Cytotoxicity of *Klebsiella oxytoca* Isolated from Patients with Antibiotic-associated Hemorrhagic Colitis

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**Abstract** Sixteen strains of *Klebsiella oxytoca* isolated from fecal specimens of 11 patients with acute and convalescent phases of antibiotic-associated hemorrhagic colitis (AAHC), 19 strains of *K. oxytoca* from clinical specimens of patients with other diseases, and 2 type strains were examined for cytotoxicity and enterotoxicity. Twenty-one (57%) out of 37 culture supernatants of *K. oxytoca* strains were cytotoxic to at least one of the cultured cell lines (CHO, HeLa, HEp-2, TCMK-1, and Vero) tested (maximum titer: 5 or 10). The cytotoxic substance was moderately heat-stable and its molecular weight appeared to be less than 1,000. Such cytotoxic substance was found both in the strains from AAHC patients and the control strains of *K. oxytoca*. However, none of the culture supernatants demonstrated enterotoxicity in the rabbit ligated ileal loop test and the suckling mouse assay. From our results it remained to be determined whether the cytotoxic substance of *K. oxytoca* has any role in the pathogenesis of AAHC.

**Key words**: *Klebsiella oxytoca*; cytotoxicity; antibiotic-associated hemorrhagic colitis

Antibiotic-associated colitis can be divided into two groups according to its clinical features. One group is antibiotic-associated pseudomembranous colitis, known to be caused by the toxigenic bacterium *Clostridium difficile* (2, 3, 13, 19), although rare cases are caused by staphylococci (4) or *Clostridium perfringens* (17). The other group is antibiotic-associated hemorrhagic colitis (AAHC) without pseudomembranes, which occurs after administration of broad-spectrum beta-lactam antibiotics such as ampicillin (9, 16, 21). In Japan, *Klebsiella oxytoca* has been frequently detected as a predominant organism from bloody stool specimens of patients with AAHC (16, 20, 22). Colonoscopy in cases with AAHC shows mucosal erythema and erosion without pseudomembranes. Patients recover within a short period without specific therapy after withdrawal of antibiotics. While some investigators (1, 7, 18) speculate that AAHC is a mild type of pseudomembran-
branous colitis, others have suggested that AAHC is caused by a hypersensitivity mechanism (8, 15, 22). Recently, it has been reported that the culture supernatant of K. oxytoca isolated from AAHC patients causes cell death in several cell lines (10, 14). To further investigate the possible pathogenicity of K. oxytoca isolated from stools of patients with AAHC, we examined the cytotoxicity of K. oxytoca to 5 kinds of cultured cell lines and also its enterotoxicity using the rabbit ligated ileal loop test and the suckling mouse assay.

MATERIALS AND METHODS

Bacterial strains. Thirty-seven K. oxytoca strains were used. Sixteen strains were isolated from stools of patients with AAHC in both the acute and convalescent phases at Tokyo Metropolitan Komagome and Toshima General Hospitals (5). Four strains were from fecal specimens of ulcerative colitis patients, and the other 15 strains were from non-fecal specimens such as sputum, bile, urine, and pus from non-diarrheal patients at Tokyo Metropolitan Police Hospital. Six clinical isolates of K. pneumoniae were also used. The type strains of K. oxytoca ATCC 8724 and ATCC 13182, K. pneumoniae ATCC 13883, and C. difficile ATCC 17859 were included in each test as controls. The bacterial strains were stored at -80°C in 10% skim milk (Difco Laboratories, Detroit, Mich.) until tested.

Biochemical characteristics. All strains including the controls were examined for their biochemical characteristics using the API 20E System (Analytab Products, Montalieu-Vercieu, France). Additional tests were also done to examine other biological and biochemical characteristics.

Cytotoxicity assay. The K. oxytoca strains were inoculated into brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C in a shaker running at 200 rpm for 24 hr. The supernatant fluid was obtained by centrifugation, followed by membrane filtration (pore size, 0.22 µm, Millipore Corp., Bedford, Mass.). All of the culture supernatants were adjusted to pH 7.3–7.5. One hundred microliters of 5 x 10^4 tissue culture cells per ml in Eagle’s Minimal Essential Medium (Nissui Pharmaceutical Co., Tokyo), containing kanamycin (60 µg/ml) and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) was added to the wells of 96-well microtiter plates (Costar, Cambridge, Mass.) and incubated in air containing 5% CO₂ at 37°C for 2 days. Aliquots of 25 µl of the cell-free culture supernatant of K. oxytoca diluted in a two-fold series with Dulbecco’s phosphate-buffered saline (pH 7.4, Nissui Pharmaceuticals) were then added to 100 µl of the monolayer cultures in a well, and the microtiter plates were incubated at 37°C in CO₂ incubator for 48 hr. The cultured cells were observed at 4, 12, 24, and 48 hr. The results of the cytotoxicity assay were expressed as a reciprocal of the highest final dilution of culture supernatant that caused rounding of more than 90% of cultured cells in the wells. Culture supernatant of C. difficile ATCC 17859 was used as a positive control.

Physicochemical characterization of the culture supernatant. The culture supernatants filter-sterilized through a 0.22-µm pore size membrane of K. oxytoca were treated at
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60°C for 30 min or at 100°C for 15 min. The supernatant was also filtrated sequentially through DIAFLO ultra-filtration membrane (YM-2, molecular weight cut-off, 1,000, Amicon Corp., Lexington, Mass.) and both the filtrate and the retentate were also tested for cytotoxicity.

Assay for enterotoxicity. Both live cultures and culture supernatants were tested for ability to stimulate fluid accumulation in the ligated rabbit ileal loop according to the method of De and Chatterjee (6) with a slight modification. The ileal specimens were taken from Japanese albino rabbits weighing ca. 1.8 kg (Saitama Experimental Animals Supply Co., Saitama). The results were considered valid only if the positive and negative controls showed appropriate responses.

Culture supernatants were also tested for enterotoxicity by the suckling mouse assay as previously described (9). ICR suckling mice 2–3 days old (Charles River Japan Inc., Atsugi, Kanagawa) were used in the assay.

Antimicrobial susceptibility test. The minimum inhibitory concentrations (MICs) were determined by the agar dilution method established by Japan Society of Chemotherapy (11).

Plasmid detection procedure. Plasmids were extracted using the method described by Kado and Liu (12) with a slight modification and detected by agarose gel electrophoresis.

RESULTS

Identification of K. oxytoca

There were differences among K. oxytoca strains in the production of pigment on DHL agar and the fermentation of dulcitol and melezitose. Almost all of the strains isolated from bloody diarrhea in the acute phase of AAHC produced brown pigment and fermented dulcitol and melezitose. None of the K. oxytoca strains were hemolytic on blood agar containing blood from human, sheep, horse, ox, rabbit, chicken, or guinea pig.

Cytotoxicity

Cytotoxicity of culture supernatants of K. oxytoca on five cultured cell lines is shown in Table 1. No significant differences in positive rates of cytotoxicity on the tissue culture cells except for HEP-2 cells were found between K. oxytoca strains isolated from the AAHC patients and from control patients. The cytotoxicity titers of K. oxytoca to tissue culture cells (the titer ranged from 5 to 10), however, were much lower than that of C. difficile (titer, 1280). Morphological changes in five tissue culture cells, commonly characterized by cell rounding, began at 24 hr and reached a maximum at 48–72 hr after inoculation of the preparation (Fig. 1). On the other hand, none of the 6 clinical isolates of K. pneumoniae and K. pneumoniae ATCC 13883 were cytotoxic to the five cultured cell lines used. The cytotoxicity of the culture supernatants of K. oxytoca was not affected by heat treatment at 60°C for 30 min, but abolished by treatment at 100°C for 15 min. Cytotoxicity was present in the YM-2 membrane filtrates, but none was found in the YM-2 retentates,
Table 1. Cytotoxicity of culture supernatants of K. oxytoca to cultured cell lines\(^a\)

<table>
<thead>
<tr>
<th>Source of K. oxytoca</th>
<th>CHO</th>
<th>HeLa</th>
<th>HEp-2</th>
<th>TCMK-1</th>
<th>Vero</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAHC</td>
<td>8/16</td>
<td>4/16</td>
<td>12/16(^b)</td>
<td>10/16</td>
<td>8/16</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Control(^c)</td>
<td>5/15</td>
<td>3/15</td>
<td>6/15(^b)</td>
<td>5/15</td>
<td>3/15</td>
</tr>
<tr>
<td>Reference(^d)</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

\(^a\) No. of isolates with cytotoxicity/no. tested.
\(^b\) Statistically significant (\(P<0.05\)).
\(^c\) K. oxytoca strains isolated from sputum, bile, pus and urine of non-diarrheal patients.
\(^d\) K. oxytoca ATCC 8724 and ATCC 13182.

Fig. 1. Phase-contrast photomicrographs showing the cytotoxic effect of the culture supernatant of K. oxytoca TMH5-4 isolated from an AAHC patient on HeLa and HEp-2 cells. Untreated cells: (A) HeLa and (C) HEp-2. Supernatant-treated (48 hr) cells: (B) HeLa and (D) HEp-2 (\(\times 180\)).

suggesting that the molecular weight of the cytotoxic substance is less than 1,000.

**Ligated Ileal Loop Test and Suckling Mouse Assay**

Both live organisms (\(3 \times 10^9\) cells/loop) and culture supernatants of K. oxytoca strains including the reference strains did not cause or caused only moderate and marginal accumulation of clear or a slightly bloody viscous fluid (volume of fluid/length of loop ratio: <1.0) in the rabbit ligated ileal loops. All of K. oxytoca strains or K. pneumoniae ATCC 13883 were negative in the suckling mouse assay (the ratio of gut weight to remaining body weight: <0.070).
Antibiotic Resistance and Plasmid DNA Analysis

Thirty (82%) of 37 K. oxytoca strains were resistant to ampicillin (MIC, >25 µg/ml). Thirteen of 37 K. oxytoca strains contained from one to five plasmids, ranging in molecular weight from approximately 2.2 Md (megadaltons) to 200 Md. Of 13 plasmid-containing strains, only 5 strains were cytotoxic to the cultured cell lines.

DISCUSSION

In this study we found that a considerable number of culture supernatants of K. oxytoca strains were cytotoxic to more than one of the cultured cell lines tested, although such cytotoxicity was very weak as compared to that by C. difficile cytotoxin. The cytotoxicity of the culture supernatant of K. oxytoca to the cultured cells caused morphological effect different from those induced by cholera toxin or E. coli heat-labile enterotoxin. Minami et al (14) have reported that the specific activity to CHO cell of K. oxytoca purified cytotoxin is similar to that of C. difficile toxin A. The differences in cytotoxicity patterns among the cell lines might be due to the differences in sensitivity of each cell line to the cytotoxic substance(s). We wondered if the concentrated culture supernatants of non-cytotoxic strains (titer, <5) could show cytotoxic effect. However, 10-fold concentrated culture supernatants of non-cytotoxic strains showed no specific cytotoxic effect on several tissue culture cells. Cytotoxicity was equally found in the culture supernatants of K. oxytoca not only from the patients with AAHC but also from the patients with other diseases (except for cytotoxicity to HEp-2 cells). The cytotoxic substance appeared to have a molecular weight of less than 1,000 and to be relatively heat-stable (60°C, 30 min).

Tamura et al reported that most of the strains of K. oxytoca isolated from AAHC patients were capsule-type 19, and gave a positive hemorrhagic enteritis reaction in mouse loops (in abstract form; K. Tamura, and R. Sakazaki. Jpn. J. Clin. Pathol. [in Japanese], 24: 137, 1976). On the other hand, Shimada et al (18) reported that K. oxytoca isolated from a patient with ampicillin-induced pseudomembranous colitis failed to produce cecitis in hamsters. From our results of the rabbit ligated loop test and the suckling mouse assay, K. oxytoca and its culture supernatant did not show any enterotoxic effect on these animal models.

Despite some differences in biochemical characteristics, antibiotic resistance, and plasmid profiles among K. oxytoca strains, we could not find any relationships between these characteristics and the cytotoxicity to cultured cell lines.

In conclusion, we could not find any conclusive evidence that K. oxytoca or its cytotoxic substance has any role in the pathogenesis of AAHC. Further research would be needed to understand in more detail the pathogenesis of AAHC.

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REFERENCES


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