ORIGINAL ARTICLE

The association of surface adhesin genes and the biofilm formation among *Klebsiella oxytoca* clinical isolates

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Abstract

Bacterial adhesins mediate the attachment and biofilm production leading to the persistence of colonized strains. The aim of this study was evaluation of the association of surface adhesin genes with the biofilm formation among *Klebsiella oxytoca* isolates. Among 50 isolates of *K. oxytoca* from patients with antibiotic-associated diarrhoea, the susceptibility test, MIC (according to CLSI 2016) and phenotypic biofilm formation (with microtitre tissue-plate assay) were performed. The presence of adhesins was investigated using PCR. Thirty-three (66%) isolates produced moderate-level biofilms, but none of them exhibited strong biofilm formation. The presence of adhesins was as follows: *fimA*, 60% (*n* = 30), *mrkA*, 42% (*n* = 21), *matB*, 96% (*n* = 48) and *pilQ*, 92% (*n* = 46). The biofilm formation was related to the presence of *fimA* (odds ratio (OR) 0.8571, 95% CI 1.733–6.267, p <0.0001), *mrkA* (OR 0.2462, 95% CI 2.723–4.622, p 0.001), *matB* (OR 0.4521, 95% CI 1.353–5.332, p 0.008) and *pilQ* (OR 0.1481, 95% CI 1.691–6.117, p <0.0001). The *npsB* toxin-encoding gene was detected among 46 (92%) isolates. Resistance to non-β-lactam antibiotics was significantly associated with the presence of adhesin-encoding genes. The presence of adhesins and the capsular encoding gene was significantly associated with biofilm formation among *K. oxytoca* isolates. The presence of surface adhesin-encoding genes was significantly associated with the biofilm formation and also with resistance to non-β-lactam antibiotics among *K. oxytoca* clinical isolates. In addition, biofilm production was not significantly associated with β-lactam resistance among the isolates.
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Introduction

Klebsiella oxytoca is one of the agents causing antibiotic-associated haemorrhagic colitis [1,2]. On the other hand, *Klebsiella* spp. produce biofilms via several types of adhesive structures [3] found in both *Klebsiella pneumoniae* and *K. oxytoca*, mostly including capsule, and type 1 and type 3 fimbria. The type 3 fimbria subunit proteins constitute the major bacterial adhesins encoded by the *mrkABCDF* (mannose-resistance adhesins of *Klebsiella* spp.) genes, among which *mrkA* and *mrkD* are the main subunits and attachment subunits, respectively [4]. The type 3 fimbrial genes are encoded by chromosomal, conjugative plasmids and transposons [5–8]. These binding structures have been mainly detected among biofilm producer isolates. The *mrkD* subunit contains sequence variations among isolates due to mutations in this region [9]. The attachment of *K. oxytoca* isolates to the epithelial cells leads to biofilm formation and persistence of infection, or difficulty in eradication of the infection. The relation of adhesive genes and the antibiotic resistance pattern of isolates has not been fully revealed [10]. The toxin-producing isolates are identified by the cell culture and PCR amplification of related *npsA* and *npsB* genes [11,12]. Screening of biofilm-associated genes and evaluation of their relation to the biofilm formation would help decisions on eradication of biofilm-related infections.

In recent years, isolates with resistance to third- and fourth-generation cephalosporins have spread around the world.

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These isolates produce extended-spectrum β-lactamases. In addition, isolates expressing extended-spectrum β-lactamases have shown multiple resistance to fluoroquinolones and aminoglycosides. In addition, carbapenemase enzymes cause difficulty in infection eradication in *K. oxytoca* [13,14]. The purpose of this study was to evaluate the relationship between biofilm formation and the presence of surface adhesin genes in *K. oxytoca*.

Materials and methods

A total of 50 *K. oxytoca* were isolated from faecal samples from hospitalized patients with haemorrhagic colitis during 2013–2016. The isolates were inoculated onto MacConkey and blood agar media (Merk, Darmstadt, Germany) and identified with biochemical and molecular (amplification of *pehX* gene) tests.

The susceptibility of isolates was implemented with the Kirby Bauer method. For each isolate, a bacterial suspension equal to the turbidity of half McFarland was prepared and spread on Müller–Hinton agar medium (Merk, Germany). The plates were observed with the naked eye and the zones were interpreted during 18–22 h. The phenotypic extended-spectrum β-lactamases and carbapenemase production was investigated with combined disc and Carba-NP tests, respectively, according to the CLSI 2016 advice [15].

The MIC for ceftazidime, cefotaxime and imipenem were investigated using the agar dilution method (Sigma Aldrich, St Louis, MO, USA). Briefly, a bacterial suspension equal to the opacity of the half McFarland standard was prepared and 10 μL was inoculated onto Müller–Hinton agar containing dilutions of antibiotics. After culture, plates were incubated for 18–24 h. Any spotted growth was considered a positive result.

The phenotypic biofilm formation was assessed with a microtitre tissue-plate assay using 96-well plates according to previous publications. Each isolate was cultured in trypticase soy broth for 24 h, then diluted 1:100 and 20 μL was used to inoculate into 180 μL trypticase soy broth in each well of a 96-well plate (in triplicate for each isolate) and incubated overnight. The wells were washed and 10% crystal violet (volume/volume) was added for the staining of precipitated and attached cells for 15 min. Next, the wells were washed with sterile distilled water and methanol (99%) was used for fixation of biofilms; the plate was left to dry for up to 24 h. Thereafter, the biofilms were solubilized with 96% ethanol and assessed under the ELISA reader at an OD for 490 nm [16–18]. For measurement of biofilm formation, the test OD was compared with the control OD (OD_c); where $OD>4\times OD_c$ means strong biofilm formation, $2\times OD_c<OD\leq 4\times OD_c$ means moderate biofilm formation, $OD_c<OD\leq 2\times OD_c$ means weak biofilm formation and $OD \leq 0.08324$ means no biofilm formation [19].

The PCR was applied to amplify the *fimA*-, *mrkA*-, *matB*-, *pilQ*- and *pilL*-encoding adhesins and the *npsB* toxin-encoding gene for which specific primers (TAKARA, Seoul, South Korea; Table 1) were designed in this study. For the amplification of genes, the thermal profile included 94°C for 4 min, 30 cycles of 94°C for 30 s, annealing temperature (Table 1) for 30 s, 72°C for 30 s and a final extension of 72°C for 10 min.

The analysis of data in which the association of adhesin genes and biofilm formation was considered, 95% CI and error <5% ($p < 0.05$) were significant in the unpaired *t*-test and analysis of variance (ANOVA) test. SPSS software version 21 was used for the data analysis.

Results

Of 50 isolates, the majority were resistant to trimethoprim-sulfamethoxazole and tetracycline (50% and 40%, respectively) and 45 (90%) of them were susceptible to both piperacillin-tazobactam and amikacin. In addition, resistance was observed to ceftazidime (28%), cefepime (20%), cefotaxime (28%), imipenem (18%), meropenem (14%), cefoxitin (26%),

TABLE 1. The specific primer sequences used in this study

Primer	Sequence 5′–3′	Annealing temperature (°C)	Amplicon (bp)	Reference
mrkA	F: CTGGCCGGCGCTACTGCTAAG R: CACCCGGGATGATTTTGTTGG	60	127	This study
fimA	F: GCACCGCGATTGACAGC R: CGAAGGTTGCGCCATCCAG	59	132	This study
matB/ecp	F: GTACTGGGCGGCAACCTTAG R: GTGCCGCTGATGATGGAGAA	61	98	This study
pilL	F: TCTATGCCGCCTCTCCTGAAGTTG R: TCGGCGATAATGACACGGGGATAC	60	150	This study
pilQ	F: TCCGCCAGGCTCCACTTC R: GCTCGCGGGCATCTGAC	61	194	This study
npsB	F: CCCGTTGGCCGCTCATCACCTAT R: GCGCCGCACAATTTCCCTTCCTC	60	470	This study

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TABLE 2. The rate of resistance to antibiotics related to surface adhesion genes

Antibiotics	*fimA* (*n* = 30)	*mrkA* (*n* = 21)	*pilQ* (*n* = 46)	*matB* (*n* = 48)	p Value
CAZ	16 (32%)	9 (18%)	16 (32%)	16 (32%)	0.212
FEP	12 (24%)	7 (14%)	12 (24%)	12 (24%)	0.104
CTX	15 (30%)	6 (12%)	16 (32%)	15 (30%)	0.132
IPM	4 (8%)	3 (6%)	4 (8%)	4 (8%)	0.195
MEM	3 (6%)	3 (6%)	4 (8%)	5 (10%)	0.191
PITZ	2 (4%)	2 (4%)	3 (6%)	4 (8%)	0.106
FOX	12 (24%)	9 (18%)	13 (26%)	12 (24%)	0.351
AN	2 (4%)	1 (2%)	3 (6%)	3 (6%)	0.011
GN	4 (8%)	3 (6%)	5 (10%)	6 (12%)	0.002
CP	10 (20%)	11 (22%)	5 (10%)	12 (24%)	<0.001
TE	16 (32%)	14 (28%)	17 (34%)	17 (34%)	0.004
SXT	20 (40%)	18 (39%)	21 (42%)	21 (42%)	0.001

Abbreviations: CAZ, ceftazidime; FEP, cefepime; CTX, cefotaxime; IPM, imipenem; MEM, meropenem; PITZ, piperacillin-tazobactam; FOX, cefoxitin; AN, amikacin; GN, gentamicin; CP, ciprofloxacin; TE, tetracycline; SXT, tetracycline.

gentamicin (16%) and ciprofloxacin (22%). using ANOVA test, resistance to ciprofloxacin, tetracycline, gentamicin, amikacin and trimethoprim-sulfamethoxazole was significantly associated with the presence of all adhesin genes.

Thirty-three (66%) isolates produced moderate-level biofilms, but none of them exhibited strong biofilm formation. There was no significant difference between β-lactam (cephalosporins and carbapenem) resistant and susceptible isolates of *K. oxytoca* regarding biofilm formation (p >0.05). Fourteen of 16 ciprofloxacin-resistant and seven of eight gentamicin-resistant *K. oxytoca* produced moderate-level biofilms (p 0.0001, using the ANOVA test).

The presence of adhesins was as follows: *fimA* (60%, *n* = 30), *mrkA* (42%, *n* = 21), *matB* (96%, *n* = 48), and *pilQ* (92%, *n* = 46). The biofilm formation was related to the presence of *fimA* (odds ratio (OR) 0.8571, 95% CI 1.733–6.267, p <0.0001), *mrkA* (OR 0.2462, 95% CI 2.723–4.622, p 0.001), *matB* (OR 0.4521, 95% CI 1.353–5.332, p 0.008) and *pilQ* (OR 0.1481, 95% CI 1.691–6.117, p <0.0001). All the isolates were cytotoxin-positive (*npsB* gene) *K. oxytoca*.

The relation of resistance to antibiotics and presence of adhesin genes is depicted in Table 1 and the relation of phenotypic biofilm formation and presence of *fimA* and *mrkD* adhesin-encoding genes is shown in Table 2. A significant difference was observed among fimbria adhesins and resistance to non-β-lactam antibiotics (Table 2). Multivariate analysis showed that the presence of *fimA*, *pilQ*, *matB* and *mrkA* was significantly associated with resistance to ciprofloxacin, tetracycline, gentamicin, amikacin and trimethoprim-sulfamethoxazole.

The association of adhesin genes and biofilm formation by *K. oxytoca* is displayed in Table 3. The analysis demonstrated that there is a relationship between adhesins and biofilm formation (Table 3).

TABLE 3. The association of surface adhesion genes with the biofilm formation (one-way analysis of variance)

Biofilm level	*fimA* (*n* = 30)	*mrkA* (*n* = 21)	*matB* (*n* = 48)	*pilQ* (*n* = 46)	p value
Moderate	29 (58%)	19 (38%)	32 (64%)	32 (58%)	0.003
Weak	1 (2%)	2 (4%)	15 (30%)	13 (26%)	<0.001
No biofilm	0.00	0.00	1 (2%)	1 (2%)	<0.001

Discussion

The presence of adhesive structures enables the bacteria to colonize and produce biofilm, and in addition to restrict antibiotic penetration into the cells. Type 1 and type 3 fimbria play a key role in the attachment of *Enterobacteriaceae* to the host epithelial and endothelial cells [20–22]. The biofilm formation has been less studied among *K. oxytoca* isolates. In this study, 33 isolates produced moderate-level biofilm and all were *npsB*-positive, which is important for colonization in the intestine and for toxin production. Furthermore, the presence of adhesin genes was significantly associated with biofilm formation (p <0.05). As shown, 29 of 30 of *fimA*-positive isolates, 19 of 21 *mrkA*-positive isolates, 32 of 48 *matB*-positive isolates and 32 of 46 *fimA*-positive isolates produced moderate-level biofilms; however, the gene expression of adhesins is yet to be revealed.

The presence of surface adhesion genes was significantly associated with resistance to the non-β-lactam antibiotics, suggesting the inhibitory role of adhesins in the drugs' infiltration. The presence of adhesion genes was independently associated with resistance to ciprofloxacin, tetracycline, gentamicin and trimethoprim-sulfamethoxazole discs. Several previous studies have shown the relation between resistance to antibiotics and presence of surface adhesive genes. Vuotto *et al.* showed that antibiotic resistance increases in *K. pneumoniae* when the isolates grow in biofilm mode [23]. Therefore, it is suggested that these antibiotics should be used with caution in the presence of biofilm formation or in biofilm-related infections.

Another study indicated the role of type 1 and type 3 fimbria of *K. pneumoniae* in the attachment to the murine urinary tract [20]. It has been shown that *K. pneumoniae* growth on abiotic and human cell surfaces is mainly mediated by the *mrkA* gene [24–26]. Twenty isolates contained all the adhesive genes and 19 of them produced moderate-level biofilms. Furthermore, nine isolates were multidrug-resistant *K. oxytoca* and could amplify all the adhesin-encoding genes. The results suggested that β-lactam resistance is not associated with the presence of surface adhesive structures, but resistance to other antibiotics is possibly related to these surface adhesins. In contrast, a significant relation was observed between *fimA*$^{+}$ *mrkA*$^{+}$ and *fimA*$^{-}$ *mrkA*$^{-}$ isolates and resistance to tetracycline and trimethoprim-

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sulfamethoxazole (p 0.0271). Investigation of the expression of the biofilm-related genes by quantitative real-time PCR will be helpful. In addition, more investigations are needed regarding biofilm formation and antibiotic resistance to allow more careful prescription of specific antibiotics.

The results showed the relation between the presence of surface adhesin-encoding genes and biofilm formation and also resistance to non-β-lactam antibiotics among *K. oxytoca* clinical isolates. In addition, biofilm production was not significantly associated with the β-lactam resistance among the isolates. Bacterial adhesion and colonization is related to biofilm formation and drug resistance, so it is essential to implement the control and prevention plans regarding biofilm-associated infections and eradication of infections.

Transparency declaration

The authors have no conflicts of interest to declare.

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