

CTX-M-Type Extended-Spectrum β -Lactamase-Producing *Klebsiella pneumoniae* Isolated from Cases of Bovine Mastitis in Japan

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(Received 5 March 2013/Accepted 15 April 2014/Published online in J-STAGE 1 May 2014)

ABSTRACT. Three *Klebsiella pneumoniae* isolates producing extended-spectrum beta-lactamase (ESBL) were obtained from three dairy cows with clinical mastitis in two farms in western Japan. Two of the 3 isolates from cows in different farms were able to transfer plasmids carrying the *bla*_{CTX-M-2} gene to *Escherichia coli* recipient. Pulsed-field gel electrophoresis (PFGE) patterns of the 2 isolates were different from each other, although restricted-fragment patterns of the two conjugative plasmids were similar to each other. Additionally, PCR-based replicon typing revealed that both the plasmids belonged to type Inc.T. These results suggest that ESBL-encoding genes can be distributed in bacteria on dairy farms through the plasmids.

KEY WORDS: bovine mastitis, ESBL, *Klebsiella pneumoniae*

doi: 10.1292/jvms.13-0120; *J. Vet. Med. Sci.* 76(8): 1153–1156, 2014

Bovine mastitis due to *Klebsiella pneumoniae* causes a significant drop in milk production and high mortality of the affected cows [9]. Recently, *K. pneumoniae* strains that produce extended-spectrum beta-lactamase (ESBL) were isolated from cases of bovine mastitis in Europe [6, 13]. ESBLs hydrolyze not only 1st- and 2nd-generation cephalosporins but also 3rd- and 4th-generation cephalosporins, and association of ESBL genes with transferable plasmids has often been reported [8]. The emergence of bacteria that produce ESBLs in domestic animals is a threat to animal hygiene and public health [16]. In the present study, we isolated *K. pneumoniae* from 20 dairy cows with clinical mastitis in multiple farms in western Japan. Three of the isolates were found to be resistant to ceftiofur, a 3rd-generation cephalosporin. Thus, the isolates were further characterized.

From April to September in 2011, milk samples were obtained from 20 dairy cows with clinical mastitis that were raised on 6 farms (farms A, B, C, E, F and G) located in western Japan. All the samples were collected before starting antimicrobial treatment. Rearing practices and the treatment for mastitis were reported from the farms. Farms A and C were located within 1.5 km of each other. The other 4 farms were more than 7 km west of farms A and C. Clinical symptoms observed included heat, induration and swelling of udders, fever, anorexia and reduced milk production. Aerobic culture on DHL agar plates yielded a heavy growth of pinkish red colonies in pure culture. These colonies were identified

as *K. pneumoniae* by the API 20E test (bioMérieux, Marcy l'Etoile, France). A representative isolate was selected randomly from 1 sample, and a total of 20 isolates were used for further examinations. All the isolates were subtyped using pulsed-field gel electrophoresis (PFGE) with *Xba*I-digested chromosomal DNAs as described elsewhere [17].

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method using Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD, U.S.A.) plates according to the recommendations given in document M07-A8 of the Clinical and Laboratory Standards Institute (CLSI) [4] and interpreted using the resistance breakpoints defined in the previous study [15] as follows: ampicillin (AMP) and cefazolin (CEZ), 32 μ g/ml; ceftiofur (CTF), 8 μ g/ml; dihydrostreptomycin (DSM), 32 μ g/ml; gentamicin (GEN), 16 μ g/ml; kanamycin (KAN), 64 μ g/ml; oxytetracycline (OTC), 16 μ g/ml; chloramphenicol (CHL), 32 μ g/ml; nalidixic acid (NAL), 32 μ g/ml; enrofloxacin (ERFX), 2 μ g/ml; and trimethoprim (TMP), 16 μ g/ml. Each antibiotic reagent, except for CTF, was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, U.S.A.). CTF was obtained from Pfizer Inc. (Tokyo, Japan). ESBL-production among CTF-resistant isolates was screened and confirmed using the disc diffusion method based on the criteria provided by the CLSI [5]. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as quality control strains.

ESBL-producing strains were tested by PCR targeting the *bla*_{CTX-M} gene using the specific primer set as follows: CTX-M-1: 5'-GACGATGTCACCTGGCTGAGC-3' and 5'-AGCCGCCGACGCTAATACA-3', CTX-M-2: 5'-GC-GACCTGGTTAACTAACAATC-3' and 5'-CGGTAGTATTGCCCTTAAGCC-3', CTX-M-3: 5'-CGCTTTGCCATGTGCAGCACC-3' and 5'-GCTCAGTACGATCGAGCC-3', CTX-M-4: 5'-GCTGGAGAAAAGCAGCGGAG-3' and 5'-GTAAGCTGACGCAACGTCTG-3', CTX-M-5: 5'-AT-

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Table 1. Characteristics of *K. pneumoniae* isolates obtained from milk samples of mastitic dairy cows

Isolate*	Isolation date	MIC value ($\mu\text{g/ml}$)**						ESBL <i>bla</i> gene	Conjugation to <i>E. coli</i>
		AMP	CEZ	CTF	DSM	KAN	OTC		
A45	Aug 9, 2011	<u>32</u>	4	0.25	2	2	0.25	n/a***	n/a***
A46	Aug 17, 2011	<u>512</u>	<u>256</u>	<u>8</u>	<u>128</u>	2	2	<i>bla</i> _{CTX-M-2}	+
A48	Aug 22, 2011	<u>32</u>	1	0.5	<u>128</u>	2	2	n/a	n/a
A49	Aug 22, 2011	16	1	0.5	<u>256</u>	4	2	n/a	n/a
B29	Jul 1, 2011	16	4	0.5	<u>128</u>	2	2	n/a	n/a
B30	Jul 5, 2011	<u>32</u>	2	0.5	<u>256</u>	2	<u>512</u>	n/a	n/a
B31	Jul 13, 2011	<u>32</u>	1	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B32	Jul 13, 2011	<u>32</u>	1	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B34	Jul 14, 2011	<u>32</u>	2	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B35	Jul 15, 2011	<u>32</u>	1	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B36	Jul 15, 2011	<u>32</u>	1	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B40	Jul 22, 2011	<u>32</u>	1	0.5	<u>256</u>	2	<u>>512</u>	n/a	n/a
B44	Aug 16, 2011	<u>32</u>	2	0.5	<u>128</u>	2	<u>512</u>	n/a	n/a
B50	Sep 15, 2011	<u>32</u>	2	1	<u>256</u>	2	<u>512</u>	n/a	n/a
B51	Sep 15, 2011	<u>32</u>	2	1	<u>256</u>	2	<u>512</u>	n/a	n/a
C22	Apr 21, 2011	<u>512</u>	<u>128</u>	<u>8</u>	<u>128</u>	2	1	<i>bla</i> _{CTX-M-2}	-
C52	Sep 21, 2011	<u>>512</u>	<u>256</u>	<u>16</u>	<u>128</u>	2	2	<i>bla</i> _{CTX-M-2}	+
E33	Jul 13, 2011	<u>32</u>	<u>256</u>	1	<u>64</u>	2	2	n/a	n/a
F42	Jul 26, 2011	<u>256</u>	1	0.5	<u>512</u>	<u>512</u>	<u>256</u>	n/a	n/a
G53	Sep 20, 2011	<u>32</u>	1	0.5	2	2	2	n/a	n/a

*The first letter (alphabet) indicates each of the farms where cows were raised. **AMP, ampicilin; CEZ, cefazolin; CTF, ceftiofur; DSM, dihydrostreptomycin; OTC, oxytetracycline; KAN, kanamycin, Underlined numbers in boldface represent resistance to each of the antimicrobials. ***Not applicable.

GATGAGAAAAAGCGTAAGG-3' and 5'-TTAATAAC-CGTCGGTGAC-3'). Amplified products were sequenced using the same primer pair.

Plasmid DNAs were extracted from ESBL-producing strains using Plasmid Midi Kit (Qiagen, Hilden, Germany) and electrophoresed in an agarose gel in Tris-acetate-EDTA buffer. For southern blot analysis, PCR-amplified ESBL gene fragments from one of the ESBL-producing strains, *K. pneumoniae* A46 (see below or Table 1), were labeled with digoxigenin using DIG High Prime Labeling and Detection Starter Kit (Roche, Indianapolis, IN, U.S.A.).

In the transfer experiments [17], ESBL-producing strains were used as donor strains, and *E. coli* ML1410 was used as a recipient. Overnight cultures of donor and recipient strains were co-cultured in freshly prepared broth and incubated at 37°C for 6 hr. Transconjugants were selected on DHL plates containing 50 $\mu\text{g/ml}$ of AMP and 25 $\mu\text{g/ml}$ of NAL. To compare restriction fragment length patterns (RFLPs) of plasmids conjugated into *E. coli* ML1410, the plasmid DNAs were extracted, digested with *DraI* or *PstI* and electrophoresed. These RFLPs were compared with that of the plasmid harboring the *bla*_{CTX-M-2} gene originated from *E. coli* strain D1633 that was isolated from a commercial broiler chicken with colibacillosis. Plasmids of parent ESBL-producing strains and the transconjugants were typed by PCR-based replicon typing [3].

Among the 20 isolates, a total of 11 PFGE patterns were distinguished by more than 6-band differences (Fig. 1A). Ten of 11 isolates obtained from cows on farm B had identical PFGE patterns. Rates of resistance to AMP and DSM

were 90% and 90%, respectively (Table 1). All the isolates were susceptible to GEN (0.5–1), CHL (2–8), NAL (4–16), ERFX (<0.13) and TMP (0.5–2) with the MIC ranges ($\mu\text{g/ml}$) described in parentheses. Three isolates (A46, C22 and C52) that were resistant to CTF (MICs: 8, 8 and 16 $\mu\text{g/ml}$, respectively) were confirmed to be ESBL producers and harbored the ESBL-encoding gene *bla*_{CTX-M-2}. A southern blot analysis confirmed that the gene was located on plasmids with more than 50 kb plasmids in size (data not shown). Transferability tests demonstrated that 2 of the 3 ESBL-producing *K. pneumoniae* isolates (A46 and C52) were able to transfer the R plasmids to the *E. coli* recipient (Table 1). *DraI*- and *PstI*-digested patterns of the plasmids obtained from 2 transconjugants were apparently similar to each other and were distinguishable from those of the plasmid obtained from *E. coli* strain D1633 (Fig. 1B). The plasmids from the two transconjugants, which were derived from A46 and C52, and those from the parental strains were confirmed to be type Inc.T. On the other hand, the plasmid from strain C22 belonged to type Inc.FIC.

In the present study, three isolates of ESBL-producing *K. pneumoniae* were obtained from cows with clinical mastitis that were reared on 2 farms (farms A and C). The *bla*_{CTX-M-2} gene was detected in transferable plasmids in 2 of the 3 ESBL-producing *K. pneumoniae*. The PFGE patterns of the 2 isolates were distinguishable, although the 2 conjugative plasmids carrying the *bla*_{CTX-M-2} gene had similar RFLP profiles and belonged to an identical replicon type. These results suggest that ESBL genes can be distributed in bacteria on dairy farms through the plasmids. According to the reports

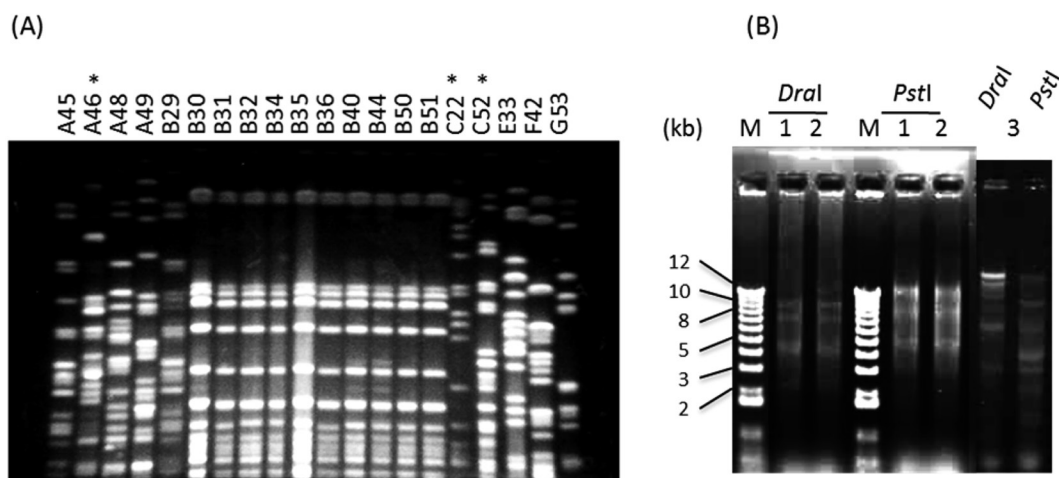


Fig. 1. (A) PFGE patterns of *Xba*I-digested chromosomal DNA of *Klebsiella pneumoniae* isolates obtained from milk samples of mastitic dairy cows. The isolates are described in Table 1. Asterisks above the isolate number indicate ESBL-producing isolates. (B) Restriction profiles of *Dra*I- and *Pst*I-digested plasmids from *bla*_{CTX-M-2}-positive trans-conjugants. Lanes 1 and 2, plasmids from *K. pneumoniae* A46 and C52, respectively, conjugated into *E. coli*. Lane 3, plasmids from *E. coli* strain D1633, conjugated into ML1410 laboratory strain.

from the farms, animals in farm A were administered cefazolin and cefuroxime for 6 and 9 days, respectively, and those in farm C were administered cefazolin, cefuroxime and cefepirin for 3 days. The 3 cows infected with ESBL-producing *K. pneumoniae* were not recovered. Prescriptions or information in the other farms were unavailable. The farmers and employees in farms A and C did not visit each other, and no common veterinarians attended animals in both the farms. Companies supplied cattle feed to these farms were different from each other. Other environmental factors may contribute to occurrence of the R-plasmid carrying the *bla*_{CTX-M-2} gene in *K. pneumoniae*, because the geographical distance between farms A and C was relatively close. Dahmen *et al.* [6] showed low molecular diversity of ESBL-producing strains causing cattle mastitis in France with respect to ESBL genes, plasmids and clones. In a study of ESBL-producing *K. pneumoniae* and *E. coli* isolates obtained from rectal samples of broiler chickens from different farms in Japan [10], it was suggested that transferable plasmids carrying the *bla*_{CTX-M-2} or *bla*_{CTX-M-14} gene are distributed among those species.

In the present study, 10 of 11 isolates obtained from cows in farm B had identical PFGE patterns, suggesting that the strain with this PFGE profile had spread clonally. Munoz *et al.* [14] reported that one *K. pneumoniae* mastitis outbreak on a single dairy farm was attributed to contagious transmission or to exposure of multiple cows to a common source, because the isolates had identical random amplified polymorphic DNA (RAPD) types.

E. coli harboring the *bla*_{CTX-M-14} gene was detected in milk from mastitic cows in Switzerland [7]. The *bla*_{SHV-12} and *bla*_{CTX-M-15} genes were reported in *Enterobacter* and *Klebsiella* isolates from clinical bovine mastitis in Egypt [1]. According to reports of monitoring of ESBL-producing

bacteria from food-producing animals in Japan, *K. pneumoniae* harboring the *bla*_{SHV-60} gene were isolated from raw milk from a healthy cow [10], and bovine isolates of *E. coli* with the *bla*_{CTX-M-2} gene were obtained from 1.5% (6/396) of fecal samples and surfaces of carcasses of cattle (0.7%, 2/270) [16] and animals with colibacillosis (2.8%, 2/72) [2]. In Japan, cephalosporins including ceftiofur are approved for parenteral use in cattle. Although the association of the use of ceftiofur with the emergence of ESBL producers in cattle is controversial [11, 12, 16, 18], continuous monitoring and molecular characterization of ESBL producers remain important to reveal the factors involved in introducing these strains into farms and the genes responsible for the resistance.

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