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Prevalence of *Pseudomonas fluorescens* Producing Extended-Spectrum β-lactamases Genes from Sepsis Patients in Al-Najaf-Iraq

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ABSTRACT— Resistance to numerous antibiotics has emerged as a result of the development of extended-spectrum β-lactamases (ESBLs) generated in *Pseudomonas fluorescens*. As a result, failing to diagnose ESBL strains properly can result in treatment failure. The goal of this research was to look at the development of ESBLs in *P. fluorescens* isolated from sepsis blood patients in Al-Najaf. Sensitivity testing demonstrated that 32 (100%) of the *P. fluorescens* isolates were resistant to at least one of the β-lactam antibiotic. Of 32 isolates, 22 (68.6%) isolates were bearing bla_{TEM} gene and 19 (59.4%) isolates were bearing bla_{SHV} gene, of these isolates, 19 isolates carried the two genes together, but only 16 (50%) and 13 (40.6%) had $bla_{CTX\text{-}M}$ and bla_{OXA} genes, respectively. The high rate of ESBL genes found in *Pseudomonas fluorescens* isolates and these instances is concerning, according to the current investigation.

KEYWORDS: *P. fluorescens*, ESBLs, bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M}$.

1. INTRODUCTION

Pseudomonas fluorescens is infrequent causes of soft tissue and skin infections (SSTIs). It's a gram-negative bacillus that's linked to *P. aeruginosa* additionally can be present in the environment. There are some case reports of bacteremia in the literature, although pathogenicity is less and death is uncommon. The preponderance of patients who are impacted by the aforementioned is had comorbidities that cause a weakened immune response or are immunocompromised patients [28].

Interventions targeted at reducing the risk of ESBL acquisition, difficult treatment owing to multi-resistance, and the lack of advanced diagnostic laboratories all contribute to a reduction in the quality of healthcare available to battle ESBLs. As a consequent, *P. fluorescens* have maintained one of the most common causes of community-acquired infections and nosocomial, and have been the subject of several clinical guidelines and research investigations [15].

ESBL enzymes are plasmid-based enzymes that destroy and inactivate β-lactam antibiotics such as penicillin, third-generation cephalosporins, and aztreonam. These enzymes are formed as a result of genetic alterations in bacteria, which occur most commonly as a result of the misuse or extensive utilize of β-lactam antibiotics to infections treatment in humans and animals [7], [16].

The most commonly founded ESBLs belong to the CTX-M, SHV, OXA and TEM families. These enzymes producers are usually multiplied drug-resistant [20]. The phenotypic and genetic studies on the presence of ESBLs possessed by these bacteria in Iraq is limited compared to other countries, therefore, the aim of current research is to know the emergence of the four main ESBLs genes in *P. fluorescens* isolated from human blood sepsis in Al-Najaf/ Iraq.

2. Methods

2.1 Specimen Collection and Bacterial Isolation

Blood samples were collected from patients attended consultation clinics in Al-Najaf hospitals who suffer from sepsis. Each blood specimen (2-3 ml) was added to 10-15 ml of brain heart infusion (BHI) broth. The bottle of BHI broth was incubated at 37°C for 5-7 days. Each BHI was sub-cultured on blood agar and then incubated at 37°C for 24 hr to give microorganism more chance to grow [1].

A loopful from colonies of blood agar colony was sub-cultured on the selective media, MacConkey agar additionally blood agar, and incubated at 37˚C under the aerobic condition for 24 hr.

2.2 Identification of P. fluorescens

The identification of P. fluorescens from blood samples was done with the gram stain, IMViC tests and then were confirmed by using Vitek 2 compact system [3], Christner et al., 2010).

2.2.1 Antibiotic Profile of P. fluorescens Isolates

The disk diffusion method, recommended by the Clinical and Laboratory Standards [10], was used to determine the susceptibility of all *P. fluorescens* isolates to β-lactam antibiotics including the classes penicillins (ampicillin, ticarcillin additionally piperacillin), cephems (cefotaxime, ceftazidime, cefoxitin, ceftriaxone, cefepime, and cefixime), monobactams (aztreonam) and β-lactam/β-lactamase inhibitor combinations (piperacillin-tazobactam additionally amoxicillin-clavulanic acid) (Cyprus Company, Belgium and Bioanalyse Company, Turkey).

Overnight-grown cultures of each isolate in Luria–Bertani broth were prepared. The turbidity of the broth was checked to 0.5 McFarland standard tube. Each isolate was spread on the surface of Mueller-Hinton (MH) agar by sterile cotton swab and after 15 minutes, antibiotic discs were placed on plates (the distance between discs was 15 mm at least), then incubated the plates at 37°C for 18-20 hrs. The zone diameter around each disc was measured and compared with CLSI guidelines to interpret results [10].

2.2.2 Primers oligonucleotide design

The oligonucleotide sequences of bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ genes were obtained from [4]. bla_{OXA} gene was obtained from [12]. As in the following sequences of the bla_{TEM} gene F- TTTCGTGTCGCCCTTATTCC, R- ATCGTTGTCAGAAGTAAGTTGG with **product size 403 bp;** bla_{SHV} gene F- CGCCTGTGTATTATCTCCCT, R- CGAGTAGTCCACCAGATCCT with **product size 293 bp**; $bla_{CTX\text{-}M}$ gene F- CGCTGTTGTTAGGAAGTGTG, R- GGCTGGGTGAAGTAAGTGAC with product size 754bp finally bla_{OXA} gene F- ACCAGATTCAACTTTCAA and R- TCTTGGCTTTTATGCTTG with size amplicon 598bp.

2.2.3 DNA extraction

P. fluorescens DNA was extracted with a Promega Wizard Genomic DNA Purification Kit and used as the template from which to amplify the β-lactamase genes. These templates were stored in a sterile Eppendorf tube at -20°C.

2.2.4 Amplification of β-lactamase genes by PCR

To analyze the β-lactam resistance determinants, the, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M}$ and bla_{TEM} genes were amplified by PCR technique. PCRs were accomplished in total volumes of 25µl including 2.5 µl of each primer, 5 µl of template DNA, 12.5 µl of Taq Master Mix (Bioneer AccuPower Gold PreMix, Korea), and

2.5 µl PCR grade water. Thermocycler Biosystem was adjusted the initial denaturation at 94˚C for 5 min, 35 cycles of 30 sec at 94˚C, 30 sec at 60˚C, and 50 sec at 72˚C, the final extension at 72˚C for 5 min.

The PCR products and DNA-size ladder (GeneDireX INC, USA) were analyzed with electrophoresis on 1% agarose gels. Electrophoresis was accomplished at 60-70 V for 90 minutes. The bands of PCR products and ladder were visualized with ultraviolet light to determine the target product size according to the ladder.

3. Results and Discussion

3.1 Isolation and Identification of P. fluorescens

During the collection period, a sum of 200 samples were gathered from the patients with suspected sepsis from the main hospitals in Al-Najaf Al-Ashraf. After observing the culture and morphological characteristics of bacterial isolates and performing the classical IMViC tests, then confirmed by the Vitek 2 compact system. A total of 32 (16%) isolates had been identified as *P. fluorescens*.

The gram-negative bacillus bacteria *P. fluorescens* can be found in soil and water. It's been identified as an opportunistic human pathogen that can cause nosocomial infection, particularly in immunocompromised people. Because of their rarity and low pathogenicity rate, clinical data on these organisms is scarce [14]. Three previous case reports of *P. fluorescens* infections linked to immunocompromised oncology and contaminated infusions patient were found by [19]. Although bacteremia, shock, and mortality of *P. fluorescens* are uncommon, risk factors for all patients should be recognized. The most frequents cause of infections with these isolates is catheter-related bloodstream infections, immunosuppression, or contaminated blood product infusions [31].

3.2 Antimicrobial Susceptibility of P. fluorescens Isolates

All of the 22 *P. fluorescens* isolates were evaluated for susceptibility to 12 different antibiotics belonged to β-lactam classes. A summary of susceptibility rates (according to [10] guidelines as resistant, intermediate resistant, and susceptible) for all antibiotics against *P. fluorescens* is given in the following table.

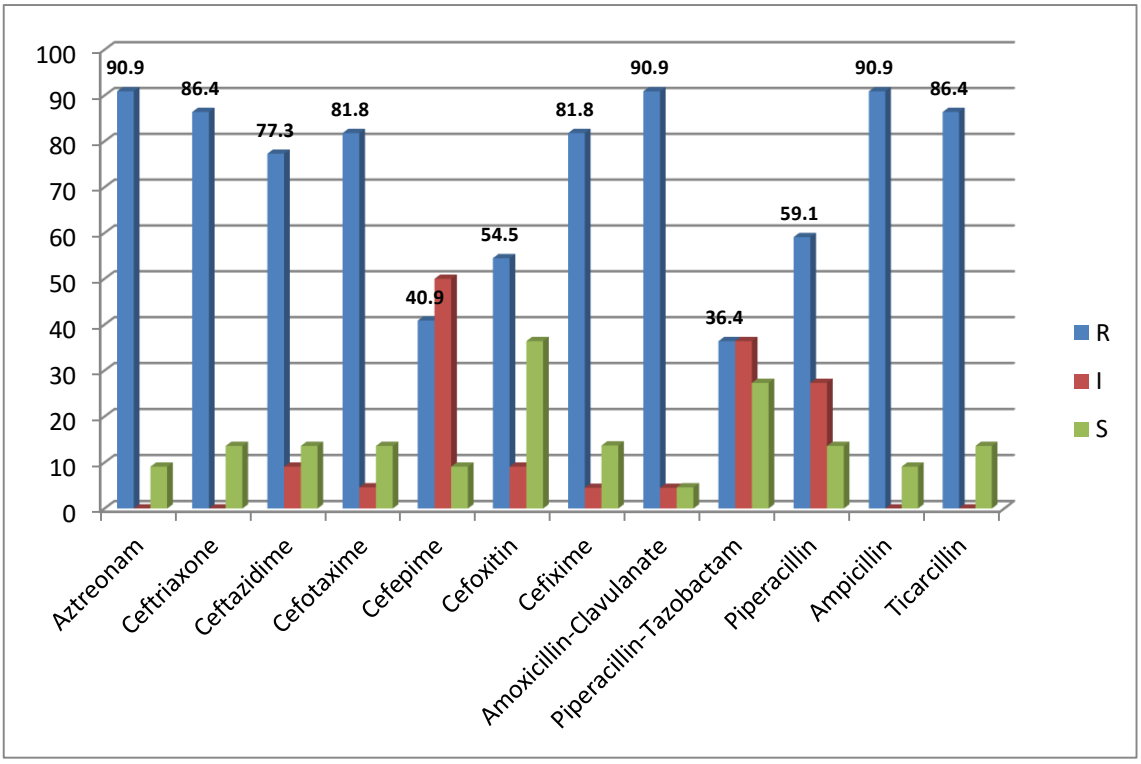

Figure (3): Antimicrobials Profile of *P. fluorescens* Isolates From Sepsis Infections

Patients with shock, and bacteremia should be treated with comprehensive antibiotic coverage for gram-negative, gram-positive, and gas-producing bacteria at first, and then deescalate when cultures and sensitivities improve. Patients with the positive results of cultures for *P. fluorescens* and *P. putida* were studied by von Graevenitz *et al*., who discovered that both species were sensitive to tetracycline, colistin, piperacillin/tazobactam, higher generation cephalosporins, aminoglycosides, carbapenems, and polymyxin. However, research have revealed that multidrug resistant carbapenem species exist [14]. Aggressive source control, in addition to antibiotics, was discovered to be important for successful therapy in these individuals [29].

This is due to the ability of bacterial β-lactamases to hydrolyze β-lactam antibiotics, rendering them ineffectual as therapeutic agents. The bacterium will eventually develop resistance to a diversity of β-lactam antibiotics, including cephalosporins, monobactams, and carbapenems. ESBL-producing organisms will have potentially enormous implications unless and until early screening is initiated, considering treatment failure, laboratory diagnosis, and infection control concerns [8]. The most common antibiotics utilized to cure infections caused by ESBL-PE are β-lactam antibiotics such as extended-spectrum penicillins, carbapenems, monobactams, and cephalosporins. Despite the fact that β-lactam antibiotics are the high frequently given antibiotics by many clinicians, ESBL-PE continues to cause a number of hospital and community-acquired illnesses around the world. ESBL-PE is now accountable for a number of spread of disease, providing difficult infection control difficulties and negatively impacting many clinical outcomes [27].

3.3 ESBLs Genes (bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M})

The 32 (100%) *P. fluorescens* isolates appeared resistant to at least one agent of penicillins, monobactams, cephems and β-lactam/β-lactamase inhibitor combinations were chosen for further studies. These isolates were indicated to reveal the existence of ESBLs genes by detecting the bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M}

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genes.

The findings of current research indicated that 22/32 (68.8%) isolates yielded amplification products with the *bla*$_{TEM}$ gene (Figure 2) and 19/32 (59.4%) isolates yielded amplification products with *bla*$_{SHV}$ gene (Figure 3). While *bla*$_{CTX\text{-}M}$ and *bla*$_{OXA}$ genes were 16/32 (50%) and 13/32 (40.6%) detected in resistant *P. fluorescens* isolates (Figure 4 and Figure 5, respectively).

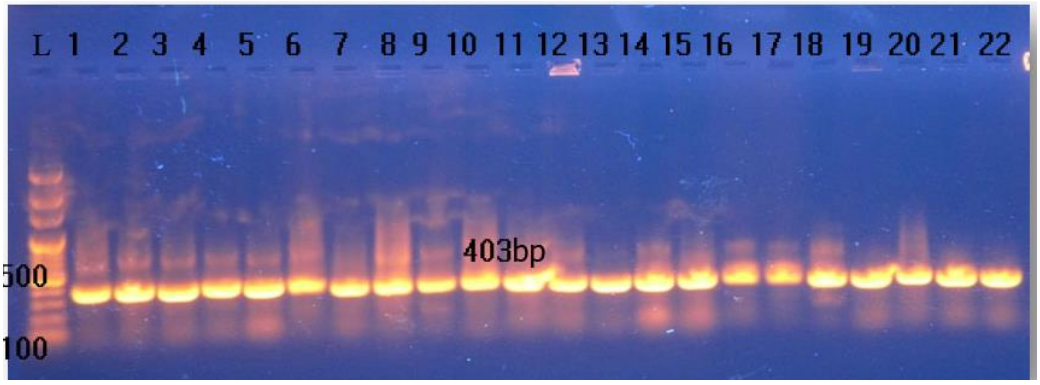

Figure (2): Ethidium bromide-stained 1% agarose gel of monoplex PCR amplified products from extracted DNA of *P. fluorescens* isolates and amplified with *bla*$_{TEM}$ genes primers. The electrophoresis was performed at 70 volt for 1:30 hr. Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (1-22) show positive results with *bla*$_{TEM}$ gene.

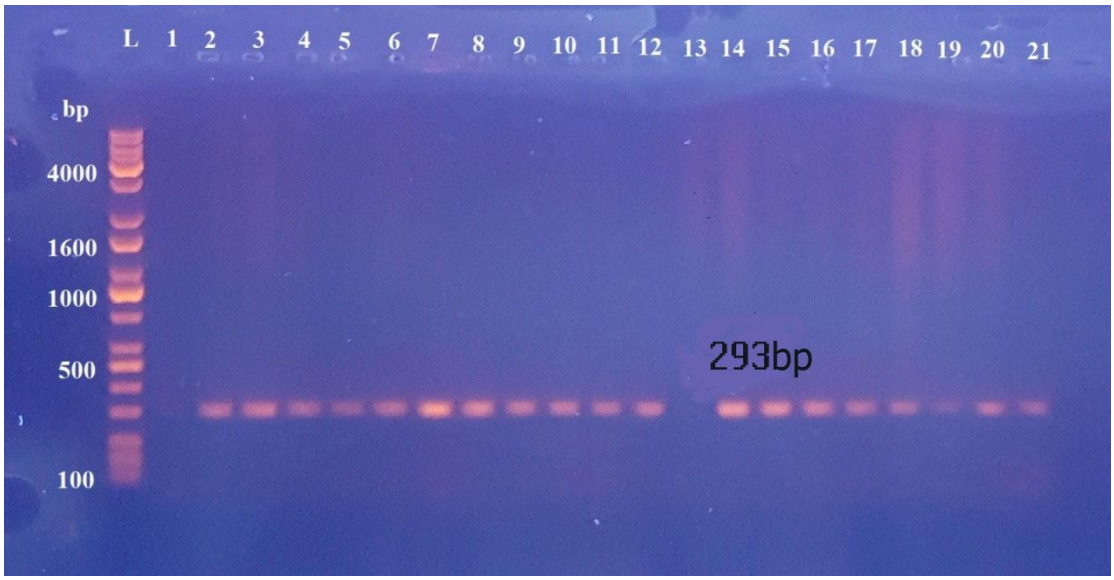

Figure (3): Ethidium bromide-stained 1% agarose gel of monoplex PCR amplified products from extracted DNA of *P. fluorescens* isolates and amplified with *bla*$_{SHV}$ genes primers. The electrophoresis was performed at 70 volt for 1:30 hr. Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (2-12 and 14-21) show positive results with *bla*$_{SHV}$ gene.
Lanes (1, 13 and 22) show negative results with *bla*$_{SHV}$ gene.

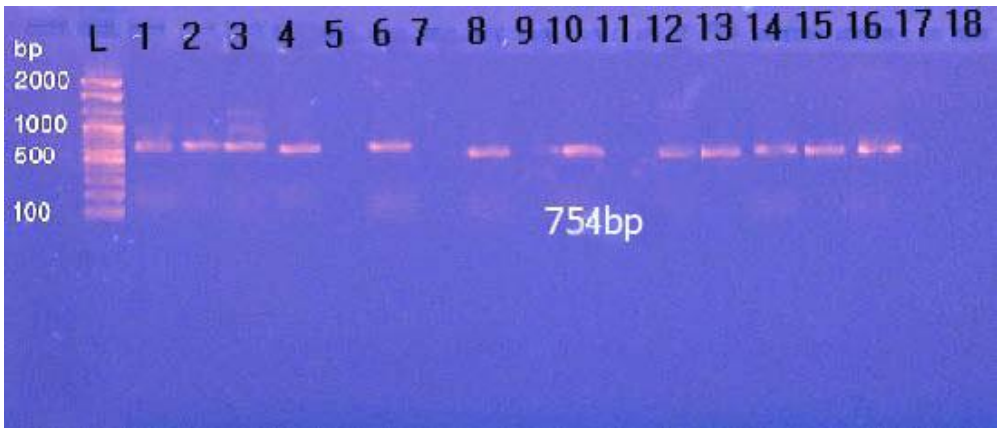

Figure (4): Ethidium bromide-stained 1% agarose gel of monoplex PCR amplified products from extracted DNA of *P. fluorescens* isolates and amplified with $bla_{CTX\text{-}M}$ genes primers. The electrophoresis was performed at 70 volt for 1:30 hr. Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (1-4, 6, 8, 10 and 12-16) show positive results with $bla_{CTX\text{-}M}$ gene.
Lanes (5, 7, 9,11, 17 and 18) show negative results with $bla_{CTX\text{-}M}$ gene.

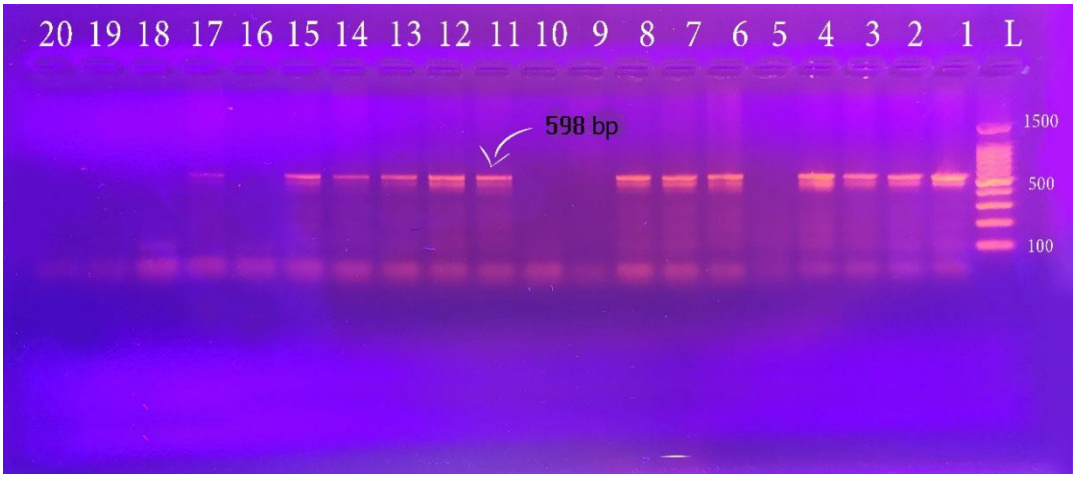

Figure (5): Ethidium bromide-stained 1% agarose gel of monoplex PCR amplified products from extracted DNA of *P. fluorescens* isolates and amplified with bla_{OXA} genes primers. The electrophoresis was performed at 70 volt for 1:30 hr. Lane (L), DNA molecular size marker (100 bp GeneDireX INC ladder), Lanes (47, 88, 36, 31, 28, 50, 49 and 45) show positive results with bla_{OXA} gene.
Lanes (21, 11 and 37) show negative results with bla_{OXA} gene.

The emergence of *P. fluorescens* β-lactam resistant strains was of great concern particularly after appearing extended-spectrum β-lactamases (ESBLs).

ESBL-generation pathogens, primarily Gram-negative bacteria, are offering significant problems to those working in global health to diagnose, treat, prevent, and control infections, as well as create a new antimicrobial drug to combat the devastating effects of AMR [17].

$bla_{CTX\text{-}M}$ is considered as predominant gene [24]. So, current research attempts to discover the existence of β-lactam resistant genes like bla_{TEM}, bla_{SHV}, bla_{OXA} and $bla_{CTX\text{-}M}$ (belong to ESBLs).

In this study, out of *P. fluorescens* isolates, 22 (68.8%) isolates carried bla_{TEM} genes. The present of bla_{SHV} gene is 19 (59.4%) of *P. fluorescens* isolates.

bla$_{TEM}$ is considered one of the plasmid-mediated β-lactamases which are found in the Enterobacteriaceae family [18]. In a study conducted by who former, *bla*$_{TEM}$ gene was widely distributed in both *Escherichia coli* and *Salmonella* spp. isolates [30].

The incidence of *bla*$_{CTX-M}$ gene group in the bacteria that confers resistance against ceftriaxone [2], [11], [21]. The present research revealed that the *bla*$_{CTX-M}$ gene was the high fequence16 (50%). Additionally, the existence of several *bla* genes is frequently reported all throughout the world [25]. TEM-1, the most common *bla* expressed enzyme found in human clinical isolates around the world [9], [25], is not an ESBL. Some TEM-1 derivatives, on the other hand, have ESBL features. Various researchers have reported the predominance of *bla*$_{CTX-M}$ genes in combination with the *bla*$_{TEM}$ gene [26].

CTX-M may be linked to the widespread utilize of 3th-generation cephalosporins, particularly cefotaxime, and ceftriaxone, or to a high mobilization of the encoding genes [5]. The *bla*$_{CTX-M}$ genes have been deployed to plasmid about 10 times most common than other Class A β-lactamases, according to [6].

Gram-negative bacteria produce β-lactamases as a key resistance mechanism to overcome antibiotics derived from penicillin, and bla$_{TEM}$ and bla$_{CTX-M}$ ESBLs can hydrolyze cephalosporins (the 3th and 4th generation) [32].

Although Gram-negative bacteria are acquired OXA enzymes (oxacillinases) generally, nevertheless the current investigation revealed the 13 (59.1%) of a *bla*$_{OXA}$ gene particularly. These enzymes are resistant to cefepime [13] and embedded into gene cassettes of class 1 integrons [23], which are present among isolates. Resistance to carboxy- and amino-penicillins, as well as narrow-spectrum cephalosporins, is conferred by Class D oxacillinases [22].

4. Conclusion

The results of this investigation indicated that 68.8% and 59.4% of isolates carried *bla*$_{TEM}$ and *bla*$_{SHV}$ genes, respectively while 50%, 40.6% of isolates carried the *bla*$_{CTX}$-M and *bla*$_{CTX}$-M genes respectively and these cases are considered alarming.

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