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Research Article

Antibiotic resistance in *Enterobacter cloacae* from Anbar hospitals

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Abstract

This study aimed for isolation and Identification of the *E. cloacae* from clinical sources. The results showed that fifteen isolates out of 150 samples were all *E.cloacae* identified by morphological, microscopic, and biochemical tests and confirmed by Vitek II system. These results showed a correlation between OMPs (OmpA, OmpX, OmpF, Ompc) and antibiotic resistance in ECC clinical isolates. The OmpX gene was present in each isolate, while 12 isolates were positive for the OmpA gene, and ten isolates were positive for the OmpF, and OmpC genes. The isolates were β- lactam; cephalosporin resistance exhibited resistance to Cefoxitin, Ceftazidime, Cefazolin, Ceftriaxone, Cefepime, meropenem, Amoxicillin/clavulanic, Ertapenem, and Imipenem tested antibiotics accordingly could be categorized as MDR, These findings suggested the possible establishment of multi-drug resistance *Enterobacter* bacteria in clinical settings.

Keywords: *Enterobacter cloacae* complex, hospital-acquired infections, OMPs

Introduction

The bacterium known as *Enterobacter cloacae* is widespread throughout the natural world, where it coexists with humans as a commensal organism in their intestines and as a saprophyte in the environment (Mezzatesta et al.,2012). Infections caused by *E. cloacae* are most frequently brought on by healthcare-associated pathogens in immunocompromised patients, such as the elderly and those suffering from many medical conditions. *E. cloacae* has also emerged as a serious nosocomial pathogen in neonatal intensive care units over the past few years(Dalben et al.,2008).

Numerous innate and acquired resistance mechanisms to antibiotics necessary for treating human disease have been discovered in this organism, and selective factors push *E. cloacae* toward a multidrug-resistant phenotype (Conlan et al.,2014). Fluoroquinolones are frequently employed in the clinical therapy of persistent Enterobacteriaceae infections; nevertheless, The *E. cloacae* complex has evolved resistance to several different types of antibiotics, and this class of antibiotics is just one of them. These antibiotics operate on bacteria by interacting with type II topoisomerases (DNA gyrase and topoisomerase IV). The bacteria have evolved resistance mechanisms that either result in reduced access to the target itself by either decreasing permeability or increasing expression of efflux pumps, such as AcrAB and MexAB, or in target mutations, such as GyrA/GyrB for DNA gyrase and ParC/ParE for topoisomerase IV(Munita and Arias 2016; van Eijk et al. 2017).

OMPs have several different physiological roles in bacteria in addition to their duties as solute carriers. For example, OmpC and OmpF are responsible for the influx of antibiotics and other types of solutes. At the same time, OmpX inhibits the host's defensive mechanisms, and OmpA establishes a physical connection between the outer membrane and the peptidoglycan layer. While the expression of some porins, such as OmpA, remains constant in cells, other porins, including LamB, PhoE, and FhuA, are triggered by either the presence of a particular substrate or external stimuli(Masi et al. 2019). The aim of this research is study a correlation between OMPs (*OmpA, OmpX, OmpF, Ompc*) and antibiotic resistance in ECC clinical isolates hospitals in Anbar Governorate.

Martial and methods

in this study have collected about 150 samples from patients suffering from urinary tract infections, burns and wounds. The ages included in the study were between (15-64) years old for both sexes. The period of collection of pathological samples extended from April 2022 to January 2023. collected from Al-Fallujah and Al Ramadi Teaching Hospitals from hospitalized patients. Smear from the infectious area was taken by sterilized cotton swab, while urine samples from midstream urine in a clean container and then samples were inoculated on the culture media (MacConkey, chocolate and Blood agar) and incubated aerobically at 37°C for 24 hrs.

After the positive culture appeared. Then reward of Microscopic identification by using gram stain:. Biochemical Identification included the indole test, Methyl red test, Voges –Proskauer test

and Citrate utilization test (Hemraj et al. 2017). The diagnosis was confirmed by using the Vitek II system(Fritsche et al. 2011)**.**

Antibiotic susceptibility

The susceptibility test was performed by the automated Vitek-2 system using an AST card and also by using the disc diffusion method (Kirby-Bauer technique)**.** The FavorPrepTM / Cultured Cell Genomic DNA Extraction Mini Kit (Taiwan) is used to extract genomic DNA from *Enterobacter cloacae* Complex isolates. The thermocycler (Bioapplied cycler®, USA) was used to create and amplify the PCR reactions. The PCR program for detection of OmpX was initially denaturated 95°C for 3 min. 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45s, and extention 72°C for 60 s with a final elongation step at 72°C for 7 min. The PCR program for the detection of OmpA was initially denatured 95°C for 3 min. 35 cycles of denaturation 95°C for 45 s, annealing at 63°C for 45s, and extension 72°C for 45s with a final elongation step at 72°C for 7 min. The PCR program for detection of OmpF, OmpC was initial denaturation 95°C for 3 min. 40 cycles of denaturation 95°C for 30 s, annealing 61°C for 45s, and extention 72°C for 45s with a final elongation step at 72°C for 7 min. Following PCR amplification was confirmed using agarose gel electrophoresis. The criteria based on the extracted DNA were completely reliable for PCR.

Table 1. Reaction components of genes

Component	25µL (Final volume)
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	16.5 µl

Results and Discussion

In this study 150 samples were collected, as were as following fifty midstream urine samples and fifty burns swabs, fifty wound swabs. These samples were isolated showed that 120/150 (80%)

samples were positive bacterial cultures, while the remaining 30/150 (20%) showed no growth, as shown in Figure 1

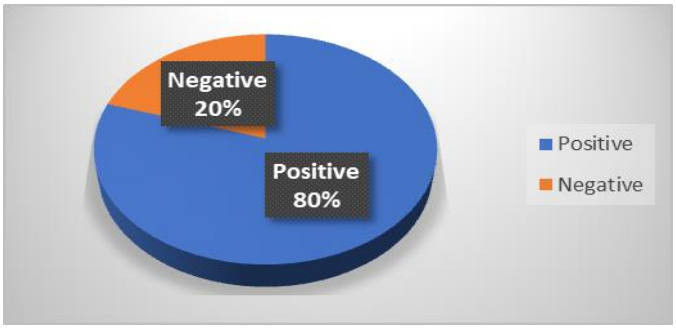

Figure 1. Distribution of positive and negative cultures of bacteria

Only fifteen (12.5%) of the positive culture samples were identified as Enterobacter and the remaining 105/120 (87.5%) contained isolates from other bacterial genera, as shown in Figure 2.

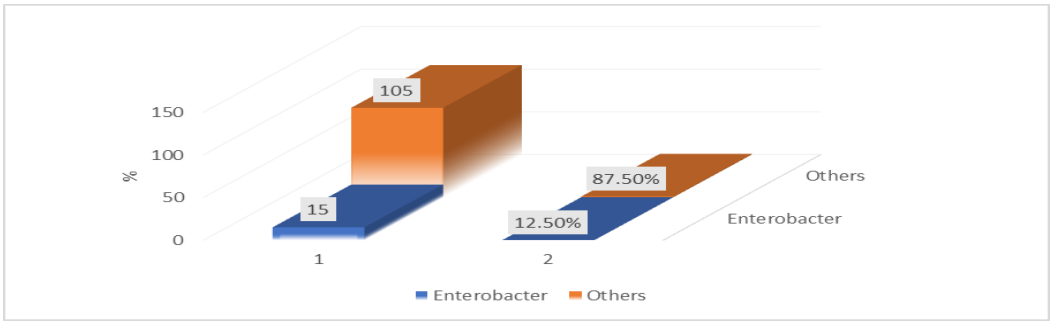

Figure 2. Bacterial isolates recovered from the samples

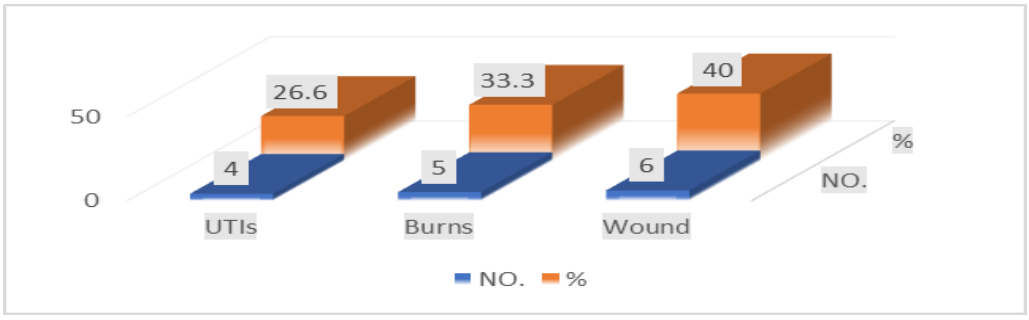

Figure 3. Distribution of isolated bacteria according to the source

According to Fig. 3, swabs from wound infection showed a higher percent of Enterobacter isolates 6/15(40%), while the UTI samples showed less distribution of bacteria 4/15(26.6%). To confirm the initial diagnosis, Enterobacter were first characterized by morphological, microscopical, and

being cultivated on MacConkey agar and Blood agar under aerobic conditions. When grown on the MacConkey agar medium, results showed that the colonies appear as pink to red, lactose-fermenting, slightly mucoid colonies similar in appearance to *Klebsiella pneumoniae* as illustrated. On Blood agar media they grow as non-hemolytic grey-white colonies after 24 hrs of incubation (Bayramoglu,2020). Also, several biochemical tests were done for further identification and characterization of *Enterobacter,* the results of biochemical tests and the result of Gram stain were summarized in Table 2.

Table 2. Biochemical tests for characterization of *Enterobacter cloacae* complex

Enterobacter	Indole	Methyl red	VP	Citrate	Urase
	-	-	+	+	+

(+) a positive result, (-) negative result, (VP) Voges –Proskauer test

Using Finegold and Marti, Bergey's Manual of Systemic Bacteriology and the biochemical processes (Tindall,2007) .were utilized to determine the type of bacteria. For extra confirmation, specific biochemical tests were carried out, including gram-negative rods. Fifteen specimens show Negative for indole, positive for citrate, positive for vp, and negative for methyl red and gram stain. The IMViC tests are a collection of unique examinations used to identify organisms in the microbiology lab and biotechnology department. *Enterobacter cloacae* complex (ECC) was identified successfully by using the VITEK 2 compact System GN cards (BioMerieux France) (Tindall, 2007).

Test for Antibiotic Susceptibility

The Vitek 2 Technology was used to verify the antibiotic sensitivity of all Fifteen *Enterobacter cloacae* complex (ECC) samples from patients with wound, burn, and urinary tract infections. Susceptibility was examined to seventeen antimicrobials including Piperacillin/Tazobactam, Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, Imipenem, Gentamicin, Ciprofloxacin, Levofloxacin, Tigecycline, Nitrofurantoin, Trimethoprim/ Sulfamethoxazole, Meropenem, Amoxicillin/clavulanic acid, and norfloxacin. The results of antimicrobial susceptibility test rates of the fifteenth ECC isolates revealed various resistance levels to antimicrobial agents as following: Piperacillin/Tazobactam 40%, Cefazolin 100%, Cefoxitin100%, Ceftazidime 100%, Ceftriaxone 100%, Cefepime 100%, Ertapenem 6.66%,

Imipenem 40%, Gentamicin 40%, Ciprofloxacin 33.3%, Levofloxacin 33.3%, Tigecycline 0%, Nitrofurantoin0%, Trimethoprim/ Sulfamethoxazole 66.6%, Meropenem 100%, Amoxicillin/clavulanic acid 100%, and norfloxacin 33.3% as showed in Figure 4.

The above findings showed that the isolates were resistant to every antibiotic

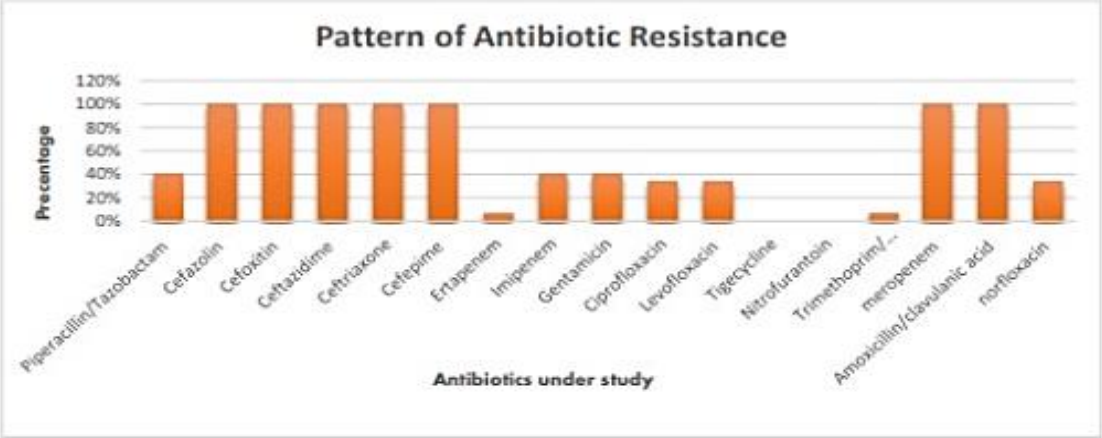

Figure 4. Pattern of Antiibiotic Resistance in Enterobacter cloacae complex

The current study showed that ECC possess the highest resistance (100%) toward the Cephalosporins class, which includes several generations like Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone and Cefepime, Fifty strains were resistant to ceftazidime, while 54 were resistant to ceftriaxone, according to a study conducted in Twain. There were three groups of 50 ceftazidime-resistant isolates: One was susceptible, two were immediately susceptible, and 47 were resistant to ceftriaxone. According to reports, third-generation cephalosporins were resistant to more than 70% of *Enterobacter* isolates in Poland; similarly, data from France and Guadeloupe showed that *E. hormaechei* had greater rates of resistance to third-generation cephalosporins when compared to other *Enterobacte*r clusters (Chang et al,.2022). In contrast, our results revealed that the resistance rate for Amoxicillin/clavulanic acid was 100%. Augmentin belongs to the penicillin-like antibiotics class of medicines. It works by preventing bacteria from growing. In Figure (4), ECC was 40% resistant to piperacillin/tazobactam and toward

Meropenem was 100% resistant, ECC was resistant to Imipenem at 40%, and less resistant to Ertapenem was 6.66%. These are intravenous-lactam antibiotics from the Carbapenems class, Carbapenem-resistant Enterobacteriaceae (CRE) have emerged as a significant global public health concern, and E. cloacae complex that was carbapenem-resistant was concentrated in the

Southwest and Pacific Coast by 2014-2015. It is challenging to choose an effective treatment for *Enterobacter* spp. because they have an inherent resistance to ampicillin and broad-spectrum cephalosporins and have acquired genetic mobile elements that make them resistant to many antibiotics, including third-generation cephalosporins and carbapenems(Pot et al.,2021). The result revealed that the resistance of ECC isolates toward the Quinolones class of antibiotics which include Ciprofloxacin, Levofloxacin and Norfloxacin was the same in percentage 33.3%, while no resistance or low MICs against ciprofloxacin and Levofloxacin found in a study conducted by (Wilson et al.,2017).

In Figure 4, Resistance of ECC isolates to Gentamicin was (40%). A similar tendency was observed in a prior study where their ECC clinical isolates showed approach results to Gentamicin by percentages (50%) (Davin-Regli et al.,2019). The current study also demonstrated that ECC resistance to Trimethoprim/ Sulfamethoxazole was 66.6%. Similar findings were observed in Iran, where clinical ECC resistance to Trimethoprim/ Sulfamethoxazole was 60.4% (Esteban-Cuesta et al.,2019).It is worth mentioning that in this study all our ECC isolates were susceptible to the Tigecycline and Nitrofurantoin, which can be considered the most effective agents against ECC under study. When treating ECC infection, antibiotics should be given in a systematic, nonrandom manner. Multi-drug resistant (MDR) ECC strains, which constitute 65 to 75 percent of Enterobacter infections, have emerged and spread as a result of antibiotic use (Hariharan et al., 2019).

Detection of OmpX gene

By employing a particular primer that focuses on the precise sequence of the target gene, the OmpX gene was detected using the conventional PCR approach for all of the isolates currently under study. Then, 1.5% agarose gel was used to place the amplified products For 1.5 hours afterwards. Results showed that 15/15 (100%) of the isolates had this gene, and the bands for all positive isolates were within the range of the gene's predicted size (200 bp)—figure 5

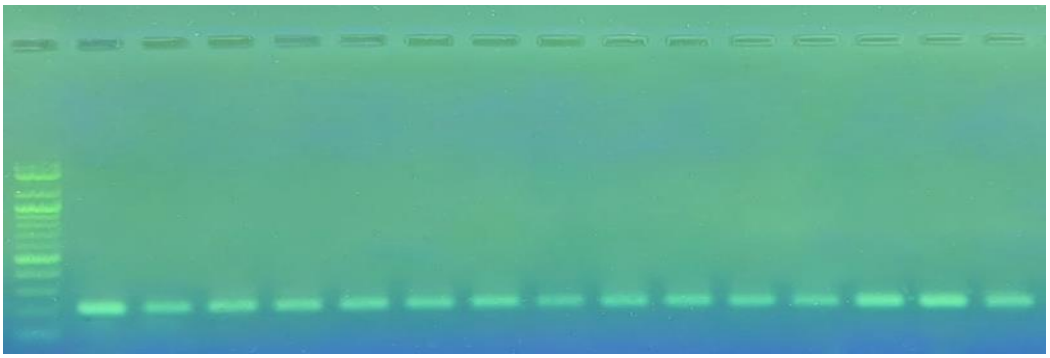

Figure 5. Traditional PCR amplification fragments (200 bp) for OmpX gene identification. Lanes 1–15 contain Enterobacter cloacae, a 100-bp DNA ladder, and negative control (NC). After 1.5 hours of electrophoresis on agarose gel (1.5%) at 70 V/cm, amplicons were examined using a UV transilluminator documentation system.

Detection of ompA gene

By employing a particular primer that focuses on the precise sequence of the target gene, the OmpA gene was detected using the conventional PCR approach for all of the isolates currently under study. Then, 1.5% agarose gel was used to place the amplified products For 1.5 hours afterwards. Results showed that 12/15 (80%) of the isolates had this gene, and the bands for all positive isolates were within the range of the gene's predicted size (396 bp)—figure 6.

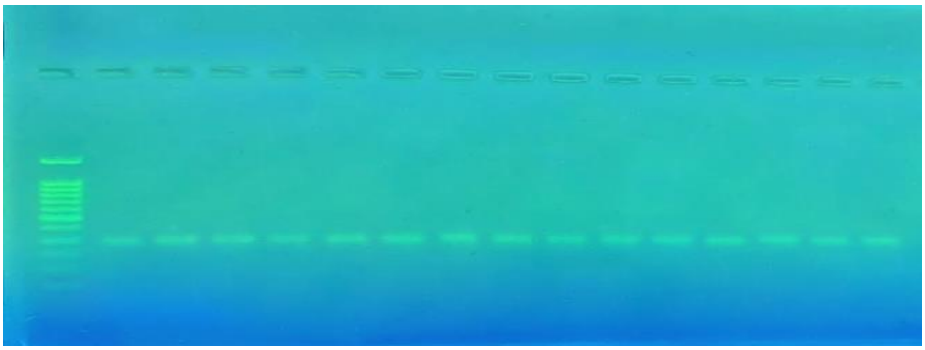

Figure 6. Traditional PCR amplification fragments (396 bp) for OmpA gene identification. Lanes 1–15 contain Enterobacter cloacae, a 100-bp DNA ladder, and negative control (NC). After 1.5 hours of electrophoresis on agarose gel (1.5%) at 70 V/cm, amplicons were examined using a UV transilluminator documentation system.

Detection of OmpF gene

By employing a particular primer that focuses on the precise sequence of the target gene, the OmpF gene was detected using the conventional PCR approach for all of the isolates currently under study. Then, 1.5% agarose gel was used to place the amplified products For 1.5 hours

afterwards. Results showed that 10/15 (66.6%) of the isolates had this gene, and the bands for all positive isolates were within the range of the gene's predicted size (285 bp)—figure 7.

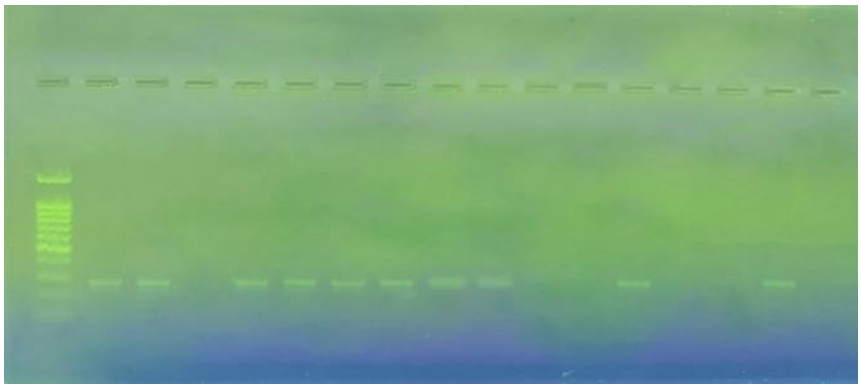

Figure 7. Traditional PCR amplification fragments (285 bp) for *OmpF* gene identification. Lanes 1–15 contain *Enterobacter cloacae*, a 100-bp DNA ladder, and negative control (NC). After 1.5 hours of electrophoresis on agarose gel (1.5%) at 70 V/cm, amplicons were examined using a UV transilluminator documentation system.

Detection of ompC gene

The OmpC gene was detected using the conventional PCR approach for all of the isolates currently under study by employing a particular primer that targets the focus on the sequence of the target gene. Then, 1.5% agarose gel was used to place the amplified products For 1.5 hours afterwards. Results showed that 10/15 (66.6%) of the isolates had this gene, and the bands for all positive isolates were within the range of the gene's predicted size (515 bp)—figure 8.

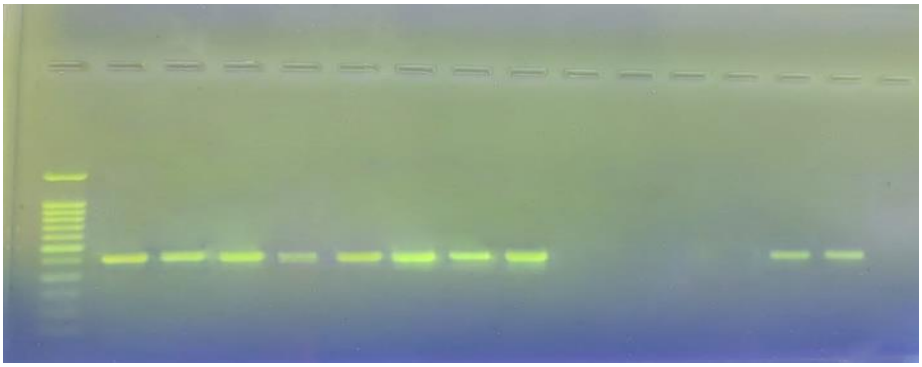

Figure 8. Traditional PCR amplification fragments (515 bp) for OmpC gene identification. Lanes 1–15 contain Enterobacter cloacae, a 100-bp DNA ladder, and negative control (NC). After 1.5 hours of electrophoresis on agarose gel (1.5%) at 70 V/cm, amplicons were examined using a UV transilluminator documentation system.

This study, which was conducted for the first time in Al-Anbar Governorate, revealed the detection of *Enterobacter cloacea* Complex genes. The results showed that OmpX gene was present in all

fifteen ECC isolates. Overproduction of OmpX can lead to antibiotic resistance, and the presence of this protein may indicate pathogenic potential (Arabi et al.,2017).The OmpA gene present in 12/15 ECC isolatese . In Gram-negative bacteria, OmpA porins are extremely prevalent OMPs that play a variety of pathogenic activities, including adhesion, invasion, biofilm, serum resistance, evading host defences, and antimicrobial resistance (Liu et al.,2021). By comparing our result with another previous study, most isolates (n = 13) were positive for the OmpA gene(Liu et al.,2021). The results showed that *OmpC and OmpF* genes were present in the same number and percentage, 10/15 75%; a previous study showed the different percentages of distribution of OmpC and OmpF genes(Pompilio et al.,2021). While in another study found a low expression level of OmpC and OmpF(Mishra et al.,2020). The OmpC and OmpF are responsible for the influx of antibiotics and other solutes. As for the five samples low expression was observed of OmpF,OmpC.By comparing our results with a previous study to detect carbapenemase and AmpC β-lactamase Most isolates showed decreased expression of ompC and/or ompF, and contained a broad distribution of ESBLs and AmpC β-lactamases (Mishra et al.,2020; Majewski et al.,2016; Cai et al.,2019).

Conclusion

This thesis sheds light on the *Enterobacter cloacae* complex in particular. It showed its resistance to antibiotics and the possibility of establishing multidrug resistance, which makes it a severe nosocomial infection in intensive care units. This study also revealed outer membrane proteins. Where it detected *OmpX*, *OmpA*, *OmpF* and *OmpC* genes, OmpX might imply that E. cloacae play a part in bacterial pathogenicity in addition to the role of other genes' adhesion, invasion, biofilm formation, and antimicrobial resistance.

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