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Original Article

Molecular characterisation of *Klebsiella oxytoca* strains isolated from patients with antibiotic-associated diarrhoea

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ABSTRACT

Background and study aim: Colitis is a common complication after treatment with antibiotics such as β-lactams, quinolones, and aminoglycosides. Recently, *Klebsiella oxytoca* has been implicated in this type of diarrhoea. The prevalence and characterisations of *K. oxytoca* isolated from patients with antibiotic-associated diarrhoea were investigated. The *K. oxytoca* isolates were also tested for cytotoxin production.
Patients and methods: This study was conducted from May 2011 to Dec 2013. Faecal samples were collected from hospitalised patients receiving antibiotic treatment. Initial cultivation was performed on specific media. The clinical isolates were confirmed by polymerase chain reaction (PCR) using the specific *K. oxytoca* polygalacturonase (pehX) gene. The double-disc diffusion test was used to detect extended-spectrum beta-lactamase (ESBL)-producing strains. Tracking of ESBL-encoding genes was performed via PCR. The organism was cultured on Hep-2 cell lines for cytotoxin production.
Results: Out of 331 samples collected from patients, 40 were confirmed molecularly to be clinical isolates of *K. oxytoca*. Fourteen (35%) ESBL-producing strains were isolated using the double-disc diffusion method. Among the molecularly confirmed *K. oxytoca* isolates, seven (17.5%) tested positive for the *bla*SHV gene, 12 (30%) for *bla*TEM, 10 (25%) for *bla*CTX-M, three (7.5%) for *bla*OXA, nine (22.5%) for *bla*CTX-M-15, and seven (17.5%) for *bla*TEM-1. Five (12%) isolates showed cytotoxin activity below 30%, 12 (30%) strains showed moderate cytotoxin activity between 30% and 60%, and 23 (58%) strains showed cytotoxin activity ⩾60%.
Conclusions: The cytotoxin-producing *K. oxytoca* is found to be one of the causes of antibiotic-induced colitis. Discontinuing treatment and allowing normal intestinal flora to be established or prescribing appropriate medication after antibiogram can help patients with antibiotic-induced haemorrhagic colitis in a timely manner.

Introduction

Antibiotic-associated diarrhoea frequently occurs during antibiotic treatment [1]. In some patients, antibiotic treatment causes the selection and overgrowth of toxin-producing bacteria, which in turn causes antibiotic-associated colitis (AAC). Toxin-producing *Clostridium difficile* is a significant bacterium that causes pseudomembranous colitis [2]. It is the main cause of infectious diarrhoea in hospitals and long-term care facilities [3]. However, this bacterium is seen only in 10–20% of cases of colitis. Antibiotic-associated haemorrhagic colitis (AAHC) is another type of colitis not caused by *C. difficile* [4]. AAHC was first reported in 1978 by Toffler and colleagues from Austria [5]. This form of colitis occurs concurrent with or after treatment with beta-lactam antibiotics, cephalosporins, and quinolones as well. Suspected haemorrhagic colitis caused by antibiotics has been found to resolve spontaneously after antibiotic therapy was discontinued. These complications are a result of various mechanisms such as allergic reactions, mucosal ischaemia, and infections of bacterial or viral origin [5,6].

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Recently, AAHC was shown to be caused by cytotoxin-producing *Klebsiella oxytoca*. Unlike the colitis caused by *C. difficile*, this form of colitis often begins with abdominal pain, bloody diarrhoea, and sudden onset requiring hospitalisation [1,7]. *K. oxytoca* can colonise healthy individuals (1.6–9%), and it has been proven to be an opportunistic pathogen of the intestine [2].

Cytotoxins are key to the pathogenesis of *K. oxytoca*. In a patient with AAHC, cytotoxicity varies with different species of bacteria [8,9]. In the majority of patients with AAHC, a significant number ($>10^6$ CFU/ml) of *K. oxytoca* are observed in the stool [6]. Cephalosporin antibiotics are used for the first-line treatment of *K. oxytoca* infections. In the case of resistance to cephalosporins, aminoglycosides (usually gentamicin), and fluoroquinolones (e.g., ciprofloxacin), carbapenems can be used [10]. *Klebsiella* spp. are able to hydrolyse cephalosporins and even carbapenems [11,12]. The production of β-lactamase enzymes is the most important mechanism of resistance to β-lactam antibiotics [13]. Resistance to aminoglycosides occurs via the methylation of 16S ribosomal RNA (rRNA) gene, which reduces its affinity for aminoglycosides. Several studies confirm that most *arm*A methylase genes, with *bla*CTX-M-15 and *bla*TEM-1 genes, are simultaneously transmitted by conjugation to *K. oxytoca* [14]. Studies have also reported the prevalence of extended-spectrum beta-lactamases (ESBLs) such as TEM, SHV, PER, VEB, CTX-M, and OXA in *K. oxytoca* [15,16].

In our region, no comprehensive study has been conducted on *K. oxytoca*, which is known to induce AAC. Hence, the aim of this study was to determine the prevalence, characteristics, and cytotoxin production of *K. oxytoca* isolated from patients with colitis caused by antibiotic therapy.

Patients and methods

Stool samples

A total of 331 stool samples were collected over an 18-month period (from May 2011 to December 2013) from hospitalised patients (Taleghani, Milad, and Shariati) including children and adults (male and female). The protocol was approved by the ethics committee of the Hamadan University of Medical Sciences, Iran. The majority of patients presented with diarrhoea or bloody diarrhoea and a history of β-lactam antibiotic treatment from 1 week to 2 months, for example, penicillin, imipenem, and meropenem; cephalosporins in general such as ceftriaxone and ceftazidime; and aminoglycosides such as gentamicin and amikacin. The patients were divided into four groups: 259 patients receiving antibiotic therapy and having diarrhoea (A^+D^+, 78%), 39 (12%) patients receiving antibiotic therapy but not having diarrhoea (A^+D^-), 30 (9%) patients not receiving antibiotic treatment but having diarrhoea (A^-D^+), and three patients who did not receive antibiotic treatment and did not suffer from diarrhoea (A^-D^-, 1%) [4].

Isolation and identification of bacteria

The stool samples were transferred to Cary Blair transport medium (Merck, Darmstadt, Germany) via swabs. The demographic features of patients such as sex, age, and duration of antibiotic use were recorded in a questionnaire. The transferred stool samples were then cultured on MacConkey agar medium (Merck, Darmstadt, Germany) and incubated at 37 °C for 18 h. All of the samples for identification of *K. oxytoca* were done on Lactose-positive colonies; biochemical tests such as indole production, Simmons' citrate, triple sugar iron (TSI) agar, lysine decarboxylase, ortho-nitrophenyl-β-galactopyranoside (ONPG), and malonate tests were also performed on the samples [17].

DNA extraction

For this purpose, 2 mL of the overnight bacterial culture was centrifuged, and the pellet was resuspended in 620 mL of lysis buffer (10 mM Tris–HCl, 50 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl; pH 8) containing 1% sodium dodecyl sulphate (SDS) and 0.4 mg/mL of proteinase K (Sigma, USA). The mixture was incubated for 1 h at 56 °C and then for 1 h at 100 °C. An equal volume of phenol/chloroform/isoamyl alcohol (BDH, UK) was added to the mixture and centrifuged at 10,000 rpm for 10 min. The supernatant was added to an equal volume of chloroform. After centrifugation at 10,000 rpm, the top layer was collected, and DNA was precipitated with two volumes of cold isopropanol (Merck, Darmstadt, Germany) at −20 °C for 10 min. The pellet was obtained by centrifugation for 20 min and then washed with 1.5 mL of 70% cold ethanol (Merck, Darmstadt, Germany). Finally, the pellet was resuspended in 100 mL of TE 1× buffer (10 mM Tris–HCl and 1 mM EDTA; pH 8) and 1 μL was used for the polymerase chain reaction (PCR) mixture [18].

K. oxytoca isolates confirmed by PCR assay

The *K. oxytoca* strains isolated by biochemical tests were molecularly confirmed by the PCR assay using the polygalacturonase pehX gene, a species-specific *K. oxytoca* gene (Table 1) [14,19–21].

The *K. oxytoca* ATCC 13182 strains, provided by the Pasteur Institute of Iran, were used as a positive control. The PCR reaction conditions were as follows: initial denaturation at 95 °C for 2 min

Table 1
Primer sequences.

Gene	Primer name	Primer sequences	Size bp	References
SHV	bla_{SHV} R	5′-GCCTTTATCGGCCTTCACTCAAG-3′	868	[19]
	bla_{SHV} F	5′-TTAGCGTTGCCAGTGCTCGATCA-3′		
TEM	bla_{TEM} R	5′-GAGTATTCAACATTTCCGTGTC - 3′	931	[19]
	bla_{TEM} F	5′-TAATCAGTGAGGCACCTATCTC -3′		
CTX-M	bla_{CTX-M} R	5′-ACCGCGATATCGTTGGT-3′	909	[19]
	bla_{CTX-M} F	5′-CGCTTTGCGATGTGCAG-3′		
OXA	bla_{OXA} R	5′-AGTGTGTTTAGAATGGTGATC-3′	846	[19]
	bla_{OXA} F	5′-ACACAATACATATCAACTTCGC-3′		
CTX-M-15	$bla_{CTX-M-15}$ R	5′-ACCGTCGGTGACGATTTTAG-3′	876	[20]
	$bla_{CTX-M-15}$ F	5′-AGAATAAGGAATCCCATGGTT-3′		
TEM-1	bla_{TEM-1} R	5′-CTG ACA GTT ACC AAT GCT TA-3′	858	[20]
	bla_{TEM-1} F	5′-ATG AGT ATT CAA CAT TTC CG-3′		
*arm*A methylase	*arm* A R	5′-GGAGAAGGGAATGGAAGAGA-3′	592	[14]
	arm A F	5′-AGGTTGTTTCCATTTCTGAG-3′		
peh X	*peh* X C	5′-GATACGGAGTATGCCTTTACGGTG-3′	344	[18]
	peh X D	5′-TAGCCTTTATCAAGCGGATACTGG-3′		