Cytotoxic Effects of *Klebsiella oxytoca* Strains Isolated from Patients with Antibiotic-Associated Hemorrhagic Colitis or Other Diseases Caused by Infections and from Healthy Subjects

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Antibiotic-associated hemorrhagic colitis (AAHC) is associated with *Klebsiella oxytoca*. This study analyzed whether cytotoxic properties are linked to specific subtypes of *K. oxytoca*. *Klebsiella* isolates from stools of AAHC patients, healthy carriers, and diarrhea patients as well as from infections of other organs were investigated. Cytotoxic effects on human epithelial cells were limited to the species *K. oxytoca* and were not detectable for any other *Klebsiella* species. Isolates from AAHC patients and from stools showed the highest proportion of cytotoxic strains. Urinary or respiratory tract isolates exhibited no cytoxicity. Macrorestriction profiling of strains revealed no genetic relationships of AAHC isolates or the cytotoxic phenotype but identified that different *K. oxytoca* strains with different cytotoxic behaviors may be prevalent in the same AAHC patient. Under laboratory conditions, cytotoxicity was maximally effective after exponential bacterial growth and then declined despite the continued viability of *K. oxytoca* cells in culture. Given its capacity to induce AAHC and that a high proportion of stool isolates tested cytoxin positive, we argue that *K. oxytoca* should be considered an opportunistic pathogen if detected in stools. The ability to induce disease after antibiotic treatment most likely represents an overgrowth of the toxin-producing bacterium due to an alteration of the normal colonic microflora.

Antibiotic-associated colitis (AAC) is a frequent adverse effect observed when the normal bacterial flora is altered due to antibiotic therapy. Most cases of AAC are caused by infection by and extensive growth of *Clostridium difficile*, leading to pseudomembranous colitis. A special form of AAC is antibiotic-associated hemorrhagic colitis (AAHC), which was first described in 1978 (21) and has since been ascribed specific clinical, endoscopic, histopathological, and microbiological characteristics (9, 10). AAHC is not associated with *C. difficile* and was only recently shown to be caused by *Klebsiella oxytoca* (9, 10). AAHC is typically observed after a brief therapy with penicillins, with a sudden onset of bloody diarrhea often in combination with severe abdominal cramps, which often requires hospitalization. The key features of AAHC upon endoscopy are mucosal hemorrhage and mucosal edema, usually with segmental distribution, commonly affecting the ascending colon and the cecum (9). Histology typically resembles that of colitis induced by toxin-producing bacteria (10).

For the majority of patients with AAHC, stool testing reveals *K. oxytoca* in significant amounts (>10^6 CFU/ml) (9, 23). This Gram-negative rod is ubiquitous in the environment (e.g., soil and water) but can also be isolated from skin, mucous membranes, and the intestines of humans and animals (19). Human infections with *K. oxytoca* resemble those with *Klebsiella pneumoniae*; i.e., respiratory and urinary tracts are commonly affected (e.g., nosocomial pneumonia), in addition to soft tissue and hepatobiliary infections (6). Until recently, *K. oxytoca* was not considered to be an intestinal pathogen, and its presence in stool has placed the organism as a constituent of the normal gut microflora. For the healthy population, colonization of the intestine with *K. oxytoca* has been reported for 1.6% to 9% of subjects (1, 4, 10, 23). It is important that *K. oxytoca* constitutively produces β-lactamases conferring resistance to amino- and carbapenem β-lactam antibiotics (13), agents typically used before the onset of AAHC. The association of *K. oxytoca* with AAHC was previously established by an animal model using a *K. oxytoca* strain isolated from a patient with AAHC in combination with an antibiotic to induce right-sided hemorrhagic colitis (10).

In the 1990s, two independent groups reported that *K. oxytoca* strains isolated from patients with AAHC produce a cytotoxin, which caused cell death in cultured Hep2, Vero, CHO-K1, and HeLa cell lines as well as in an isolated intestinal-loop

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model (8, 15–17). In contrast, two laboratory strains of *K. oxytoca* exhibited no cytotoxicity, indicating that cytotoxin production might be strain specific (16, 17). The cytotoxin was reported to be heat labile, insensitive to proteinase digestion, and of a low molecular mass (8, 16, 17). A detailed analysis of the chemical nature and molecular structure of the cytotoxin is not yet available. Moreover, a causal link between toxin production in *K. oxytoca* and AAHC has not been established.

The aim of the current study was to assess whether cytotoxin production is specific to certain subtypes of *K. oxytoca* and to test the hypothesis that AAHC uniquely correlates with those strains. A total of 121 *Klebsiella* isolates were investigated, including *K. oxytoca* strains isolated from stool samples from patients with AAHC, healthy carriers, and patients with colitis/diarrhea of other causes. *K. oxytoca* strains from infections involving other body sites and other *Klebsiella spp.* were analyzed in comparison. The characterization of all isolates was performed by using genotypic and biochemical methods, and the capacity of each isolate to induce cytotoxic effects on cultured eukaryotic cells was measured. A subset of strains was genotyped by macrorestriction profiling to assess their genetic relatedness.

**MATERIALS AND METHODS**

**Bacterial strains.** The *Klebsiella* isolates used in this study are listed in Table S1 in the supplemental material. The isolates were obtained from patients treated at the Medical University of Graz and from healthy volunteers. The study was approved by the local institutional review board, and written informed consent was obtained from all subjects. Two cytotoxin-negative reference isolates, *K. oxytoca* DSM 4796 (ATCC 8724) and DSM 5175 (ATCC 13182) (DSMZ, Braunschweig, Germany), and cytotoxin-producing strain MH 43-1 (kindly provided by T. Chida, Department of Microbiology, Medical and Dental University, Tokyo, Japan) (8) were served as controls. The viewer was blinded to the sources of isolates. All strains were grown in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) (8) served as controls. The viewer was blinded to the sources of isolates. All strains were grown in tryptic soy broth (TSB) (Merck, Germany) and M9 minimal medium (Invitrogen, Lofer, Austria) at 37°C under gentle shaking overnight or on tryptic soy agar (Merck).

All *Klebsiella* isolates were subjected to biochemical analyses by using the API 20E test (bioMérieux, Marcy l’Etoile, France). Indole production was assessed three times independently with each isolate by using James (R2) reagent according to the manufacturer’s specifications (bioMérieux). The polygalacturonase (pghK) gene was previously shown to be specific for *K. oxytoca* among *Klebsiella spp.*; thus, a 344-bp PCR amplification product of the *pghK* gene was employed for the identification of all isolates according to a previously reported procedure (11). Tag polymerase (NEB, Bedford, MA) was applied according to the manufacturer’s specifications.

Isolates that were not unambiguously identified by means of API testing, indole production, and PCR were subsequently subjected to 16S rRNA gene sequence analyses. The differentiation of species by 16S rRNA gene sequence analysis was performed according to a method described previously by Boye and Hansen (3). This approach differentiates between *Klebsiella* species according to species-specific base changes (3, 20). PCR products were cycle sequenced with the BigDye termination cycle sequencing ready reaction kit (v3.1; Applied Biosystems, Foster City, CA) and resolved by use of an ABI Prism 310 genetic analyzer. Sequence data analysis was performed by using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/), and multiple sequence alignments were performed with SeqMan II (DNASTar Inc.). Reference sequences were *Escherichia coli* (GenBank accession no. J01695), *K. oxytoca* ATCC 13182 (GenBank accession no. Y17655), *P. pneumotropica* ATCC 13883T (GenBank accession no. Y17656), *K. planticola* ATCC 3553T (GenBank accession no. Y17659), *K. ornithinolytica* 590861 (GenBank accession no. Y17662), and *K. terrigena* ATCC 33257T (GenBank accession no. Y17668).

**Cytotoxin tissue culture assay.** Cytotoxicity production was monitored in a modified cell culture assay as described previously (16). Hep2 cells were grown in minimal essential alpha medium with Earle’s balanced salt solution (Invitrogen, Lofer, Austria) and with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37°C with 5% CO₂ in 95% humidity. Cells were newly seeded every 48 h as recommended by the supplier (European Collection of Cell Culture [ECACC], Wiltshire, United Kingdom). Thirty milliliters of TSB (Merek, Darmstadt, Germany) was inoculated with a single bacterial colony and incubated for 14 to 16 h at 37°C with gentle agitation (180 rpm). Cytotoxic effects were measured for cultures grown to an optical density at 600 nm (OD₆₀₀) of 4 to 6. Cultures were then centrifuged at 5,000 rpm at 4°C for 20 min, and the supernatants were filtered through 0.2-μm cellulose acetate filters (Millipore, MA). Dilutions of the filtered supernatant were prepared in phosphate-buffered saline (PBS). A total of 1.5 × 10⁴ Hep2 cells in 100 μl culture medium were seeded per well in 96-well tissue culture plates (Greiner, Kremsmünster, Austria). Equivalent volumes (50 μl) of pure and diluted supernatants were added before incubation for 48 h. Eukaryotic cells were then assessed microscopically for cytotoxic effects as described previously (8, 16). The viability of Hep2 cells was assessed spectrophotometrically by using 3-(4,5-di-methyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO). Cells were washed once with 200 μl PBS per well and incubated with 200 μl of PBS containing 5 mg/ml MTT for 2 h at 37°C. The MTT solution was removed by aspiration, and cells were lysed with a 1:25 (vol/vol) solution of 96% acetic acid and 2-propanol (18). Absorbance units in the wells were then determined at a 595-nm wavelength (microplate reader 550; Bio-Rad, Hercules, CA). Each dilution was measured in triplicate and compared to controls treated with PBS only. The 50% cytotoxic dose (CD₅₀) was expressed as the final dilution of the bacterial supernatant wherein viability decreased to 50% of that of PBS-treated Hep2 cells. Statistical significance was determined by using the Fisher exact test to compare the AAHC isolates with *Klebsiella* isolates from other test groups (SigmaPlot 11.0; Systat Software Inc., San Jose, CA).

**Analysis of cytotoxin production during bacterial growth.** Cells of clinical isolate *K. oxytoca* 04/1O and the type strain *K. oxytoca* ATCC 13182 were grown in 2 ml TSB at 37°C for 48 h under gentle agitation. Bacterial cultures were harvested at regular time points, and the cells were collected by centrifugation at 5,000 rpm at 4°C for 20 min. Pellets were resuspended in 1 ml PBS and plated onto agar plates in serial dilutions. Bacterial supernatants were filtered and examined by the MTT test as described above. Values are expressed as means ± standard deviations (SD).

**Strain typing of isolates by PFGE.** Seventy *Klebsiella* isolates were genotyped by macrorestriction profiling with the restriction endonuclease XbaI and resolved by pulsed-field gel electrophoresis (PFGE) analysis as described recently (7, 12). Briefly, bacterial cells grown overnight in TSB were pelleted, washed once with 2 ml PBS, and diluted into PBS to an OD₆₀₀ of 0.2. The suspension was mixed with an equal amount of 2% low-melting agarose (FMC BioProducts, Rockland, ME) prepared in 0.5× Tris-borate-EDTA (TBE) buffer. Solidified blocks were incubated in 1.3 ml of lysing buffer (0.25 mM EDTA [pH 8], 1% SDS) containing 100 mg/ml protease K for 48 h at 50°C under gentle shaking. After 24 h, the buffer was removed, and fresh buffer was added. Agarose blocks were washed three times with 1.3 ml Tris-EDTA (TE) buffer containing 2 mM Pefabloc SC (Roche, Basel, Switzerland) at 37°C for 30 min. Subsequently, blocks were incubated with 40 U XbaI (Merck, Germany) for 48 h at 37°C under gentle shaking. After 24 h, the culture was harvested, and the supernatant was extracted as described previously (7, 12). A total of 121 *Klebsiella* isolates were genotyped by macrorestriction profiling with the restriction endonuclease XbaI and resolved by pulsed-field gel electrophoresis (PFGE) analysis as described recently (12). Bacterial cells grown overnight in TSB were pelleted, washed once with 2 ml PBS, and diluted into PBS to an OD₆₀₀ of 0.2. The suspension was mixed with an equal amount of 2% low-melting agarose (FMC BioProducts, Rockland, ME) prepared in 0.5× Tris-borate-EDTA (TBE) buffer. Solidified blocks were incubated in 1.3 ml of lysis buffer (0.25 mM EDTA [pH 8], 1% SDS) containing 100 mg/ml protease K for 48 h at 37°C under gentle shaking. After 24 h, the buffer was removed, and fresh buffer was added. Agarose blocks were washed three times with 1.3 ml Tris-EDTA (TE) buffer containing 2 mM Pefabloc SC (Roche, Basel, Switzerland) at 37°C for 30 min. Subsequently, blocks were incubated with 40 U XbaI (Merck, Germany) for 48 h at 37°C under gentle shaking. After 24 h, the culture was harvested, and the supernatant was extracted as described previously (7, 12).

**RESULTS**

Cytotoxic properties detected at the early stationary phase of *K. oxytoca* growth. The capacity of toxin-producing AAHC *K. oxytoca* isolate 04/1O to impair Hep2 cell viability compared to laboratory control strain ATCC 13182 was monitored over 48 h of bacterial growth. Cytotoxic properties were first observed for the conditioned culture medium of the clinical isolate at the end of the logarithmic growth phase (Fig. 1B). The capacity to mediate substantial cell killing was maintained throughout an additional 30-h period. At this point, after 44 h
of cultivation, no variation in the viability of isolate 04/1O was observed (Fig. 1A), but the culture medium no longer exhibited cytotoxic effects on Hep2 cells. Cytotoxin production by isolate 04/1O was not observed under similar conditions in M9 minimal medium (not shown).

**Clinical source of *Klebsiella* isolates.** The clinical diagnoses for the patients and the sources of isolation of *Klebsiella* strains are shown in Table 1 and Table S1 in the supplemental material. Fifteen *K. oxytoca* isolates from 13 patients with AAHC (group I) were isolated during the active phase of colitis. AAHC was diagnosed based on typical clinical and endoscopic and/or radiological features (9, 10). All AAHC patients tested negative for *Clostridium difficile* and other intestinal pathogenic bacteria. *K. oxytoca* strains of group II were obtained from patients with *C. difficile*-negative AAC or from other forms of diarrhea or colitis with negative stool cultures for intestinal pathogens. Thirteen *K. oxytoca* isolates (group III) were derived from stools of healthy carriers without intestinal symptoms or preceding antibiotic therapy. For comparison, *K. oxytoca* isolates from other organ infection sites were also tested. These included isolates from the urinary tract (group IV; n = 10), the respiratory tract (group V; n = 16), bacteremia (group VI; n = 13), and mucocutaneous infections (group VII; n = 16). *K. pneumoniae* isolates (group VIII; n = 19) and other *Klebsiella* species (group IX; n = 5) originated from stool samples of either healthy volunteers or diarrhea patients.

**Klebsiella identification.** The results for the three different methods applied for strain identification are compared in Table S1 in the supplemental material. Results for API 20E biochemical testing, and the *K. oxytoca*-specific *pheX* PCR analysis (11) were consistent for 105 of 124 strains (121 clinical strains and 3 control strains). Discrepancies were observed for 21 isolates. 16S rRNA gene analysis was used to clarify the taxonomy of uncertain strains (Table S1). Four of the 21 isolates were identified as *K. oeni* and one was identified as *K. planticola* based on 16S rRNA gene analyses. Cytotoxin production by the 21 aberrant isolates was monitored with the cell culture assay. The five isolates found to reduce the viability of cultured Hep-2 cells were all identified as being *K. oxytoca* isolates by 16S rRNA gene sequencing (see below).

**Cytotoxin production by *K. oxytoca* isolates.** The capacity of these bacterial isolates to produce a cytotoxic substance was assessed by the cultivation of Hep2 cells in standard medium supplemented with cell-free supernatant of the bacterial isolate grown for 14 to 16 h. As illustrated in the optimized assay in Fig. 1, conditioned culture medium from a toxin-producing strain grown to early stationary phase contains sufficient cytotoxin for obvious detection even after extensive dilution. A range of pure and serially diluted aliquots of filtered culture medium from each isolate grown to this stage was tested in cell culture. Eukaryotic cell viability was evaluated microscopically (Fig. 2). The cytotoxic effect was evident by cell rounding and detachment from the substratum, indicating cell death. Hep2 cells also exhibited cell fragmentation typically observed for apoptosis. Comparative cell viability was determined quantitatively based on MTT uptake and reduction with a colorimetric assay (18). The bacterial supernatant was defined as being toxin positive when the aliquot of undiluted supernatant added to the tissue culture medium was sufficient to reduce the viability of the Hep2 cells by ≥50% compared to a supplement of PBS alone. No discernible effects on Hep2 cells were observed upon microscopic evaluation or for the MTT test following cultivation in the presence of PBS or conditioned growth medium from the toxin-negative control (Fig. 3A and B). Ninety test strains (73%) showed no cytotoxin production. In contrast, cytotoxic effects were detectable for cultures containing the medium of 31 (27%) bacterial isolates. The 31 strains inducing positive toxic effects were evaluated further and assigned various levels of cytotoxicity, as defined in Fig. 3. Based on these results, 11 strains were assigned low toxin production (Fig. 3C). Fourteen strains induced medium cytotoxic effects (Fig. 3D), and 6 were identified as being highly cytotoxic (Fig. 3E). Notably, all toxin-producing isolates were identified as being *K. oxytoca* isolates. From the total number of *K. oxytoca* isolates investigated (97), 66 (68%) exhibited no evidence of toxin
production and 31 (32%) reduced the viability of the cultured human cells (Fig. 3F).

Cytotoxicity of *K. oxytoca* from patients with AAHC and other infections. To examine whether cytotoxin production was correlated with other shared properties of *Klebsiella* strains, isolates were classified into nine groups according to their clinical diagnosis and the source of bacterial strains (Table 1). *K. oxytoca* isolates from AAHC patients showed the highest proportion of toxin-producing strains (69%) (Fig. 4). Cytotoxin production was also a common finding for other *K.

### TABLE 1. *Klebsiella* isolates used in this study

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Diagnosis (no. of patients)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Isolation source</th>
<th>No. of Patients</th>
<th>No. of cytotoxic isolates/no. of cytotoxin-negative isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>AAHC</td>
<td>AAHC (13)</td>
<td>Stool culture</td>
<td>13/15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9/6</td>
</tr>
<tr>
<td>II</td>
<td>Diarrhea</td>
<td>AAC (7), diarrhea (4), IBD (2), hemorrhagic colitis (1)</td>
<td>Stool culture</td>
<td>14</td>
<td>8/6</td>
</tr>
<tr>
<td>III</td>
<td>Healthy carrier</td>
<td>Asymptomatic carriers (13)</td>
<td>Stool culture</td>
<td>13</td>
<td>6/7</td>
</tr>
<tr>
<td>IV</td>
<td>Urinary tract</td>
<td>UTI (7), complicated UTI (3)</td>
<td>Urine culture</td>
<td>10</td>
<td>0/10</td>
</tr>
<tr>
<td>V</td>
<td>Respiratory tract</td>
<td>VAP (7), pneumonia (5), exacerbated COPD (2), bronchitis/bronchiectasis (2)</td>
<td>Respiratory culture</td>
<td>16</td>
<td>0/16</td>
</tr>
<tr>
<td>VI</td>
<td>Blood</td>
<td>Bacteremia (10), CRBSI (3)</td>
<td>Blood culture</td>
<td>13</td>
<td>2/11</td>
</tr>
<tr>
<td>VII</td>
<td>Skin</td>
<td>DFS (6), CSSTI (3), other mucocutaneous infections (7)</td>
<td>Wound swab</td>
<td>16</td>
<td>6/10</td>
</tr>
<tr>
<td>VIII</td>
<td><em>K. pneumoniae</em></td>
<td>Asymptomatic carriers (9), AAC (7), IBD (2), hemorrhagic colitis (1)</td>
<td>Stool culture</td>
<td>19</td>
<td>0/19</td>
</tr>
<tr>
<td>IX</td>
<td>Other <em>Klebsiella</em> species</td>
<td>Hemorrhagic colitis (2), AAC (1), IBD (1), diarrhea (1)</td>
<td>Stool culture</td>
<td>5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

<sup>a</sup> In total, 121 *Klebsiella* isolates were divided into 9 test groups according to the clinical diagnosis of patients and the source of isolation.  
<sup>b</sup> If not otherwise indicated, isolates were classified as being *K. oxytoca* strains.  
<sup>c</sup> Abbreviations: AAHC, antibiotic-associated hemorrhagic colitis; AAC, antibiotic-associated colitis; IBD, inflammatory bowel disease; UTI, urinary tract infection; VAP, ventilator-associated pneumonia; COPD, chronic obstructive pulmonary disease; CRBSI, central venous catheter-related bloodstream infection; DFS, diabetic foot syndrome; CSSTI, complicated skin and skin structure infection. The numbers of patients from each subclass are noted in parentheses.  
<sup>d</sup> Fifteen isolates were derived from 13 patients.

FIG. 2. Cytotoxic effects of bacterial supernatants on cultured human cells. (A and B) Hep2 cells cultivated in medium supplemented with supernatant (1/48 dilution) from toxin-negative *K. oxytoca* laboratory strain ATCC 13182 shown at ×100 (A) and ×400 (B) magnifications. (C and D) Hep2 cells incubated with bacterial supernatant obtained from *K. oxytoca* strain 04/1O isolated from a patient suffering from AAHC (1/48 dilution) shown at ×100 (C) and ×400 (D) magnifications.
oxytoca strains isolated from stools. Fifty-seven percent of strains originating from diarrhea cases (acute and chronic forms of colitis) as well as 46% of strains isolated from asymptomatic carriers displayed cytotoxicity. Isolates originating from skin infections also contained a high proportion of cytotoxic strains (37%). In contrast, only two isolates originating from bloodstream infections showed cytotoxicity. One strain was isolated from a catheter-related bloodstream infection (CRBSI), and the second strain was isolated from a patient with cholangitis. None of the isolates originating from the respiratory and urinary tracts exhibited a cytotoxic phenotype. Also, none of the Klebsiella pneumoniae strains or any other Klebsiella species, all of which were isolated from stool samples, exhibited cytotoxic activity.

Quantification of the cytotoxic effects induced by the isolates revealed that the isolates originating from AAHC cases and from skin infections had the highest toxin levels, detectable at least until a dilution of 1/48 of the bacterial supernatant. One isolate of the highest cytotoxicity class was obtained from a non-AAHC diarrhea patient. Isolates from other organs did not reach the highest level of cytotoxicity (Fig. 4).

Genotyping revealed no clonal relationship among K. oxytoca isolates from AAHC cases. A representative number of 70 isolates, including 13 strains from AAHC cases, were analyzed by macrorestriction profiling by means of PFGE. No clonal relationship of the AAHC isolates or of any other group of isolates in relation to their isolation source or cytotoxic prop-
Cytotoxic effects of *Klebsiella* were evident by XbaI macrorestriction profiling (data not shown).

**Simultaneous isolation of cytotoxin-positive and cytotoxin-negative strains from a patient with AAHC.** Figure 5 shows typing results and cytotoxin testing results for five *K. oxytoca* isolates obtained from the same AAHC patient during the active phase of the disease. Macrorestriction profiling demonstrated that three genetically different strains were present within the five isolates. One strain displayed cytotoxin production, while the two other strains exhibited no cytotoxic effects in the cell culture assay (Fig. 5). This finding indicates that cytotoxin-positive and cytotoxin-negative *K. oxytoca* strains can be present simultaneously in the intestines of patients with AAHC.

**DISCUSSION**

This study characterized the cytotoxic phenotypes of 121 *Klebsiella* isolates, comprising 97 *K. oxytoca* isolates and 24 isolates of other *Klebsiella* species, including *K. pneumoniae* (*n* = 19), *K. ornithinolytica* (*n* = 4), and *K. planticola* (*n* = 1). The isolates originated from AAHC patients as well as from other isolation sources. The analysis revealed that cytotoxin production was limited to *K. oxytoca* and was not detectable for other *Klebsiella* species tested, including *K. pneumoniae*. We also observed a strong association between the cytotoxicity of *K. oxytoca* and AAHC. Sixty-nine percent of the *K. oxytoca* isolates obtained from AAHC patients produced the cytotoxin. Not all AAHC isolates were cytotoxin positive. Importantly, however, we observed that a single AAHC patient carried multiple, genetically distinct *K. oxytoca* strains, which were toxin positive as well as toxin negative. If generally true, this finding may explain why the available isolates for some AAHC patients did not score positively in the cytotoxicity test. Moreover, it follows that more than one *K. oxytoca* stool isolate needs to be tested for cytotoxicity production if this bacterium is suspected to be the cause of intestinal disease. An alternative pathophysiological mechanism other than the toxin production of *K. oxytoca* in inducing colitis cannot be ruled out by this study. However, taken together with the typical histological features of toxin-induced colitis seen in AAHC and in colitis induced by *K. oxytoca* in animal models of this disease (10), the current results support the hypothesis that the *K. oxytoca* cytotoxin is associated with AAHC. Future studies need to clarify this issue.

Cytotoxic effects of *K. oxytoca* isolates from AAHC patients were reported previously (1, 8, 10, 15–17). To date, however, those reports were not extended to include other *Klebsiella* species or *K. oxytoca* strains from infections of other organs. An important finding of the current study was that the majority of toxin-positive strains were obtained from stool cultures regardless of whether they were isolated from symptomatic or asymptomatic carriers. Accordingly, we propose that *K. oxytoca* should not be viewed as a naive constituent of the normal bowel microflora, in contrast to other *Klebsiella* species. Given its capacity to induce AAHC, resident *K. oxytoca* should be classified as an opportunistic pathogen that is able to induce disease under certain circumstances, such as an alteration of the normal colonic microflora due to antibiotic treatment and the subsequent overgrowth of the toxin-producing bacterium (5). This proposal is supported by the finding that despite the
fact that a high proportion of isolates originating from symptomatic carriers showed cytotoxicity, their amounts in stools were significantly lower than amounts in stools from symptomatic patients (\(< 10^3\) CFU/ml compared to \(4 \times 10^6\) CFU/ml for AAHC patients) (23). The coexistence of toxin-positive and toxin-negative isolates in patients and in healthy individuals is consistent with the hypothesis that colitis may result from toxin production during the outgrowth of these strains following antibiotic treatment. Moreover, for the patient group with acute or chronic diarrheal diseases, more than half of the isolates were cytotoxic positive. Thus, the role of cytotoxic-producing \(K.\) \(oxytoca\) in intestinal disease other than AAHC will require further investigation (23).

Interestingly, cytotoxicity was also detected for a large proportion of skin isolates, which were derived primarily from foot ulcers. These wound infections are thought to be derived via fecal contamination (2). None of the isolates originating from typical \(Klebsiella\) infection sites, i.e., the urinary and respiratory tracts, showed a cytotoxic phenotype. Two isolates from bloodstream infections were cytotoxic positive: one was isolated from a patient with CRBSI, and the second was obtained from a patient with cholangitis. It is reasonable to propose that the origin of these infection-causing strains was the skin or intestine. In summary, we found that cytotoxic-positive \(K.\) \(oxytoca\) isolates most frequently originated from body sites with high rates of bacterial colonization, such as the intestine and skin, whereas isolates from the urinary and respiratory tracts were cytotoxic negative.

A rigorous identification of the isolates used here was performed with four different phenotypic and genotypic methods. These included API 20E testing, indole production, pehX PCR, and, ultimately, 16S rRNA gene analysis for ambiguously identified isolates. The “gold standard” for differentiation between \(K.\) \(pneumoniae\) and \(K.\) \(oxytoca\) detects the tryptophanase-catalyzed conversion of tryptophan to indole in \(K.\) \(oxytoca\). Importantly, the phenotypic test can yield false-negative results due to the loss of activity by some strains of \(K.\) \(oxytoca\). Despite this, the phenotypic test can yield false-negative results due to the loss of activity by some strains of \(K.\) \(oxytoca\).

We thank Susanne Häusler for her assistance in performing the PFCs and Christina Strempfl and Bernadette Neuhold for their technical assistance.

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