**First Characterization and Emergence of SHV-60 in Raw Milk of a Healthy Cow in Japan**

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**ABSTRACT.** During monitoring of raw milk samples from healthy cows for the presence of antibiotic resistant bacteria, one isolate of *Klebsiella pneumoniae* strain HUF-100 was found to be resistant to oxyimino-cephalosporins and aztreonam. It was found to carry a chromosomally-encoded extended-spectrum β-lactamase that has not been described previously, namely SHV-60. Thus, it must be expected that this strain will spread further among food-producing animals and thereby constitute a reservoir of this resistant strain and resistance gene that can transfer to and cause treatment problems for humans. The present study confirms the hypothesis that some of multiple antibiotic resistant zoonotic bacterial pathogens may initially emerge from food animals and reports, for the first time, this type of emergence in Japan.

**KEY WORDS:** *Klebsiella pneumoniae*, milk, SHV-β-lactamase.

Colonization of the udders of the healthy cows with multidrug-resistant zoonotic pathogens during lactation or dry periods is one of the most important threats affecting not only the dairy industry but also public health. Recently, sections of the population in developed nations have been misinformed by many sites on the internet and regard raw milk as more nutritious and better tasting than pasteurized milk [12]. Therefore, we conducted a study to monitor multidrug-resistant bacteria in the raw milk of California mastitis test negative healthy cows that had not received any antibiotics in their feed and that had not been treated with antibiotics for more than 3 months, factors not analyzed previously.

Twenty milk samples were collected aseptically into sterile screw-capped bottles after discarding three streams of milk and were directly transported to the laboratory in an ice box. Twenty-five ml of individual milk samples was added to 225 ml of buffered peptone water and incubated at 37°C with agitation for 24 hr. Following incubation, the enriched samples were subcultured onto MacConkey agar plates and incubated for 24 hr at 35°C. An API-20E system (bioMérieux, Marcy l’Etoile, France) was used for biochemical identification of isolated strains. The characterized strains were tested for susceptibility to antimicrobials by disk diffusion and MIC methods using Muller-Hinton agar (Nissui Pharmaceutical Co., Tokyo, Japan) and Muller-Hinton broth (Oxoid), respectively, according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2005) [6]. A strain identified as *Klebsiella pneumoniae*, KPHUF-100, exhibited an uncommon phenotype, suggesting the presence of an extended-spectrum β-lactamase (ESBL) with a spectrum extended to oxyimino-cephalosporins and aztreonam. MIC determination in the presence of a fixed concentration of clavulanic acid (4 µg/ml; Table 1) confirmed that this isolate was an ESBL producer and excluded the likelihood that resistance was due to the presence of AmpC-type β-lactamase or permeability effects resulting from changes in outer membrane porins [15].

β-lactamase production was first confirmed by isoelectric focusing that was performed in a 111 Mini IEF Cell apparatus (Bio-Rad Laboratories) on polyacrylamide gel in the presence of amphotericin in the pH range 3.5–10. Broad-range pl markers (Bio-Rad Laboratories) were used. Crude β-lactamase preparations [14] from *K. pneumoniae* strains known to harbor SHV-1 and SHV-26 were used as controls. After isoelectric focusing, β-lactamases were detected by spreading nitrocefin (100 µg/ml) on the gel surface.

The SHV gene was first amplified by PCR using SHV-specific primers SHV-F1 and SHV-R1 [2], and then the entire SHV gene was amplified using the following pair of primers: SHV-F2 (5’-CGGCCCTCCTAAGGATGTA-3’) and SHV-R2 (5’-CAATAGCCGCGTGCCTG-3’). The SHV gene was sequenced on both strands with SHV-specific primers using an ABI automatic DNA sequencer (model 373, Perkin-Elmer). The purified PCR fragment obtained from KPHUF-100 using the SHV-F2 and SHV-R2 oligonucleotide primers was cloned into the Smal digested pBluescript II SK(−) (Stratagene, U.S.A.) using a TaKaRa Ligation Kit. The ligation mixtures were then used to transform *E. coli* TG1 competent cells. Transformants were selected on LB agar containing 50 µg/ml of ampicillin. The recombinant plasmid DNA was isolated from the transformed cells using a QIAprep Spin Miniprep Kit (Qiagen KK, Japan), and the DNA sequence of the inserts was determined for both strands using universal vector primers T7.
(5'-TAATACGACTCACTATAGGG-3') and T3 (5'-ATTAACCCTCACTAAAG-3') to give the polishing protocol extra DNA.

A 1,026-bp nucleotide sequence was determined. This sequence contained an 861-nucleotide reading frame with a G+C content of 63.33%. From the putative reading frame, the precursor form of SHV-60 seemed to consist of 286 amino acid residues. ESBLs differ from their parent enzymes TEM-1, TEM-2 and SHV-1 enzymes by 1 to 7 amino acid substitutions that alter the configuration and properties of the active site. In most cases, the amino acid changes work in concert and have a cooperative, positive effect on the ability of the enzyme to hydrolyze the newer cephalosporins [19]. In this strain, there were two amino acid changes in comparison with SHV-1, namely, L35Q and A187T expanded its spectrum of hydrolysis to oxyiminocephalosporins and aztreonam. The combination of these two mutations has not been described before. It should be noted that our SHV gene has the same mutations as SHV-60 (accession number AJ866283).

The PCR product was ligated with the phagemid vector pBAD33 [8]. This cloning vector has a chloramphenicol resistance gene and the P_EBAD promoter of the araBAD (arabinose) operon for gene expression. The cloning site was SmaI. Ligated vectors were transformed into E. coli TG1 competent cells. Transformants were selected on LB agar plates to which 10 μg/ml of chloramphenicol and 2 μg/ml of cefoperazone were added and were then checked by PCR and endonuclease digestion.

Upon isoelectric focusing, with reference to the control isolates, K. pneumoniae KPHUF-100 showed a band of pl 7.6 similar to the isolate carrying SHV-1 (Fig. 1). The MICs of both strains were quite similar but differed sharply from those of the E. coli TG1/vector for all β-lactams tested (Table 1).

To determine the location of this gene, Southern blot hybridization was used according to the instructions provided in a Non-radioactive Detection Kit (GE Healthcare). After agarose gel electrophoresis of the plasmid purified by the alkaline lysis method, the DNA fragments in the gel were transferred onto Hybond-N+ nylon membranes (GE Healthcare). The DNA fragment containing the whole SHV gene was amplified by PCR using the SHV-F2 and SHV-R2 primers and purified. The purified fragment was labelled with alkaline phosphatase using an AlkPhos Direct Labeling System (GE Healthcare) and was subsequently used as a DNA probe. A single hybridization signal that corresponded to the chromosomal DNA was detected (Fig. 2).

Conjugation experiments were carried out by broth mating as described previously [13] using a rifampicin-resistant mutant of E. coli HB101 obtained in vitro [17] as the recipient strain and selection with rifampicin (150 μg/ml) and cefoperazone (2 μg/ml). Electroporation of the total DNA preparation of K. pneumoniae KPHUF-100 in electrocompetent E. coli TG1 was carried out using a MicroPulser apparatus (Bio-Rad Laboratoties) according to the manufac-

### Table 1. MICs for Klebsiella pneumoniae KPHUF-100, E. coli TG1/vector and E. coli TG1/pSHV-60

<table>
<thead>
<tr>
<th>Antimicrobial(s)</th>
<th>KPHUF-100</th>
<th>E. coli TG1/vector</th>
<th>E. coli TG1/pSHV-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>&gt;128</td>
<td>4</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Ampicillin-clavulanic acid</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>32</td>
<td>&lt;0.125</td>
<td>64</td>
</tr>
<tr>
<td>Aztreonam-clavulanic acid</td>
<td>4</td>
<td>&lt;0.125</td>
<td>8</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>64</td>
<td>0.25</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefoperazone-clavulanic acid</td>
<td>0.5</td>
<td>&lt;0.125</td>
<td>4</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>8</td>
<td>0.25</td>
<td>8</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2</td>
<td>&lt;0.125</td>
<td>16</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2</td>
<td>&lt;0.125</td>
<td>16</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>8</td>
<td>&lt;0.125</td>
<td>8</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>8</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>Imipenem</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
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</tbody>
</table>

Fig. 1. Isoelectric focusing of the new β-lactamase and reference β-lactamases. Lane 1: SHV-1. Lane 2: SHV-60. Lane 3: SHV-26. An arrow indicates pl 7.6.
ture’s instructions and selection with ampicillin (50 µg/ml). Transfer of β-lactam resistance to E. coli was not detected either in the conjugation assay or following electroporation of the total DNA preparation, which confirmed the chromosomal localization of this gene.

*K. pneumoniae* is among the most common gram-negative bacteria encountered by physicians worldwide and has been a recognized pulmonary pathogen since its discovery over 100 years ago; however, the reason for the geographic preponderance of severe manifestations of *K. pneumoniae* infections including bacteremic liver abscess and community-acquired bacterial meningitis in Asia is unknown [9]. In Japan, there has been a recent increase in the incidence of infection caused by multidrug-resistant *K. pneumoniae* [14, 21] constituting a threat not only by making it more difficult to select an effective antibiotic treatment for individual patients, but also by being a frequent cause of nosocomial outbreaks in hospitals [7, 11]. However, the origins of these bacteria are unknown.

Our findings suggest that under certain conditions, the udders of healthy animals may be colonized by multidrug-resistant bacteria and raised a complex question: What are the mechanisms involved in selection of multidrug-resistant bacteria in the udders of healthy animals? We assume that selection occurs outside the udder, and after invasion through the teat sphincter, these multidrug-resistant bacteria remain dormant. Perhaps even more surprising is the fact that only 8% of new coliform infections contracted during the dry period are ever translated into clinical mastitis [3]. β-lactam-producing microorganisms are found naturally in soil, suggesting that the intrinsic chromosomal antibiotic resistance originates in the soil in response to the inhibitory environment generated by such antibiotic-producing microorganisms [10]. Moreover, the fecal and urine waste of the animals contains both resistant bacteria and antibiotic residues. Whether commercially made or naturally occurring, stable antibiotics accumulate in soil inhabited by dairy animals [4]. It is possible that environmental antibiotic pressure selects some clustered point mutations that can be chromosomally or plasmid encoded [5]. This type of environment favors the emergence of resistance, transfer of genes and amplification of resistant strains [1] that can colonize the teat canal and teat cistern of the udder during lactation or the dry period for months [18] and provide a reservoir for multidrug-resistant bacteria in raw milk even in healthy animals. This assumption does not require that every lactating animal carrying resistant bacteria must have been treated with an antimicrobial drug. Resistant organisms can be acquired from an environment contaminated by drug use in a previous herd or flock of animals [20]. In addition, resistant microorganisms have been found on conventional and organic farms, which illustrates how widespread the phenomenon of bacterial resistance is on farms [1, 16].

It must be expected that this strain will spread further among food-producing animals and thereby constitute a reservoir of this resistant strain and resistance gene that can transfer to and cause treatment problems for humans. This study confirms the hypothesis that novel ESBL producers, which have the potential to cause treatment problems in humans may initially emerge from food-producing animals and reports, for the first time, this kind of emergence in Japan. Surveys are needed to assess if this is a unique situation on a single farm or if ESBL-mediated resistance is emerging within other animal populations in Japan.
The nucleotide sequence of the coding region of the β-lactamase gene has been deposited in the EMBL-GenBank nucleotide sequence data banks through the DNA Data Bank of Japan (accession number AB302939).

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REFERENCES