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Detection of a New SHV-Type Extended-Spectrum β-Lactamase, SHV-31, in a *Klebsiella pneumoniae* Strain Causing a Large Nosocomial Outbreak in The Netherlands[▽]

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A *Klebsiella pneumoniae* strain resistant to third-generation cephalosporins was isolated in the eastern Netherlands. The strain was found to carry a novel extended-spectrum β-lactamase, namely, SHV-31. The combination of the two mutations by which SHV-31 differs from SHV-1, namely, L35Q and E240K, had previously only been described in association with one or more additional mutations.

The Netherlands is well known for its low antibiotic consumption (4) and low prevalence of multidrug resistance, including that caused by gram-negative bacteria producing extended-spectrum β-lactamases (ESBLs) (3, 5, 13). The overall prevalence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates (TEM- and SHV-type ESBLs) was less than 1% in 1997 (13). In a study designed to determine the incidences of ESBL-producing vancomycin-resistant *Enterococus faecium* and methicillin-resistant *Staphylococcus aureus* isolates from 38 centers across the world during 2001 to 2002, the overall ESBL production rate for the combined *Enterobacteriaceae* isolates was lowest in The Netherlands, amounting to 2.0%, as against an average of 10.5% in other countries (1).

Among intensive care unit (ICU) isolates collected for a European multicenter study, the prevalence of ESBL-producing *Klebsiella* strains in three Dutch hospitals was as high as 16% (eight times higher than the general prevalence), but still far lower than the average prevalence in other European countries (7).

A *K. pneumoniae* strain, KPN15-NL, resistant to extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones, was isolated in August 2001 from a wound culture from an ICU trauma patient admitted to a tertiary-care 1,000-bed teaching hospital (18-bed ICU) situated in the easternmost part of The Netherlands. The strain was identified with biochemical tests by means of API ID32E (bioMérieux). The patient had never been hospitalized before. By the end of 2002, *Klebsiella* strains with the same antibiotic resistance profile and with the same randomly amplified polymorphic DNA and restriction fragment length polymorphism patterns were isolated from a total of 85 ICU patients, thus making KPN15-NL the index isolate of a large epidemic.

Susceptibility testing, carried out according to the latest CLSI (formerly NCCLS) guidelines (2), proved *K. pneumoniae* KPN15-NL to be resistant to extended-spectrum cephalosporins. The Kirby-Bauer test revealed features typical of an ESBL-producing strain, namely, resistance to ceftazidime, cefotaxime, and aztreonam, but susceptibility to cefepime, and reversal of these resistances by clavulanic acid. The isolate was also tested for its antimicrobial susceptibilities by broth microdilution. The resulting β-lactam MICs are reported in Table 1. The isolate also proved resistant to gentamicin (64 μg/ml), but not to amikacin (8 μg/ml), and resistant to fluoroquinolones (ciprofloxacin, >128 μg/ml, and levofloxacin, 128 μg/ml).

The β-lactamase production was first confirmed by isoelectric focusing, performed in a precast polyacrylamide (5%) gel containing ampholines (pH range, 3.5 to 9.0) (Amersham Pharmacia Biotech, Uppsala, Sweden) on a biophoresis apparatus (Bio-Rad, Hercules, CA). The enzyme activity was revealed by overlaying the gel with a paper filter soaked in 250 μM nitrocefin (Oxoid, Basingstoke, Hampshire, England). When inhibition with clavulanic acid was tested, the gel was previously overlaid with a paper filter soaked in 2 μg/ml of clavulanic acid. The strain showed two bands, at pI 5.4 and 7.8; both bands were inhibited by clavulanic acid in the overlay test (data not shown).

The presence of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} resistance genes was checked by PCR using recombinant *Taq* polymerase (Amersham Biosciences) and specific oligonucleotide primers as follows: TEM-FW and TEM-REV, specific for bla_{TEM} (8); SHV-FW and SHV-REV, specific for bla_{SHV} (10); CTX-MU1 and CTX-MU2, specific for $bla_{CTX\text{-}M}$ (9); and OXA1F/R, OXA2F/R, and OXA10F/R, specific for bla_{OXA} (12).

PCR products were purified using a QIAGEN microspin (QIAGEN GmbH, Hilden, Germany). We obtained products for bla_{TEM} and for bla_{SHV}. The entire coding regions were amplified. DNA sequencing of the amplicons obtained was performed on both strands and analyzed in an ABI PRISM 377 DNA sequencer. The TEM product proved to be a TEM-1 enzyme, which was consistent with the pI 5.4 isoelectric focus band. The SHV product showed two amino acid changes compared with SHV-1, namely, L35Q and E240K. Thus, KPN15-NL turned out to harbor a novel SHV enzyme, which

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TABLE 1. MICs for *K. pneumoniae* KPN-15-NL and *E. coli* XL10

Antimicrobial(s)	MIC (μg/ml) for:		
	KPN15-NL	*E. coli* XL10/vector	*E. coli* XL10/pSHV-31
Ampicillin	>128	2	>128
Amoxicillin	>128	16	>128
Amoxicillin-clavulanic acid	32	8	8
Piperacillin	>128	1	64
Piperacillin-tazobactam	16	1	4
Cefoxitin	16	1	1
Ceftazidime	128	0.12	32
Ceftazidime-clavulanic acid	8	0.12	4
Cefotaxime	8	≤0.06	4
Cefepime	1	≤0.06	0.5
Aztreonam	>128	0.12	>128
Imipenem	≤0.06	≤0.06	≤0.06
Ertapenem	≤0.06	≤0.06	≤0.06

has been termed SHV-31 by the Lahey Clinic (http://www.lahey.org/studies).

The SHV gene was cloned in the phagemid vector pPCR script Cam SK$^+$ (Stratagene, La Jolla, CA). The entire SHV gene was amplified by PCR with the following primers: SHV-CF (5′ GGGGAATTCTTATTTGTCGC) and SHV-CR (5′ CAGAATTCGCTTAGCGTTGCCAGT). The primers extended beyond the coding sequence to give the polishing protocol some extra DNA.

The PCR product was ligated with the phagemid vector pPCR script Cam SK$^+$. This cloning vector has a chloramphenicol resistance gene and a *lac* promoter for gene expression (also driving bla_{SHV-31} expression). The cloning site was SfiI. Ligated vectors were transformed in *E. coli* XL10 ultracompetent cells by the ligation kit polishing protocol (Stratagene, La Jolla, CA). Transformants were selected on LB agar plates with 30 μg/ml of chloramphenicol and 2 μg/ml of ceftazidime added and then checked by PCR and endonuclease digestion.

Upon isoelectric focusing, the *E. coli* XL10/pSHV-31 strain showed a band of pI 7.8, and its MICs for extended-spectrum cephalosporins paralleled those of *K. pneumoniae* KPN15-NL, differing sharply from those of *E. coli* XL10 for all β-lactams tested (ampicillin, >64-fold; ceftazidime, 256-fold; cefotaxime, >64-fold; cefepime, >8-fold; and aztreonam, >1,000-fold).

We also performed bacterial conjugation experiments using *E. coli* J53 AzR as the recipient strain and selecting with 100 μg/ml of sodium azide and 2 μg/ml of ceftazidime. After all attempts at conjugation had failed, including those involving electroporation, and in order to investigate where the SHV-31 gene of *K. pneumoniae* KPN15-NL was located, plasmid DNA was extracted from both *K. pneumoniae* KPN15-NL and *E. coli* XL10/pSHV-31.

Plasmid extraction from KPN15-NL and XL10/pSHV-31 was performed by means of the alkaline lysis method. One-half of each plasmid sample was treated with plasmid-safe DNase (Epicenter Technologies) to remove contaminating chromosomal DNA. The plasmids were separated by electrophoresis in a 0.6% agarose gel. Plasmid profile gels were stained with ethidium bromide (Fig. 1A). Southern analysis was performed by standard methods (11) (Fig. 1B).

The bla_{SHV-31}-specific probe was synthesized by using the SHV-31 primers and incorporating digoxigenin-11-dUTP (Roche, Mannheim, Germany) into the PCR product. The probe reacted only with the plasmid preparation not treated with plasmid-safe DNase, and therefore, we assume that the SHV-31 gene has a chromosomal localization.

The two amino acid mutations found in the SHV gene, namely, L35Q and E240K, have been found previously in other SHV genes either alone or as a pair. However, in the latter case, one or more further mutations were also reported.

The outbreak of the *K. pneumoniae* strain resistant to extended-spectrum cephalosporins mentioned in the present study started in August 2001 and by the end of 2002 had involved a total of 85 ICU patients. After that, the strain became hyperendemic in the center and is presently still a frequent cause of nosocomial infection in the affected hospital.

Outbreaks of *K. pneumoniae* producing ESBLs are very rare in The Netherlands. Gruteke et al. (5) previously reported an outbreak of multiresistant *K. pneumoniae* harboring SHV-5 in a Dutch 250-bed care facility between March 1997 and June 1999.

Recent data from The Netherlands clearly show the great diversity of ESBL genes in 34 nonduplicate *Salmonella enterica* strains isolated in 2001 and 2002 from poultry, poultry products, and human patients and seem to confirm that many of these genes appear to spread rapidly (6).

The occurrence of clinical outbreaks, the diversity of the genes involved and their rapid spread, the finding of novel enzymes, and the presence of ESBLs also in animals and in animal products all contribute to making ESBLs a matter of serious concern even in The Netherlands.

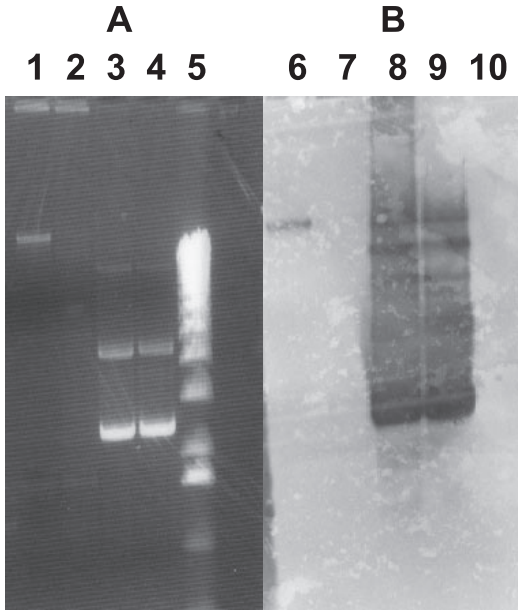

FIG. 1. Analysis of localization of the bla_{SHV-31} gene. The figure shows an agarose gel stained with ethidium bromide (A) and a Southern blot (B). Lanes 1 and 6, plasmid DNA extracted from KPN15-NL; lanes 2 and 7, plasmid DNA extracted from KPN15-NL, treated with plasmid-safe DNase; lanes 3 and 8, DNA from a plasmid carrying the bla_{SHV-31} gene, extracted from the host strain *E. coli* XL10; lanes 4 and 9, DNA from a plasmid carrying the bla_{SHV-31} gene, extracted from the host strain *E. coli* XL10 and treated with plasmid-safe DNase; lanes 5 and 10, 1-kb marker (Invitrogen).

Protein structure accession number. The GenBank accession number for the novel SHV enzyme termed SHV-31 is AY277255.

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