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Prevalence of β-lactamase produced in *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from gingivitis in Al-Najaf Province, Iraq

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Abstract. *Motaweq ZY. 2022. Prevalence of β-lactamase produced in* Klebsiella pneumoniae *and* Enterobacter cloacae *isolated from gingivitis in Al-Najaf Province, Iraq. Nusantara Bioscience 14: 78-83.* This study provides phenotypic and genotypic β-lactamase formation data on 26 isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from patients with gingivitis checked at Al-Kafeel clinic and private clinic in Al-Najaf Province-Iraq during the period from September 2020 to February 2021. In this study, some were detected by traditional phenotypic methods, while others were detected by phenotypic and then genotypically by using the monoplex-PCR technique. The results revealed that out of 14 β-lactam resistance *K. pneumoniae* isolates, eight isolates (57.1%) gave positive results with the direct capillary tubes method, while 12 β-lactam resistance *E. cloacae* gave 6 (50%) positive results. This result indicated that enzymatic resistance was prevalent among isolates. Furthermore, the results showed that most isolates were ESBL producers according to initial and confirmatory methods. Molecular amplification of the β-lactamase enzyme *bla*$_{SHV}$ gene was detected in 8 (57.1%) and 5 (41.6%) for *K. pneumoniae* and *E. cloacae*, respectively. While all 100% of *K. pneumoniae* and *E. cloacae* isolates gave negative results for the *bla*$_{GES}$ gene. This study aimed to investigate the β-lactamase formation and detection of *bla*$_{SHV}$ and *bla*$_{GES}$ genes in *K. pneumoniae* and *E. cloacae* isolated from gingivitis diseases.

Keywords: β-lactamase, *bla*$_{SHV}$, *bla*$_{GES}$, Enterobacteriaceae, ESBL, oral cavity disease, gingivitis

INTRODUCTION

Enterobacteriaceae are natural human gastrointestinal tract flora. Enterobacteriaceae are transiently in the mouth and are important human body pathogens. Bad hygiene, fecal-oral contamination, self-inoculation of toothbrushes, and antibiotic usage are the critical reasons for oral infection by enterobacteria in the mouth (Lafaurie et al. 2012). The mouth is an important site for research because of its anatomical and physiological features, making it a favorable site for microbial proliferation (Rocha et al. 2006). Microorganisms can be disseminated by speaking, coughing, sneezing, or breathing through aspiration via oropharyngeal secretions or transmission through saliva droplets (Fernandes et al. 2000).

The discovery and broad diffusion of novel extended-spectrum β-lactamases (ESBLs) and carbapenemases (CPEs) has resulted in a remarkable increase in antibiotic resistance among Enterobacteriaceae over the last two decades (Bush and Fisher 2011). Because carbapenems are very stable against β-lactamase hydrolysis and preserve resistance to ESBL makers, they are the ideal medicines for treating serious infections caused by ESBL-producing *Klebsiella pneumoniae* (Schroeter, 1886) Trevisan, 1887 (Colodner et al. 2004). However, carbapenem resistance in *K. pneumoniae*, mainly attributed to the formation of *K. pneumoniae* carbapenemase (KPC), has emerged, generating significant clinical difficulty and complicating treatment (Nordmann et al. 2009). KPC-1 confers moderate to high levels of carbapenem resistance, whereas KPC-2 and KPC-3 impart high levels of carbapenem resistance only with the absence of outer membrane porins (Woodford et al. 2004). KPC producers have expanded worldwide and have been found in various Gram-negative bacteria, including *Pseudomonas aeruginosa* (Schroeter, 1872) Migula, 1900 and *Escherichia coli* Mig., 1895 (Kitchel et al. 2009). This study aimed to investigate the β-lactamase formation isolates and identify *bla*$_{SHV}$ and *bla*$_{GES}$ genes in β-lactam-resistant isolates of *K. pneumoniae* and *E. cloacae* isolated from gingivitis diseases.

MATERIALS AND METHODS

Patients and clinical specimens

A total of 120 oral cavity specimens were collected from patients with gingivitis at the Al-Kafeel clinic and private clinic in Al-Najaf Governorate, Iraq, from September 2020 to February 2021. The patients included both sexes and the age range (7 to 65 years).

Bacterial isolates

The specimens were inoculated on different types of culture media, including blood agar and MacConkey agar, then dispersed on each plate with a sterile loop. Next, plates were incubated at 37°C for 24 hours. Following that, the plates were checked for bacterial growth. Finally, a single pure isolated colony was transferred to brain heart infusion agar for preservation and morphological examination by Gram-staining and other biochemical tests that validated the isolates' identity (Macfaddin 2000).

Identification of bacteria

The identification of *K. pneumoniae* and *E. cloacae* were achieved according to Macfaddin (2000). Vitek-2 GN identification card was used to confirm *K. pneumoniae* and *E. cloacae* identification.

Phenotypic detection of β-lactamase

A direct capillary tube method was used for discovering β-Lactamase formation (Guido and Pascale 2005). This method includes: (i) Two milliliters of phenol red indicator solution (0.5%) were added to Penicillin G solution. (ii) Drops of NaOH solution (1 N) were added to this suspension until the color was changed to violet. (iii) One end of the capillary tube was immersed in the prepared solution until it reached the height of 1-2 cm, then immersed this end of the capillary tube was in the culture of bacterial colonies 24-h for making a bacterial plug (avoiding the formation of bubbles between the solution and the colonies). (iv) Incubated the capillary tubes vertically at 37ºC; the result was read within 15 minutes with a change in color from the top of colonies to yellow, indicating a positive result.

Initial screening for ESBL formation

By performing an initial screen test, all bacterial isolates that produced β-lactamase were found to produce ESBL. Furthermore, if the inhibition zone of ceftazidime (30 µg) disks were less than or equal to 22 mm, the isolate would be regarded as a probable ESBL producer (Koneman et al. 1997).

Confirmatory test for ESBL formation

All the β-lactamase generating isolates were further tested for confirmatory ESBL formation by three methods; these tests were included:

Disk combination test

The disk diffusion approach confirmed the phenotypic identity of probable ESBL-producing bacteria. Ceftriaxone and ceftazidime were examined separately and combined with Tazobactam and clavulanic acid. A 5 mm increase or equal in diameter in the inhibition zone for antibiotics tested in combination with Tazobactam and clavulanic acid compared to its zone when tested alone shows the presence of an ESBL-generating strain (Koneman et al. 1997).

Disk approximation test

All β-lactamase producing isolates were tested according to Batchoun et al. (2009).

Genomic methods

Extraction of DNA

Boiling was used to extract DNA from *K. pneumoniae* and *E. cloacae* isolates. In brief, young colonies of *K. pneumoniae* and *E. cloacae* isolates were suspended in 100 microliters of sterile distilled water and heated for 15 minutes in a water bath. Then, it rapidly cooled at -20ºC for one hour, centrifuged, and the supernatant was saved for amplification (Shah et al. 2017). The concentration and purity of extracted DNA can be determined by Williams et al. (2007).

Polymerase Chain Reaction (PCR) assay

To detect *K. pneumoniae* and *E. cloacae* β-lactamase genes, a monoplex PCR assay was employed to amplify various segments of the genes under study. Two genes from each type were chosen to be amplified individually and utilized in this work (Table 1).

PCR cycling conditions

The PCR mixture was built up in a total volume of 20 µL including five µL of PCR premix, 2.5 µL of each primer, and six µL of extracted DNA. The rest volume was completed to 20 µL of sterile dDW, then vortexes. The contents of PCR-reaction tubes were centrifuged quickly to mix and bring them to the bottom of the tubes, then placed in a thermal cycler PCR programmed as follows (Table 2).

Table 1. Illustrating bla_{GES} and bla_{SHV} genes

Genes	Primer sequence (5'-3')	Amplicon size (bp)	Reference
bla_{SHV}	F:GGCCGCGTAGGCATGATAGA R:CCCGGCGATTTGCTGATTTC	714	Ensor et al. (2009)
bla_{GES}	F:AGTCGGCTAGACCGG AAAG R:TTTGTCCGTGCTCAGGAT	307	Dallenne et al. (2010)

Table 2. PCR program that applies in the thermo-cycler

Gene	Temperature(°C)/Time					Cycles number
	Initial denaturation	Condition of one cycle			Final extension	
		Denaturation	Annealing	Extension		
bla_{GES}	95-7 min.	94 - 40 sec.	57 - 40 sec.	72-1 min.	72-7 min.	30
bla_{SHV}	95 -5 min.	94 - 30 sec.	55 - 60 sec.	75- 45 sec.	72-7 min.	30

Preparation of agarose gel and DNA loading

Agarose gel electrophoresis was done according to Mainiatis et al. (1982). First, Agarose gel was prepared by dissolving 0.8 g of agarose in TBE 1X (100 mL) in a glass bottle, melting to boiling. Then the solution was cooled to 50-60ºC., 3µL of ethidium bromide dye was added with mixing, agarose was poured out into a gel jar after fixation the comb for making wells, then left to solidify. Next, the bubbles were carefully removed; the jar was put in the electrophoresis tank. Six microliters of the 100 bp DNA ladder were placed in the first left well of the agarose electrophoresis gel. Next, five µL of the amplified PCR product were carefully transferred to a well of the agarose electrophoresis gel. The electrophoresis tank closed with its unique lid, and the electric current was matched (70 volts for 1.5-2 h).

Detection of DNA by agarose gel electrophoresis

The gel documentation system was used to detect the electrophoresis outcome. Positive findings were identified when the DNA band base pairs samples were equivalent to the desired product size (Bartlett and Stirling 2003). Finally, the Biometra gel documentation system photographed the gel (Mishra et al. 2009).

RESULT AND DISCUSSION

Phenotypic detection of β -Lactamase producing isolates

The direct capillary tubes method was employed to identify β-lactamase synthesis in β-lactam resistance 14 *K. pneumoniae* and 12 *E. cloacae* isolates. Table 3 revealed that out of 14 β -lactam resistance *K. pneumoniae* isolates, eight (57.1%) gave positive results with the direct capillary tubes method. In contrast, 12 β-lactam resistance *E. cloacae* gave 6 (50%) positive results. This result indicated that enzymatic resistance was prevalent among isolates. However, in this procedure, 6 (42.9%) and 6 (50%) were non-β-lactamase producers in *K. pneumoniae* and *E. cloacae*, respectively.

A variety of processes can cause Non-susceptibility to β-lactam antibiotics in Gram-negative bacteria; however, the enzymatic mechanism, which includes intrinsic and acquired -lactamases, is the most common (Bush 2010; Bush and Jacoby 2010). As a result, various processes, such as porin loss, efflux pumps, lack of expression of acquired genes, and the presence of other undiscovered β-lactamases, could be linked to these discrepancies between genes detected and expressed phenotype (Davin-Regli and Pagès 2015).

The current findings are consistent with those of Al-Charrakh et al. (2011), and Gamboa et al. (2013) found that 41.2% and 58.5% of β-lactam resistant *Klebsiella* spp. isolates were able to produce β-lactamases, respectively.

The results of Table 4 indicate that 6 (42.9%) *K. pneumoniae* and 6 (50%) *E. cloacae* were negative with the capillary tube method, suggesting that these isolates may be either have no β-lactamases or production of low quantities of enzymes, making its detection more difficult (Jacoby and Bush 2009). On the other hand, the negative results could be related to the fact that β-lactamase in isolates required more time to destroy their cell wall and be released. Additionally, temperature and pH may significantly affect or reduce enzyme activity (Foley and Perret 1962).

Phenotypic detection of ESBLs Enzyme

All isolates of *K. pneumoniae* and *E. cloacae* resistant to β-lactam antibiotics were tested for ESBL production. In addition, Ceftazidime disks were used to examine how well the test isolates performed in the ESBL first screen disk test. If the inhibition zone of ceftazidime disks (30 µg) was less than 22 mm, the isolate was considered a probable ESBL producer by the CLSI (2018). The investigation discovered that 11/14 (78.5%) *K. pneumoniae* isolates tested positive for ESBL during the initial screening using a ceftazidime disk. In comparison, 9/12 (75%) *E. cloacae* isolate tested positive for ESBL, indicating that the isolate is suspected of producing ESBL.

The disk combination method was used to detect ESBL-generating isolates in the study. This approach combined ceftazidime and ceftriaxone disks with clavulanic acid and Tazobactam instead of ceftazidime and ceftriaxone disks alone. When the inhibition zone of combined disks was greater than or equal to 5 mm more significant than the inhibition zone of a single disk, the isolate was classified as an ESBL producer (Figure 1).

The results showed that 9 (64.2 %) *K. pneumoniae* and 8 (66.6 %) *E. cloacae*, out of the 24 bacterial isolates β-lactamase producers, demonstrated zone enhancement with clavulanic acid, indicating their ESBL production. Additionally, all isolates were confirmed using the disk approximation method. The enhancement of the inhibitory zone between 30 µg antibiotic disks (ceftazidime, ceftriaxone, cefotaxime, and aztreonam) and a 20/10g amoxicillin-clavulanate disk was interpreted as synergy, indicating the existence of an ESBL, in this approach.

Since the inhibitory zone of synergism has been characterized, screening test findings revealed that 7 (50%) *K. pneumoniae* isolates exhibited positive ESBL production test results versus 5 (41.6%) E. cloacae isolates (Figure 1).

Table 3. β-lactamase generating isolates by direct capillary tube method

Species	No. of Isolates	No. (%) of positive *β*-lactam producing	No. (%) of negative *β*-lactam producing
K. pneumoniae	14	8 (57.1)	6 (42.9)
E. cloacae	12	6 (50.0)	6 (50.0)
Total	26	14 (53.8)	12 (46.2)

Table 4. ESBLs produced in *E. cloacae* and *K. pneumoniae* isolates by confirmation methods

Species	No. of Isolates	No. (%) of positive	
		Disk combination	Disk approximation
K. pneumoniae	14	9 (64.2)	7 (50)
E. cloacae	12	8 (66.6)	5 (41.6)

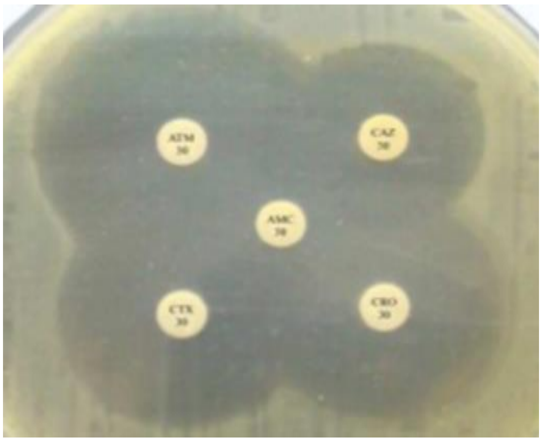


Figure 1. The positive result of ESBL formation *K. pneumoniae* on Muller Hinton agar after 24 hr of incubation at 37°C

Even though ESBLs were discovered at least three decades ago, there is still a lack of understanding of their laboratory detection and clinical importance. Failure to detect these enzymes has aided their unchecked spread and, in some cases, therapeutic failures (Yang and Zhang 2008).

The standard Kirby-Bauer disk diffusion method was used to screen for reduced sensitivity to third-generation cephalosporins and aztreonam. When the zone diameter of any of the markers matched the CLSI criteria (CLSI 2018), the isolate was termed positive for the screening test, and additional phenotypic tests were required to determine ESBL production. The discovery and broad diffusion of novel extended-spectrum β-lactamases have resulted in a remarkable increase in antibiotic resistance among Enterobacteriaceae over the last two decades (ESBLs) (Coque et al. 2008; Chong et al. 2011; Salabi et al. 2013). The most prevalent mechanism of resistance to β-lactam antibiotics among Gram-negative bacteria is the hydrolysis of the β-lactam ring by β-lactamase(s) (Bush and Jacoby 2010), however other mechanisms (e.g., changes in porin channels, efflux pumps) may contribute to or increase resistance (Bush and Fisher 2011; Canton et al. 2012).

The results partially agree with a recent study by Shakib et al. (2018) that showed that 88.6% of *K. pneumoniae* isolates produced ESBL. Moreover, Ghasemi et al. (2013) found that 60% of *K. pneumoniae* isolates ESBL producers in Shiraz, Iran. Also, agreement with Aljanaby and Alhasani (2016) pointed out that the presumptive test for ESBL production was positive for 65.5%.

The clinical microbiology laboratory faces a significant challenge in identifying ESBL producers since the affinity of ESBL-producing isolates for various substrates is varied, making detection challenging. Furthermore, in vitro, some ESBL isolates may appear susceptible to third-generation cephalosporins (Hadi 2018).

However, not all screened positive isolates were ESBL producers in the current investigation. Other mechanisms of resistance to third-generation cephalosporins and aztreonam may so exist. In organisms that produce both ESBL and AmpC, clavulanate may cause hyperproduction of the AmpC-lactamase, resulting in hydrolysis of the third-generation cephalosporin in a false-negative ESBL detection test (Thomson 2010). There are a few cases where the screening tests are positive, but the confirmatory tests are negative or inconclusive (Steward et al. 2001).

On the other hand, the coexistence of different classes of β-lactamases in a single bacterial isolate can make diagnosis difficult. The ability to detect and differentiate AmpC and ESBL-producing pathogens has epidemiological implications and may also have therapeutic implications (Al-Sehlawi 2012).

Molecular study

Molecular detection of ESBL-producing isolates

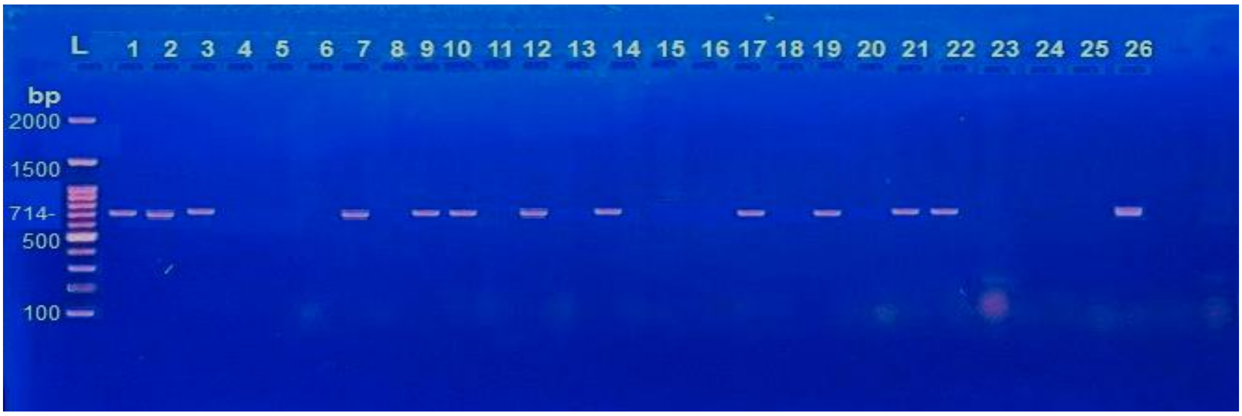
Only two genes in the families of SHV and GES were examined in the current investigation. Detection of these genes (bla_{SHV} and bla_{GES}) was performed by PCR technique. The results revealed that out of 14 *K. pneumoniae* and 12 *E. cloacae* isolates in this study were given 8 (57.1%) and 5 (41.6%) for the bla_{SHV} gene, respectively. While all 100% of *K. pneumoniae* and *E. cloacae* isolates gave negative results for the bla_{GES} gene (Figures 2A and 2B).

ESBLs have been found in *Serratia marcescens* Bizio 1823, *Enterobacter* spp., and *Citrobacter freundii* (Braak, 1928) Werkman & Gillen, 1932 isolated from many places across the world, and they are becoming increasingly common (Ferreira et al. 2010). Initially, these species' ESBLs were TEM or SHV enzymes (Dhillon and Clark 2012). However, environmental antimicrobial agent pressure could be a risk factor for ESBL gene acquisition. Furthermore, using antibiotics as feed additives in animal farming and agriculture creates selective pressure (Woodford et al. 2004).

Similar studies by Ma et al. (2005) and Szabó et al. (2005) found that 33.3% and 30.9% of *E. cloacae* isolates had the bla_{SHV} gene. In addition, Aljanaby and Alhasani (2016) showed blaSHV detected in 87.5% of K. pneumoniae isolates in Iraq.

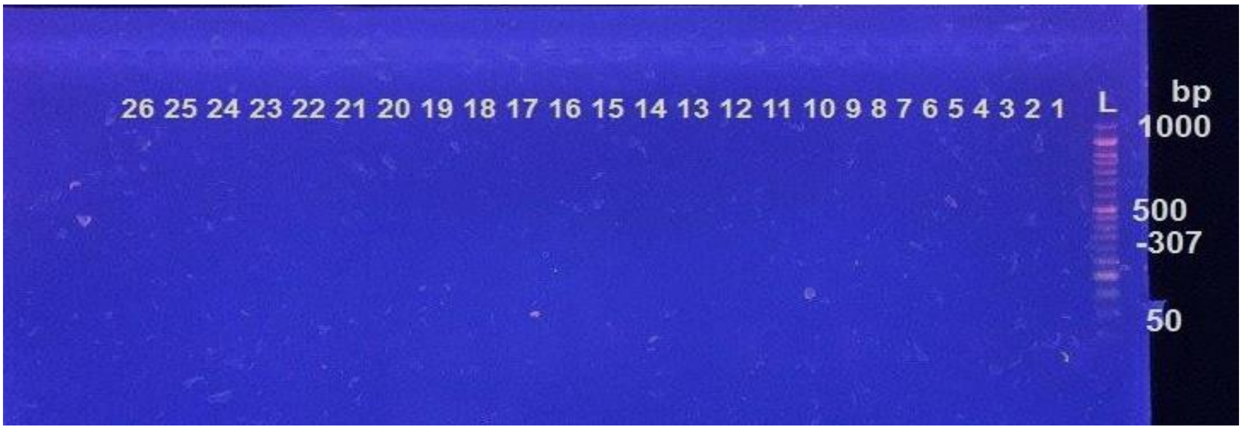
The absence of the bla_{GES} gene in all tested isolates was discovered in this investigation, which could be attributed to the absence of the bla_{GES} gene or the presence of another subtype of a gene that could not be targeted by the primer employed in this study.

The main reason for the prevalence of β-lactamases in Iraq may be due to the extensive usage of certain third-generation cephalosporin antibiotics. ESBL-producing Enterobacteriaceae are found in high numbers in Asia. The high rate of ESBL production in impoverished countries is cause for concern; a lack of money for efficient infection control and limited access to effective antimicrobials have significant implications for reducing morbidity and death associated with these infections.



bla$_{SHV}$ gene with amplified product 714 bp

A



bla$_{GES}$ gene with amplified product 307 bp

B

Figure 2. Gel electrophoresis of PCR products from DNA of *K. pneumoniae* and *E. cloacae* isolates. A. *bla$_{SHV}$* primers. Lanes (1, 2, 3, 7, 9, 10, 12, 14) positive results of *K. pneumoniae* isolates (17, 19, 21, 22, 26) positive results of *E. cloacae*, (L), DNA molecular size marker (100-bp ladder). B. *bla$_{GES}$* primers. Lane, all 100% isolates show negative results (L), DNA molecular size marker (50-bp ladder).

In conclusion, Enterobacteriaceae species is the most familiar Gram-negative bacteria isolated from three types of oral cavity diseases. The *K. pneumoniae* and *E. cloacae* isolates are the commonest Enterobacteriaceae isolated from gingivitis infections. The *K. pneumoniae* and *E. cloacae* isolates appeared with *bla$_{SHV}$* genes in β- lactam-resistant isolates, while no results for *bla$_{GES}$* genes. ESBL phenotype was highly prevalent among *K. pneumoniae* and *E. cloacae* isolates.

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