacin was (13%).This result is also identical with those of (51) who found that the susceptibility rate for ciprofloxacin was more than (85%) for *E. cloacae*. About the results of norfloxacin resistance, the results of this study with agreement with (41) in which Norfloxacin resistance was (7.4%). (15) stated that there were two mechanisms for fluoroquinolones resistance, the first one was efflux mechanism in which an activation of efflux pump that removes fluoroquinolones before intracellular concentration sufficient for inhibiting DNA metabolism can be achieved. The other mechanism (target alteration) included changes in DNA gyrase subunits decrease ability of fluoroquinolones to bind this enzyme and interfere with DNA processes.The same lower resistant expressed to Nitrofurantion was (12.5%), This result in agreement with some results such as those obtained by (52) who showed that *E. cloacae* isolates were more susceptible to Nitrofurantion therefore may still useful for treatment of cystitis and uncomplicated UTIs caused by this organism. Our result also similar to (53), who found that the resistance rate of *E. cloacae* to Nitrofurantion was (13%), while some authors decided that *E. cloacae* can be reported as resistant to Nitrofurantion without prior testing (54).Regarding to Azathromycin, the results of this study was (62%), the result of this study was similar to those performed by (41) who demonstrate that (65%) of isolates were resist to Azithromycin. The mechanism of *E. cloacae* resistance to macrolide is due to the presence of efflux pumps which is energy dependent mechanism, these pumps is multi drug systems mediated by transport proteins which confer resistance to toxic compounds (55).Furthermore, all *E. cloacae* isolates were resistant to Tetracycline (100%), this result was identical with those obtained by (56) who showed that *E. cloacae* have high resistance to this antibiotics. Tetracycline resistance can be mediated by efflux, ribosomal protection, or chemical modification, but the first two mechanisms were the most clinically significant (57). A variety of resistance determinants may encode these mechanisms. Depending on the species, the tet(A) to tet(E) efflux pump determinants were generally responsible for tetracycline resistance in Enterobacteriaceae (58). In Enterobacter spp. strain the most common efflux pump were tet (B), tet(C) and tet(D) (40).

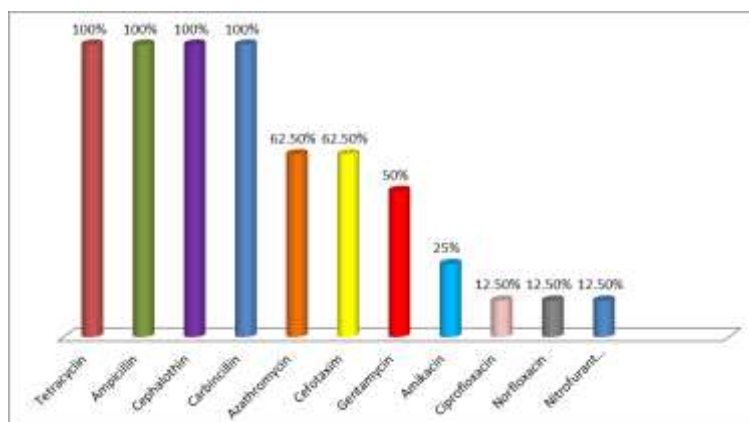


Figure (3) Antibiotics susceptibility by *E. cloacae*

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