Identification of Clinical Isolates of Indole-Positive *Klebsiella* spp., Including *Klebsiella planticola*, and a Genetic and Molecular Analysis of Their β-Lactamases

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Received 3 March 1997/Returned for modification 29 April 1997/Accepted 10 June 1997

In a collection of 43 indole-positive *Klebsiella* clinical isolates, which were initially identified as *Klebsiella oxytoca*, there were 18 isolates which exhibited a pattern characteristic of extended-spectrum β-lactamase (ESBL) resistance. This study aimed to confirm their identity by biochemical tests and by PCR and to determine the genetic basis for their resistance to the β-lactams and broad-spectrum cephalosporins. Chromosomal β-lactamase genes were analyzed by PCR, and plasmid-mediated β-lactamase genes were analyzed by conjugation and transformation. There were 39 isolates which grew on melezitose but failed to grow on 3-hydroxybutyrate, confirming them as *K. oxytoca*. PCR analysis of their β-lactamase genes divided these isolates into two groups, the bla*OXY-1* group and the bla*OXY-2* group. Each group had β-lactamases with different isoelectric points; the bla*OXY-1* group had β-lactamases with isoelectric points at 7.2, 7.8, 8.2, and 8.8, and the more common bla*OXY-2* group had β-lactamases with pIs at 5.2, 5.4 (TEM-1), 5.7, 5.9, 6.4, and 6.8. A pI of 5.2 was the most frequently detected and accounted for 59% of all the bla*OXY-2* β-lactamases. Hyperproduction of clavulanate-inhibited chromosomal β-lactamases was detected in 17 *K. oxytoca* isolates, resulting in an ESBL phenotype. *K. oxytoca* isolates having a plasmid-mediated genetic basis for their ESBL phenotype were not found, confirming that, in *K. oxytoca*, plasmids are rarely involved in conferring resistance to the newer cephalosporins. Four isolates proved to be isolates of *K. planticola* in which the β-lactamase genes failed to react with the primers used in the PCR. One *K. planticola* isolate contained a transferable plasmid harboring the SHV-5 β-lactamase gene and showed an ESBL phenotype, while the other non-ESBL *K. planticola* isolates contained chromosomal β-lactamases with isoelectric points at 7.2, 7.7, and 7.9 plus 7.2.

The genus *Klebsiella* is comprised of four species, *Klebsiella pneumoniae*, *K. oxytoca*, *K. planticola* (formerly *K. trevisanii*), and *K. terrigena* (6, 20). Modern identification systems used in many hospital diagnostic laboratories usually identify indole-negative *Klebsiella* isolates as *K. pneumoniae* and indole-positive *Klebsiella* isolates as *K. oxytoca*, unless supplementary tests are performed. However, up to 19% of *Klebsiella* isolates initially identified as *K. oxytoca* have been identified subsequently as *K. planticola* (9, 14). *K. planticola* strains were originally isolated from plant and aquatic environments (2, 6, 17) and have been associated with various human infections, including septicaemia (9).

Growth on media containing melezitose, pectate, gentisic acid, and ethanolamine and no growth on media containing 3-hydroxybutyrate or histamine differentiate *K. oxytoca* from *K. planticola* (14). Monnet and Freney (14) suggest that *K. planticola* is tentatively identified as *K. oxytoca* if the isolate contains indole-negative reactions of melezitose, pectate, gentisic acid, histamine, 3-hydroxybutyrate, melezitose, and filtrate, and DNA primers have been reported that recognize either the

bla*OXY-1* gene (668 bp) or the bla*OXY-2* gene (723 bp), one of which is present in all *K. oxytoca* isolates (8). Each *K. oxytoca* isolate produces a positive PCR fragment for either the bla*OXY-1* or the bla*OXY-2* set of primers. Therefore, an indole-positive *Klebsiella* isolate not producing a PCR product should belong to *K. planticola*. The purpose of the present study was to use PCR methods to determine if an analysis of β-lactamase genes could be used to differentiate *K. planticola* from other isolates of indole-positive *Klebsiella* spp. The location of the β-lactamase genes in those isolates with extended-spectrum β-lactamase was also examined.

**MATERIALS AND METHODS**

**Bacteria.** A total of 43 indole-positive *Klebsiella* isolates from Australian hospitals, including Sir Charles Gardiner Hospital and Fremantle Hospital (both in Western Australia), The Canberra Hospital (Australian Capital Territory), Alfred Hospital and Monash Medical Centre (both in Victoria), and Princess Alexandra Hospital (Queensland), were investigated. All strains had been identified as *K. oxytoca* with the Vitek AutoMicrobic System (Biomerieux Vitek, Sydney, Australia). A plasmid-free naldixic acid-resistant strain, *Escherichia coli* J9909, was used as the recipient in plasmid transfer experiments. *E. coli* strains harboring TEM-1, SHV-1, and SHV-5 β-lactamases were used as reference strains for isoelectric focusing (IEF) experiments.

**Carbon substrate assimilation tests.** The minimal medium, used to determine growth of indole-positive *Klebsiella* isolates on various carbon substrates, was made with the following heat-sterilized stock solutions in a final volume of 100 ml: 0.5 ml of solution A (70 g of K2HPO4 and 30 g of KH2PO4 in 100 ml of H2O), 0.5 ml of solution B (1 g of MgSO4·7H2O, 0.2 g of NaCl, and 0.15 g of MgSO4·7H2O in 100 ml of H2O), 0.05 ml of solution C (0.15 g of citric acid and 0.15 g of (NH4)2SO4·FeSO4·6H2O in 100 ml of H2O), and 1 ml of solution D (20 g of (NH4)2SO4 in 100 ml of H2O). The carbon substrates examined, ethanolamine, gentamic acid, histamine, 3-hydroxybutyrate, melezitose, and pectate, were prepared as filter-sterilized stock solutions and used at a 0.2% (wt/vol) final concentration. For assimilation tests, isolates were grown overnight in broth,
of 5.2 to 6.8 (8). PCR conditions included 35 cycles of denaturation at 94°C for

9
9
-(CCT GCT GCG GCT GGG TAA AA)-3
TTA ACA CG)-3
9
9

\textbf{TABLE 1. Resistance of 39 strains of \textit{K. oxytoca} to various \beta-lactams}

<table>
<thead>
<tr>
<th>\beta-Lactam*</th>
<th>Conc (mg/liter)</th>
<th>No. of ESBL strains (n = 17)</th>
<th>No. of non-ESBL strains (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp</td>
<td>8</td>
<td>17 (100)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>Amc</td>
<td>8 (4)*</td>
<td>17 (100)</td>
<td>6 (27)</td>
</tr>
<tr>
<td>Oth</td>
<td>8</td>
<td>17 (100)</td>
<td>8 (36)</td>
</tr>
<tr>
<td>Man</td>
<td>8</td>
<td>17 (100)</td>
<td>8 (36)</td>
</tr>
<tr>
<td>Fox</td>
<td>8</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Azt</td>
<td>8</td>
<td>17 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ctx</td>
<td>1</td>
<td>16 (94)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Imp</td>
<td>4</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ctz</td>
<td>8</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ctz</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Amp, ampicillin; Amc, co-amoxyclav; Oth, cephalothin; Man, cefamandole; Fox, cefoxitin; Azt, aztreonam; Ctx, ceftaxime; Imp, imipenem; Ctz, ceftriaxime.

washed in saline, and diluted to the opacity of a 0.5 McFarland opacity standard and 0.1 ml of diluted culture was added to 5 ml of assimilation medium. Growth was recorded after incubation for 24 and 48 h at 30 and 37°C, respectively.

Antibiotic susceptibility testing. The resistance phenotype of each strain was initially determined by agar dilution (18) and disk diffusion methods (19) with National Committee for Clinical Laboratory Standards interpretative criteria.

Clavulanate inhibition by double-disc diffusion. Strains resistant to broad-spectrum cephalosporins were tested for clavulanate inhibition of \beta-lactamase by the double-disc diffusion method described by Mulgrave and Attwood (16), which is a modification of the Casals and Pringler method (4).

PCR analysis for \beta-lactamase detection. Primer C, 5'-GCC TAG CGC TGA TTA ACA CG-3'; and primer D, 5'-(CCT GCT GCG GCT GGG TAA AA)-3', were used to detect the \textit{bla}_{OXY-2} gene, which codes for \beta-lactamases with pI in the range of 7.8 to 8.8, and primer L, 5'-CAT TCG AAG AGG GTG CA-3', and primer M, 5'-ACC TCT TTG CCG TTT TTC GC-3', were used to detect the \textit{bla}_{OXY-2} gene, which codes for \beta-lactamases with pI in the range of 5.2 to 6.8 (8). PCR conditions included 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 60°C (for \textit{bla}_{OXY-2}) or 55°C (for \textit{bla}_{OXY-2}), and extension at 72°C for 1 min. The PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and photographed with UV illumination. The PCR fragments were sized with SPP1/RI size standards.

\beta-Lactamase extraction. Cells were grown overnight at 37°C in 10 ml of brain heart infusion broth. Suspensions were pelleted, washed, resuspended in 10 mM phosphate buffer (pH 7), and chilled on ice prior to sonication (MSE, London, United Kingdom). Sonicates were cleared by centrifugation at 14,000 × g for 10 min, and the supernatants containing the crude \beta-lactamase extract were stored at -80°C.

Analytical IEF. Crude \beta-lactamase extracts were diluted with 10 mM phosphate buffer (pH 7) until the durations of the reactions with 50 mg of Nitrocefin (Oxoid, Basingstoke, United Kingdom) per liter were within the range of 15 to 30 s. IEF was carried out with a Multiphor II unit (LKB, Bromma, Sweden) according to the manufacturer’s recommendations. \beta-Lactamases with known pI in the pH range of 3.5 to 9.5 (TEM-1, 5.4; SHV-1, 7.7; SHV-5, 8.2) were included in each focusing run. The gels were overlaid with 200 mg of nitrocefin per ml in 10 mM phosphate buffer (pH 7) to visualize the \beta-lactamases.

Resistance transfer. Conjugation experiments were conducted with broth both at room temperature overnight and at 37°C for 4 h by mixing fresh overnight broth cultures in the ratio of 1 ml of donor to 10 ml of recipient (23).

Electrotransformation was conducted with a high-efficiency electrotransformation kit (Electroporation; Bio-Rad Laboratories), using the pulse controller at 25 ms, 5.5 kV, and 200 μF. The range of the time constant was 4 to 5 ms. Plasmid pUC18 was used as donor DNA in control experiments. Transformation was also conducted by osmotic shock (13). Transconjugants were selected on MacConkey agar containing nalidixic acid (100 mg/liter) and ampicillin (100 mg/liter), and transformants were selected on MacConkey agar containing ampicillin (100 mg/liter).

Preparation and electrophoresis of plasmid DNA. Plasmid DNA was extracted from \textit{E. coli} by the alkaline lysis method (3) and from \textit{Klebsiella} species according to the procedure of Domingo et al. (5). DNA was electrophoresed in 0.8% agarose gels for 2 h at 5 V cm⁻¹ and sized against four reference plasmids with molecular sizes of 170, 71, 40, and 21 kb.

**RESULTS**

Of the 43 clinical isolates initially identified as \textit{K. oxytoca}, 18 were resistant to 8 mg of aztreonam per ml and 1 mg of ceftriaxone per ml and were positive in the double-disc diffusion test. These properties are consistent with the characteristics of ESBL producers (7, 25). The remaining 25 isolates were susceptible to those cephalosporins and negative in the double-disc diffusion test and were classified as non-ESBL isolates. All isolates were examined for growth on ethanoamine, gentamic acid, histamine, 3-hydroxybutyrate, melezitose, and pectate. Two substrates, melezitose and 3-hydroxybutyrate, more reliably identified a subgroup among the indole-positive \textit{Klebsiella} isolates. By using carbon substrate assimilation tests, four isolates were identified as \textit{K. planticola}, while the remaining 39, which utilized melezitose and failed to utilize 3-hydroxybutyrate, were confirmed as \textit{K. oxytoca}.

**Antimicrobial susceptibility.** All 39 \textit{K. oxytoca} isolates were resistant to ampicillin. Seventeen isolates showed reduced susceptibility or resistance to narrow- and expanded-spectrum cephalosporins and aztreonam, and 16 exhibited resistance to ceftriaxone at 1 mg/liter. These were classified as ESBL \textit{K. oxytoca} isolates, and the remaining 22 strains were classified as non-ESBL \textit{K. oxytoca} isolates (Table 1). Clavulanate inhibition of \beta-lactamase activity was significantly greater in the non-ESBL group, as reflected in the percentage of strains resistant to co-amoxyclav (27% of non-ESBL isolates compared to 100% of ESBL isolates). In the non-ESBL group, eight (36%) strains showed resistance to cefalothin and cefamandole. Aztreonam and ceftriaxone resistance was absent from the non-ESBL \textit{K. oxytoca} isolates.

Cefotixin resistance, which is commonly associated with chromosomally mediated \beta-lactamase production, was not detected in any \textit{K. oxytoca} isolate. Also, resistance to imipenem and ceftriaxone was not detected in any indole-positive \textit{Klebsiella} isolate.

**Transfer of antimicrobial resistance.** In mating experiments with indole-positive \textit{Klebsiella} donors and \textit{E. coli} JP990, three donors produced ampicillin-resistant transconjugants. One \textit{K. planticola} donor had a transferable plasmid with an SHV-5 gene, and it transferred resistance to ampicillin, cefotaxime, ceftazidime, ceftriaxone, aztreonam, and aminoglycosides. This \textit{K. planticola} donor is an ESBL strain. Two non-ESBL \textit{K. oxytoca} donors had transferable plasmids with the TEM-1 gene. Both isolates transferred ampicillin, trimethoprim, and aminoglycoside resistance. All three transferable plasmids were extracted from the \textit{E. coli} transconjugants and appeared to be greater than 100 kb in size.

Plasmid DNA could not be detected in agarose gels from any ESBL \textit{Klebsiella} strain. In transformation experiments, three preparations of plasmid DNA extracted from 17 ESBL

**TABLE 2. \beta-Lactamase isoelectric points and antibiotic resistance patterns for \textit{K. planticola} strains**

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Resistance pattern*</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amp, Nit, Oth, Man, Azt, Ctx, Gm, Tb</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>Amp, Nit, Oth, Man</td>
<td>7.9 ± 7.2</td>
</tr>
<tr>
<td>3</td>
<td>Amp, Nit</td>
<td>7.7</td>
</tr>
<tr>
<td>4</td>
<td>Amp, Oth, Man, Fox</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* Amp, gentamicin; Tb, tobramycin; Nit, nitrofurantoin; Amp, ampicillin; Oth, cephalothin; Man, cefamandole; Fox, cefoxitin; Azt, aztreonam; Ctx, ceftaxime; Gm, ceftriaxone; Tb, tobramycin.

* pl of SHV-5.
K. oxytoca isolates produced ampicillin-resistant transformants in E. coli JP990 by either electroporation or osmotic shock. All transformants contained an identical 2.6-kb plasmid, as determined by restriction enzyme mapping (Fig. 1). The donors, whose β-lactamase had a pI of 5.2, were resistant to ampicillin, narrow- and expanded-spectrum cephalosporins, and aztreonam and showed intermediate susceptibility to ceftazidime (MICs, >1 to <8 mg/liter). By comparison, the transformants were susceptible to aztreonam and ceftazidime and resistant to ampicillin and narrow- and expanded-spectrum cephalosporins and had a β-lactamase with a pI of 5.2.

**PCR tests.** All 43 indole-positive Klesbiella isolates were analyzed by PCR with both OXY-1 gene and OXY-2 gene primers. A total of 27 strains (13 ESBL and 14 non-ESBL) were OXY-2 gene positive, while 12 strains (four ESBL and eight non-ESBL) were OXY-1 gene positive. Four strains (one ESBL and three non-ESBL) contained neither OXY-1 nor OXY-2 genes, while the donor K. planticola transformants were negative for both OXY-1 and OXY-2 genes (Fig. 2). Based on carbon substrate assimilation tests, these four PCR-negative strains were identified as K. planticola.

Three ampicillin-resistant E. coli transformants containing the 2.6-kb plasmid were negative for both OXY-1 and OXY-2 genes, while the donor Klesbiella isolates exhibited a positive reaction when tested with the OXY-2 gene primers.

**Determination of the β-lactamase isoelectric points.** The isoelectric points of the β-lactamase enzymes produced by each of the four K. planticola isolates (Table 2) and the 39 K. oxytoca isolates (Table 3) were determined. Chromosomal β-lactamase was produced in abundantly high amounts by the 17 ESBL K. oxytoca strains, based on short nitrocefin reaction times. The 22 non-ESBL K. oxytoca strains produced detectable amounts of β-lactamase. However, the crude extracts from the non-ESBL isolates with resistance to the cephalosporins required dilution to reduce activity for successful IEF.

Six β-lactamases, with pIs of 5.2, 5.4, 5.7, 5.9, 6.5, and 6.8, comprised the bla_{OXY-2} group. The bla_{OXY-2} β-lactamase with a pI of 5.2 was the predominant enzyme across all strains (59 and 47% in ESBL and non-ESBL strains, respectively). β-Lactamases with alkaline pIs of 7.8, 8.0, and 8.8 comprised the bla_{OXY-1} group. Co-amoxiclav susceptibility and prolonged nitrocefin reaction times in the crude β-lactamase extracts suggested that enzyme hyperproduction was absent in all four K. planticola isolates. The isoelectric points and resistance patterns of K. planticola strains are listed in Table 2.

**DISCUSSION**

Recently, the frequency of indole-positive Klesbiella strains identified as K. oxytoca has increased (15). Our study has shown, however, that a significant proportion (9%) of clinical isolates of indole-positive Klesbiella spp. could be identified as...
K. planticola, confirming the findings of others (9, 14). We have shown also that there was no DNA sequence homology between the β-lactamase genes of the K. planticola and K. oxytoca isolates (1). It may be important to differentiate K. planticola from other indole-positive Klebsiella species in order to ascertain their epidemiological and clinical significance.

K. planticola can be differentiated from other indole-positive Klebsiella species by carbon substrate assimilation tests, but these are not readily available to diagnostic laboratories. By using PCR, 4 of 43 indole-positive Klebsiella clinical isolates proved to be isolates of K. planticola, confirming the results of carbon substrate assimilation tests. The PCR method is not entirely suitable for distinguishing K. planticola from other indole-positive Klebsiella spp. because the conclusion depends on a negative result, but it is specific, simple, and sensitive. Such an approach could be developed by cloning the K. planticola β-lactamase gene, sequencing the clone, and devising oligonucleotides specific for the cloned gene.

A plasmid-mediated ESBL (SHV-5) gene was found in one K. planticola strain, and the ESBL phenotype was transferable by conjugation to E. coli. There were three non-ESBL K. planticola isolates which were resistant to ampicillin and had chromosomal β-lactamases with pIs of 7.2, 7.7, and 7.9 plus 7.2. Two strains were resistant to cephalothin and cefamandole, and one was resistant to ceftoxitin. It was concluded, based on co-amoxiclav susceptibility and nitrocefin reaction times, that the chromosomal β-lactamase was not hyperproduced in any K. planticola isolate.

K. oxytoca bacteria are generally moderately resistant to amoxicillin due to the activity of a chromosomally encoded β-lactamase (24). Recently, the emergence of resistance to extended-spectrum β-lactams, including aztreonam and broad-spectrum cephalosporins, has been described (16, 22). The genes responsible for extended-spectrum β-lactamase production are often located on transferable plasmids. Most of these enzymes are related to TEM or SHV β-lactamases and are characteristically inhibited by clavulanate. In addition, hyperproduction of chromosomally mediated β-lactamase has been described (1, 10, 12, 21), and the phenotype has a characteristic resistance pattern including aztreonam but not ceftazidime resistance. Hyperproduction can result from a mutation in the promoter of the β-lactamase gene (7). In K. oxytoca strains, hyperproduced chromosomally mediated β-lactamases are quite common, and 17 of the isolates in this study had this property.

The chromosomal β-lactamase genes of K. oxytoca are divided into two main groups, blaOXY-1 and blaOXY-2, as determined by PCR. The distribution of isoelectric points in our study was only slightly different from that reported by Fournier et al. (8). We found 27 blaOXY-2 and 12 blaOXY-1 β-lactamase genes, while Fournier et al. reported 35 and 20, respectively. The isoelectric points of OXY-1 β-lactamases in our collection ranged from 7.8 to 8.8, but the pl of 7.5, which accounted for 30 (42%) of the OXY-1 chromosomal β-lactamases in Fournier’s study, and the pl of 7.1, which was represented by one isolate, were not found. The isoelectric points of OXY-2 chromosomal β-lactamases ranged from 5.2 to 6.8 and were more frequently detected than those of the OXY-1 chromosomal β-lactamases. The pl 5.2 form of β-lactamase was much more frequent than any other in both collections. Originally, a plasmid-mediated K. oxytoca β-lactamase with a pl of 5.2 was identified as KH (25) and is similar to the chromosomal K1 β-lactamase.

A transferable plasmid-mediated TEM-1 β-lactamase was identified in two donor strains which were OXY-2 gene positive by PCR. However, DNA from the TEM-1 transconjugants was OXY-2 gene negative. Thus, it seems likely that these donors had a plasmid-mediated TEM-1 β-lactamase and a chromosomally encoded OXY-2 gene, but the β-lactamase produced was undetectable in the isoelectric gels due in part to dilution of crude extracts.

Plasmid DNA could not be detected in any ESBL K. oxytoca isolate, nor could β-lactamase resistance be transferred by conjugation from any donor. Nevertheless, three transformants were obtained with donor DNA from 3 of the 17 ESBL strains. In the E. coli transformants, a plasmid of 2.6 kb was detected together with a β-lactamase (pl 5.2). However, the transformants were OXY-2 gene negative by PCR, even though the K. oxytoca donors contained the OXY-2 β-lactamase gene. This indicates that K. oxytoca might also have a chromosomally mediated ESBL (pl 5.2) which conferred resistance to aztreonam and reduced susceptibility to ceftriaxone. An explanation of the failure to detect plasmid DNA in the donors might be that the copy number of the nonconjugative plasmid in the K. oxytoca hosts was too low for detection in agarose gels. Capsular polysaccharides and lipopolysaccharides may also have interfered with plasmid extraction. In the transformants, the copy number of plasmid DNA may have increased or the plasmid may have been more efficiently extracted from the new host. However, since resistance to aztreonam was not expressed in E. coli, an increase in plasmid copy number is unlikely. The absence of plasmid-mediated ESBL resistance in K. oxytoca supports a previous report by Reig et al. (21), who found that less than 10% of K. oxytoca isolates had such resistance in contrast to 92% of K. pneumoniae isolates, which relied on plasmids for their ESBL phenotype.

The data indicate significant phenotypic and genotypic differences in resistance mechanisms between K. oxytoca and K. planticola. The future development of mechanisms for rapid routine taxonomic differentiation of these species in diagnostic laboratories will be necessary to clarify whether or not significant epidemiological or pathogenetic differences exist between the indole-positive Klebsiella species.

ACKNOWLEDGMENTS

We thank David Lyon, Karen McGregor, Tom Riley, and Mark Watson for their assistance in different aspects of this study.

REFERENCES


