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The Aminoglycoside Resistance Genes, *pehX*, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{AmpC}}$, and *npsB* among *Klebsiella oxytoca* Stool Samples

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Abstract

Background*: Klebsiella oxytoca* may cause various infections, including respiratory, urinary, and bloodstream infections, often with multidrug-resistant strains posing challenges in treatment. The aim of this study was for molecular identification of *K. oxytoca* and to assess the existence of aminoglycoside resistance genes in biofilm and in toxin-producing and AmpC-positive isolates.

Methods and Results: A total of 400 non-duplicate stool samples were collected from patients with colitis from 2019 to 2020 and were immediately cultured onto McConkey and blood agar (Merk, Germany). Antibiotic discs and Mueller-Hinton agar (MHA) culture medium (Merck, Germany) were used for antimicrobial susceptibility testing. The disk diffusion was done for susceptibility examination of them using CLSI 2020. Phenotypic detection of AmpC enzymes and biofilm formation were also determined. The PCR was performed to detect polygalacturonase (*pehX*) gene, *bla*$_{\text{CTX-M}}$ gene, *npsB* toxin-encoding gene, *bla*$_{\text{AmpC}}$ gene, and the *aac(6')-lb* and *aac(3')-IIa* AMEs genes.

A total of 100 *K. oxytoca* were identified from stool samples. Most isolates were not susceptible to tetracycline, cotrimoxazole, or cefoxitin disks. Moreover, most were susceptible to amikacin and piperacillin-tazobactam disks. Among 100 isolates, 54% produced the AmpC enzyme in the combined disk method. Among them, 30 isolates were resistant to gentamicin. Strong biofilm formation was determined in 66% of isolates, and 30% of them produced moderate biofilms. Moreover, 4% of the isolates had weak biofilms. Among the 60 gentamicin-resistant *K. oxytoca*, 32 isolates had strong biofilms, and 11 isolates produced moderate ones. The *pehX* was used for the molecular identification of K. oxytoca; the results showed the presence of this gene in all isolates. The majority (98%) of *K. oxytoca* amplified the *npsB* toxin-encoding gene. The rate of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{AmpC}}$, *aac(6')-lb*, and *aac(3)-IIa* genes were 62%, 45%, 12%, 24%, respectively.

Conclusion: In our study, more than half of *K. oxytoca* showed MDR phenotype. Moreover, half of the isolates carried the *bla*$_{\text{AmpC}}$ and *bla*$_{\text{CTX-M}}$ genes. Strong biofilm formation was observed in more than 60% of them.(**International Journal of Biomedicine. 2023;13(3):127-130.**)

Keywords: *Klebsiella oxytoca* • aminoglycoside resistance • biofilm

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Abbreviations

AMEs, aminoglycoside modifying enzymes; **ESBLs**, extended-spectrum beta-lactamases; **MDR**, multidrug resistance; **PCR**, polymerase chain reaction

Introduction

Klebsiella oxytoca may cause various infections, including respiratory, urinary, and bloodstream infections, often with multidrug-resistant strains posing challenges in treatment. The genus *Klebsiella*, like *Escherichia, Salmonella, Shigella, Haemophilus*, and some other Gram-negative bacteria, belongs to the domain of *Proteobacteria* and the

branch of *Gammaproteobacteria* (class III).[1-3] This bacterium multiplies by disturbing the microbial balance of intestinal flora and causes colitis.[4] Colonization of skin and mucous membranes is the first step[5] in the pathogenicity of *K. oxytoca*. This opportunistic pathogen can generate a thick layer of biofilm as one of its important virulence factors, enabling the bacteria to attach to living or abiotic surfaces, contributing to drug resistance. The thickness of the biofilm layer extends from a simple cell layer around a bacterium to a thick layer surrounding the bacterial community. In this biofilm layer, a complex network of channels allows bacteria to access the environment. One of the benefits of this outer layer is the ability to absorb the nutrients needed and concentrate them for bacterial metabolism.[6] Biofilms make bacteria resistant to various agents, such as antibiotics, environmental stress conditions, and phagocytosis of host immune cells.[7,8] The presence of these surface components enables the bacterium to attach to various surfaces, including tissues, catheters, and other injectable medical devices. The first reported bacterial enzyme to degrade penicillin was Amp-C beta-lactamase in *E. coli*. In 1965 Swedish researchers began a genetic and systematic study of penicillin-resistant *E. coli*. The sequence of the *AmpC* gene of *E. coli* was reported in 1981. It was different from the TEM-1 beta-lactamase sequence and contained serine at its active site. AmpC β-lactamases belong to Ambler class C and Bush-Jacoby-Medeiros functional group 1.[9,10] When the functional classification scheme was published in 1995, chromosomally determined AmpC β-lactamases in Enterobacteriaceae and a few other families were known.[11,12] Aminoglycosides are important antibiotics against multidrug-resistant Gram-negative bacteria. However, resistance to these drugs has limited the choices. The aim of this study was for molecular identification of *K. oxytoca* and to assess the existence of aminoglycoside resistance genes in biofilm and in toxin-producing and AmpC-positive isolates.

Materials and Methods

A total of 400 non-duplicate stool samples were collected from patients with colitis from 2019 to 2020 and were immediately cultured onto McConkey and blood agar (Merk, Germany). Various biochemical tests were performed to confirm *K. oxytoca* isolates, in addition to the molecular test. Antibiotic discs and Mueller-Hinton agar (MHA) culture medium (Merck, Germany) were used for antimicrobial susceptibility testing. The disk diffusion was done for susceptibility examination using CLSI 2020. Antibiotic discs used were cefoxitin 30 µg (FOX), ceftazidime 30 µg (CAZ), cefotaxime 30 µg (CTX), cefepime 50 µg (FEP), meropenem 10 µg (MEM), gentamicin 10 µg (G), tetracycline 30 µg (TE), cotrimoxazole 25 µg (SXT), piperacillin-tazobactam 30 µg (PITZ), co-amoxiclav 30 µg (AMC), imipenem 10 µg (IPM), amikacin 30 µg (AN) and ciprofloxacin 30 µg (CP) (MAST, UK).[13-15]

To detect Amp-C beta-lactamase enzyme, we used ceftazidime, cefotaxime, and cefoxitin 30 µg (FOX) + clavulanic acid 10 µg and cefoxitin 30 µg plus and without boronic acid 400 µg. After incubation for 24 hours at 37°C, in the combined disk test, the diameter of the growth inhibition zone differed by 5 mm, compared to cefoxitin singly. This finding showed a positive result. Biofilm formation was performed by the microtitre-plate method and determined using an ELISA reader at OD490.

The PCR was performed to detect polygalacturonase (*pehX*) gene, $bla_{\text{CTX-M}}$ gene, *npsB* toxin-encoding gene, bla_{AmpC} gene, and the *aac(6')-lb* and *aac(3')-IIa* AMEs genes (Table 1).

Table 1.

Primer sequence.

Primer	Sequence: 5' → 3'	Amplicon size (bp)	Ref.
$bla_{\text{CTX-M}}$	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGTGCCATA	544	[16]
pehX	F: GGACTACGCCGTCTATCGTCAAG R: TAGCCTTTATCAAGCGGATACTGG	513	[17]
npsB	F: CCCGTTGGCCGCTCATCACCTAT R: GCGCCGCACAATTTCCCTTCCTC	470	[18]
bla_{AmpC}	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	462	[19]
aac(6')-Ib	F: TATGAGTGGCTAAATCGAT R: CCCGCTTTCTCGTAGCA	395	[20]
aac(3)-IIa	F: GGCAATAACGGAGGCGCTTCAAAA R: TTCCAGGCATCGGCATCTCATACG	563	[20]

Ref - Reference

Results

A total of 100 *K. oxytoca* were identified from stool samples. Most isolates were not susceptible to tetracycline, cotrimoxazole, or cefoxitin disks. Moreover, most were susceptible to amikacin and piperacillin-tazobactam disks (Table 2).

Table 2.

The antibiotic resistance profile.

Disk/Resistance (n = 100)	Susceptibility (n = %)	Intermediate (n = %)	Resistance (n = %)
CAZ	46	0.0	54
FEP	41	0.0	59
CTX	54	5	41
AMC	41	8	51
IPM	52	8	40
MEM	53	9	38
PITZ	68	2	30
FOX	63	0.0	37
AN	67	3	30
G	60	4	36
CP	41	4	56
TE	34	0.0	66
SXT	29	2	69

Among 100 isolates, 54% produced the AmpC enzyme in the combined disk method. Among them, 30 isolates

were resistant to gentamicin. Strong biofilm formation was determined in 66% of isolates, and 30% of them produced moderate biofilms. Moreover, 4% of the isolates had weak biofilms. Among the 60 gentamicin-resistant *K. oxytoca*, 32 isolates had strong biofilms, and 11 isolates produced moderate ones. The *pehX* was used for the molecular identification of *K. oxytoca*; the results showed the presence of this gene in all isolates (Figure 1). The majority (98%) of *K. oxytoca* amplified the *npsB* toxin-encoding gene. Therefore, nearly all the isolates were toxin-producing strains (Figure 2). The rate of bla_{CTX-M}, bla_{AmpC}, *aac(6')-lb*, and *aac(3)-IIa* genes were 62%, 45%, 12%, 24%, respectively. Table 3 shows the relation between biofilm formation, resistance genes, and MDR phenotype.

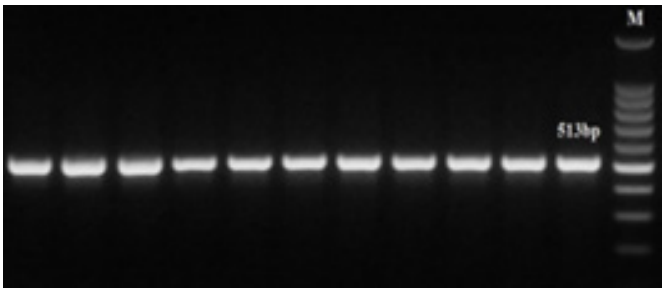

Fig. 1. *The pehX gene with 513bp size, M: 100bp DNA marker.*

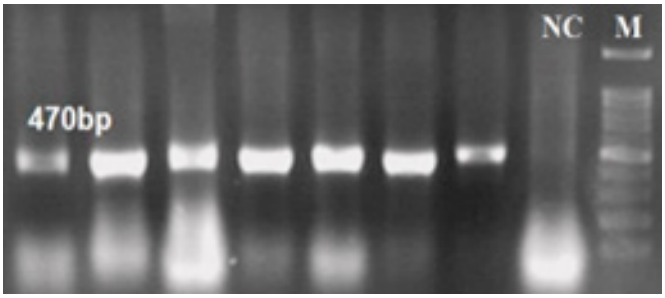

Fig. 2. *The npsB toxin gene with 470bp size, NC: negative control, M: 100bp DNA marker.*

Table 3.

The relation between biofilm formation, resistance genes and MDR phenotype.

Characteristics	bla_{CTX-M}	bla_{AmpC}	Resistance profile
Strong biofilm	35%	27%	CAZ, FEP, AMC, G, FOX, CP, SXT, TE, CTX
aac(6')-lb	6%	8%	CAZ, FEP, AMC, G, FOX, CP, SXT, TE, AN, PITZ, IPM, CTX
aac(3)-IIa	18%	16%	CAZ, FEP, AMC, G, FOX, CP, SXT, TE, AN, PITZ, IPM, MER, FEP, CTX

Discussion

We observed that 25% of stool samples were positive for *K. oxytoca* and confirmed by the presence of the *pehX* gene, 98% of which carried the *npsB* toxin-encoding gene. Most isolates were not susceptible to tetracycline, cotrimoxazole, or cefoxitin disks. However, most of them were susceptible to amikacin and piperacillin-tazobactam disks. In Cheng's study, the prevalence of K. oxytoca was that 2.1%, and 31.6% of these isolates contained toxins.[21] Also, none of the *K. oxytoca* isolates were reported from samples of hemorrhagic colitis. In 2010, Conejo et al.[22] conducted a study on multidrug-resistant *K. oxytoca* carrying the bla_{IMP-8} gene associated. A total of 9 out of 52 hospitalized patients in Spain were infected with this bacterium. In 2011, the first report was from *K. oxytoca*, new ESBLs called OXY-2, which was able to hydrolyze cefotaxime and ceftazidime.[23] This family of new broad-spectrum beta-lactamases was identified after the first report of CTX-M enzymes in 1986 in Japan and in 1989 in Germany, Argentina, France, and Italy. CTX-M beta-lactamases have now been identified in most Enterobacteriaceae species. At least 65 types of CTX-M have been identified in 5 families based on their amino acid sequences.[7,8] Recently, species with resistance to cephalosporins have been increasing rapidly, so the total samples isolated in Bulgaria, Cyprus, Romania, and Portugal, 28%, 16%, 16%, and 12%, respectively, produced ESBLs.[9,24]

In this study, 66% and 45% of isolates were contained bla_{CTX-M} and bla_{AmpC} genes, respectively. The prevalence of AmpC enzyme in *E. coli* and *K. pneumoniae* strains was 17.1% in China. In the United States, 4% of *E. coli* collected from 25 US states carried the AmpC enzyme.[25-28] In a study in India, 37.5% of *E. coli* isolates, and 24.1% of *K. pneumonia* isolates had the AmpC gene. In a study of 173 *E. coli* and *Klebsiella* isolates in the United Kingdom, 49% of *E. coli* and 55% of *Klebsiella* isolates contained the AmpC beta-lactamase enzyme, which is highly prevalent in this country.[29,30] However, biofilm formation and AMEs genes have not been investigated. We observed that 16% and 8% of AmpC-bearing *K. oxytoca* carried the *aac(3)-IIa* and *aac(6)-Ib* genes, respectively. Moreover, 27% of them were strong biofilm producers. Among 100 isolates, 54% produced the AmpC enzyme in the combined disk method. Among them, 30 isolates were resistant to gentamicin.

In conclusion, more than half of *K. oxytoca* showed MDR phenotype. Moreover, half of the isolates carried the bla_{AmpC} and bla_{CTX-M} genes. Strong biofilm formation was observed in more than 60% of them, of which 27% carried the *blaAmpC* gene, 22% carried the *aac(3)-IIa* gene, and 6% had the *aac(6)-Ib* gene. It is suggested to implement combination therapy for MDR isolates.

Competing Interests

The authors declare that they have no competing interests.

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