Introduction

Bovine mastitis is the most common and most costly disease of dairy cattle\(^\text{13}\). *Escherichia coli* and *Klebsiella pneumoniae* are most frequently isolated from acute to peracute severe mastitis, which results in high mortality and large losses in milk yield\(^\text{14,15}\). Antimicrobials approved for cattle are limited to a small number of classes, and most are used for intramammary and parenteral therapy of clinical mastitis and dry cow therapy of subclinical mastitis, possibly creating a selective pressure for drug-resistant organisms\(^\text{2}\).

Since 2000, plasmidic CTX-M-type extended-spectrum β-lactamases (ESBLs) (CTX-M) have become the predominant

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**Occurrence of Bovine Mastitis Caused by CTX-M-2 β-Lactamase Producing Klebsiella pneumoniae**

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**Summary**

The aims of this study were to determine the presence of *Enterobacteriaceae* strains producing CTX-M-type extended-spectrum β-lactamases (ESBLs) (CTX-M) involved in bovine mastitis among Japanese dairy cattle and clinical courses of cows affected by the mastitis. Between August 2006 and January 2007 we chose 51 cefazolin-resistant isolates from oxidase-negative, Gram-negative bacilli isolates obtained from 30,237 quarter milk samples from 20,194 cows with mastitis in 1,000 dairy farms in Nemuro Subprefecture of Hokkaido Prefecture, Japan. They were screened for ESBLs using the standard Clinical and Laboratory Standards Institute (CLSI) ESBLs confirmatory combination disc tests. We performed genotyping of CTX-M-, TEM-, and SHV-type β-lactamases by PCR analysis and nucleotide sequencing, and determined the minimum inhibitory concentrations (MICs) of 21 antimicrobials. We identified three *Klebsiella pneumoniae* isolates producing CTX-M-2 from three quarters of two cows with mastitis in two dairy farms. One cow had mild acute clinical mastitis (slight swelling, warmth and hardness of the udder, slightly watery foremilk with flakes) without systemic symptoms and resolved within 4 weeks of diagnosis. Another cow had severe acute clinical mastitis with systemic symptoms and resolved within 10 weeks of diagnosis. The three isolates exhibited resistance to ampicillin, cefazolin, cefuroxime, cefotaxime, ceftriaxone, cefepime, ceftiofur, ceftiraxone, cefquinome, kanamycin, and oxytetracycline. Conversely, they were susceptible to ceftazidime, cefmetazole, moxalactam, imipenem, aztreonam, gentamicin, trimethoprim-sulfamethoxazole, and enrofloxacin. This is the first report on CTX-M–producing *K. pneumoniae* involved in bovine mastitis in Japan.

**Keywords**: CTX-M type ESBLs, bovine mastitis, *Klebsiella pneumoniae*, antimicrobial susceptibility

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ESBL type detected in *E. coli* and *K. pneumoniae* isolated from humans, companion animals, and food-producing animals on a global scale, replacing TEM- and SHV-type ESBLs (TEM, SHV)\(^{3,4}\). CTX-M producers can be transmitted to humans from companion animals and from food-producing animals via food-chains\(^{5,10}\). CTX-M producers (especially *E. coli*) commonly cause community-acquired extraintestinal infections (urinary tract and bloodstream infections) in humans and their companion animals\(^{6,10}\). CTX-M-type ESBLs confer resistance against penicillins, oxyimino-cephalosporins (i.e., cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, cefotium, ceftiquimone), and monobactams, but not cephamycins and carbapenem class\(^{31}\). They pose a serious threat to oxyimino-cephalosporin therapy for *E. coli* and *K. pneumoniae* infections\(^{6,20}\). CTX-M–related genes are transferred separately from chromosomes of different *Klebsiella* species that live in water, soil, sewage, and the human intestinal tract, and are grouped into five CTX-M clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25)\(^6\). This transfer has been facilitated by genetic mobilization units such as insertion sequences (i.e., IS*ecp*1 or IS*cr1*) and subsequent incorporation into hierarchical genetic structures of the plasmid including complex class 1 integrons and transposons\(^{49}\).

CTX-M-2-producing *E. coli* strains were isolated from bovine and poultry feces in Japan between 2000 and 2001\(^{15,21}\). Because ESBL producers are not routinely screened for in veterinary mastitis laboratories, few studies have reported the incidence of *Enterobacteriaceae* producing ESBLs in bovine mastitis\(^{1,18}\). The aims of this study were to determine the presence of *Enterobacteriaceae* producing CTX-M involved in bovine mastitis and their antimicrobial susceptibility, and clinical symptoms and outcomes of cows with the mastitis.

### Materials and methods

#### Screening of ESBL-producing *Enterobacteriaceae*

We cultured mastitis milk samples from cows affected by mastitis as earlier described\(^{19}\), and performed susceptibility testing by disc diffusion according to the Clinical and Laboratory Standards Institute (CLSI) guidelines\(^7\). The mastitis milk samples were submitted to the clinical laboratory of Nemuro District Agricultural Mutual Aid Association by nine member veterinary clinics. In the first screening for ESBL producers, we chose 51 cefazolin-resistant isolates from oxidase-negative, Gram-negative bacilli isolates obtained from 30,237 quarter milk samples from 20,194 cows affected by mastitis in 1,000 dairy farms in Nemuro Subprefecture of Hokkaido Prefecture, Japan, between August 2006 and January 2007.

**ESBLs confirmatory test, PCR amplification, and DNA sequencing**

All positive isolates were screened for ESBLs using the standard CLSI\(^7\) ESBLs confirmatory combination disc tests. They were screened for metallo-β-lactamases by sodium mercaptoacetic acid (SMA) double-disc synergy test using two Kirby-Bauer discs containing cefazidime and one disc containing 3 mg SMA (Eiken Chemical Co., Ltd., Tokyo, Japan). *Enterobacteriaceae* isolates were identified using the ID 32 E API system (Sysmex bioMérieux Co., Ltd., Tokyo, Japan).

All isolates that tested positive using the ESBLs combination disc tests were PCR amplified using specific primers \(\text{bla}_{\text{CTX-M-2 group}}\), \(\text{bla}_{\text{CTX-M-9 group}}\), \(\text{bla}_{\text{CMY-1}}\), \(\text{bla}_{\text{CMY-2}}\), \(\text{bla}_{\text{TEM}}\), \(\text{bla}_{\text{SHV-1}}\), \(\text{bla}_{\text{CMY-3}-\text{ampC}}\), and \(\text{bla}_{\text{CMY-3}-\text{ampC}}\), as previously described\(^{15}\). For all isolates harboring \(\text{bla}_{\text{CTX-M-2 group}}\), \(\text{bla}_{\text{CTX-M-9 group}}\), \(\text{bla}_{\text{TEM}}\), and \(\text{bla}_{\text{SHV-1}}\), both strands of the amplicon were directly sequenced.

#### Antimicrobial susceptibility testing

The three isolates were stored at \(-70°C\) in 10% skim milk. For testing, they were subcultured on a 5% sheep blood agar plate. The minimum inhibitory concentrations (MICs) of 21 antimicrobials were determined for the three isolates by broth microdilution using a customer-designed, commercial microtiter panel (Opt Panel MP) and a Cation Adjusted Mueller-Hinton broth (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) according to the CLSI guidelines\(^7,8\). Additionally, we performed susceptibility testing for three antimicrobials by disc diffusion\(^{15,19}\). We adopted the breakpoints for veterinary pathogens to ampicillin, cefazolin, cefotiofur, cefpodoxime, imipenem, kanamycin, gentamicin, oxytetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and enrofloxacin\(^7\). However, we adopted the breakpoints for isolates from human *Enterobacteriaceae* infections for cefuroxime, ceftazidime, cefotaxime, cefepime, cefmetazole, cefotixin, moxalactam, aztreonam, and ciprofloxacin\(^7\), because the breakpoints of these 10 drugs for veterinary pathogens were not defined. No resistance breakpoint exists for ceftimoxime in the CLSI guidelines. *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as quality-control strains.

#### Mastitis cases

One cow in one dairy farm developed mild acute clinical mastitis without systemic symptoms in one quarter in August 2006. Another cow in another farm developed severe acute clinical mastitis with systemic symptoms (slight fever and anorexia) in two quarters simultaneously in January 2007. CTX-M-2–producing *K. pneumoniae* isolates were involved in these mastitis cases. The average parity of the two cows at the time of mastitis onset was 2.5 (2–3). The average number of days from parturition to onset of mastitis was 3.5 days (2–5 days). The local symptoms characterized by slight to severe swelling, warmth and hardness of the udder, slightly and considerably watery foremilk with flakes and clots, and a positive modified California Mastitis Test. Beddings were sawdust and manure-contaminated straw in the two farms in which the two mastitis cases occurred.

Affected cows were empirically treated with approved preparations for mastitis consisting of cefazolin (150 mg per quarter per day) once daily for 3 to 6 days by intramammary infusion. There-
after the cows were treated with approved preparation for mastitis consisting of kanamycin (300 mg) or a self-prepared solution of gentamicin (60–120 mg mixed with 100 ml of 5% glucose solution), each given once daily for 3 to 10 days by intramammary infusion. Cow with severe acute mastitis was given ampicillin (4 g) and trimethoprim-sulfamethoxazole (4 g/20 g); each drug was administered once daily for 1 day by intramuscular or intravenous injection. The mastitis cases clinically resolved 4 to 10 weeks after onset in terms of recovery of milk yield, resolution of clinical inflammation in milk and udders, and negative California mastitis test result. The cows with mastitis were not treated with cefotiofur, cefquinome, or fluoroquinolones.

**Results**

The ESBL confirmatory test showed that three *K. pneumoniae* isolates were positive. However, no isolates tested positive for metallo-β-lactamase according to the SMA test. The one isolate was isolated at a concentration of 100 cfu per 10 μl of a quarter milk sample in pure culture from one quarter of one cow affected by mild clinical mastitis in one dairy farm in August 2006. The other two isolates were isolated at a concentration of 20 and ∞ cfu per 10 μl of a quarter milk sample in pure culture from two quarters of another cow affected by severe clinical mastitis in another dairy farm in January 2007. Results of PCR analysis and nucleotide sequencing show that the former one *K. pneumoniae* isolate harbored blactx-m$_2$ and blashv-11, and the latter two isolates harbored blactx-m$_2$, blatem$_1$, and blashv-11.

The three isolates producing CTX-M-2 exhibited high resistance to ampicillin, cefazolin, cefuroxime, ceftriaxone, cefotaxime, cepodoxime, cefiofur, and ceftuzonome. They exhibited resistance to kanamycin, oxytetracycline, and chloramphenicol. Conversely, they were susceptible to ceftazidime, cepime, cefmetazole, cefoxitin, moxalactam, imipenem, aztreonam, gentamycin, trimethoprim-sulfamethoxazole, enrofloxacin and ciprofloxacin (Table 1).

**Discussion**

Results of our study indicate that bovine mastitis caused by CTX-M-2–producing *K. pneumoniae* had occurred in two dairy farms in Japan. The CTX-M-2 producers were isolated in pure culture, suggesting that they would have acted as a primary pathogen. To our knowledge, this is the first report on CTX-M–producing *K. pneumoniae* involved in bovine clinical mastitis in Japan. In contrast, CTX-M-1–producing *K. pneumoniae* and CTX-M-15–producing *Klebsiella oxytoca* isolates were isolated from bovine mastitis in Italy and Egypt, respectively. The dominant CTX-M type differed among regions or countries. Shiraki et al. reported that the incidence of blactx-m$_2$-positive *E. coli* in bovine feces was 6 (1.5%) of 396 bovine fecal samples in Japan between 2000 and 2001. Kojima et al. reported that blactx-m$_2$-positive *E. coli* strains were isolates from fecal samples of broilers in Japan in 2001.

Antimicrobial susceptibility of the bovine *K. pneumoniae* isolates producing CTX-M-2 was in agreement with previous study; however, they exhibited higher susceptibility to fluoroquinolones and gentamicin than did human isolates. CTX-M-type ESBLs other than CTX-M-15, CTX-M-16 and CTX-M-27 can efficiently hydrolyze cefotaxime and ceftriaxone, but not cefazidime. The blatem$_1$ and blashv-11 genes detected in this study encode non-ESBL enzymes.

No cefotiofur was administered to the cows with mastitis in this study. This antimicrobial is used primarily to treat papillomatous digital dermatitis in our clinics. Although cefotiofur has been reported to improve outcomes of severe coliform mastitis, this is an off-label use in Japan. Daniels et al. revealed that cefotiofur demonstrated a transient effect on the selection of cefotiofur-resistant strains among commensal *E. coli* populations in individual calves. The primary sources of *K. pneumoniae* were bovine feces rather than unused sawdust, and most cows were shedding *K. pneumoniae* in feces. However, the causes of the spread of CTX-M-2–producing *K. pneumoniae* strains and the sources of them could have been unidentified in this study.

The two cows with *Klebsiella* mastitis caused by CTX-M-2 producers showed slight to severe clinical symptoms. The clinical symptoms and outcomes of the cows were consistent with those of cows with conventional coliform mastitis. The two cows were resistant to β-lactams (ceftazolin) and kanamycin. However, the cows responded to gentamicin. Monitoring for CTX-M producers by screening tests and susceptibility testing is necessary to avoid prolonged empiric therapy by β-lactams.

Recently, a high incidence of blactx-m or blcmv-2-positive *E. coli* was found in retail chicken meat; however, these *E. coli* strains rarely contaminate retail beef. To our knowledge, there is no report that CTX-M producers contaminated retail dairy products, because raw milk is pasteurized. These findings suggest that bovine blactx-m$_2$-positive *K. pneumoniae* may pose a relatively low risk to public health.

In conclusion, the presence of *K. pneumoniae* producing CTX-M-2 involved in bovine mastitis was confirmed in two dairy farms in Hokkaido, Japan between 2006 and 2007. *K. pneumoniae* producing CTX-M-2 is an emerging multidrug-resistant organism and an important public health problem. Thus, routine monitoring for CTX-M producers derived from bovine mastitis and the prudent use of oxyimino-cephalosporins is necessary to prevent the clonal spread of CTX-M.
Table 1  Antimicrobial susceptibility of three CTX-M-2 β-lactamase-producing K. pneumoniae isolates from mastitis for 24 antimicrobials by broth microdilution (MIC : μg/mL) or disk diffusion method (S, I, R)

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Isolate 1 August 06 Farm 1 : Cow 1</th>
<th>Isolate 2 and 3 January 07 Farm 2 : Cow 2</th>
<th>Breakpoint(^b) (μg/mL ; mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ampicillin</strong></td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>≥32</td>
</tr>
<tr>
<td><em>Cefazolin</em></td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>≥32</td>
</tr>
<tr>
<td><em>Cefuroxime</em></td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>≥32(^a)</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>≤2</td>
<td>≤2</td>
<td>≥16(^a)</td>
</tr>
<tr>
<td>CAZ/CLA</td>
<td>≤0.25</td>
<td>≤0.25 to 0.5</td>
<td>—</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>128</td>
<td>64</td>
<td>≥4(^a)</td>
</tr>
<tr>
<td>CTX/CLA</td>
<td>≤1</td>
<td>≤1</td>
<td>—</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>≥16</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>512</td>
<td>128–512</td>
<td>≥4(^a)</td>
</tr>
<tr>
<td><em>Cefbiofur</em></td>
<td>&gt;512</td>
<td>512</td>
<td>≥8</td>
</tr>
<tr>
<td><em>Cefquinome</em></td>
<td>128</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td>Cefepime</td>
<td>≤8</td>
<td>≤8</td>
<td>≥32(^a)</td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>≤4</td>
<td>≤4</td>
<td>≥64(^a)</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>≤8</td>
<td>≤8</td>
<td>≥64(^a)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤1</td>
<td>≤1</td>
<td>≥4(^a)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>≤8</td>
<td>≤8</td>
<td>≥16(^a)</td>
</tr>
<tr>
<td><em>Gentamicin</em></td>
<td>≤2</td>
<td>≤2</td>
<td>≥16</td>
</tr>
<tr>
<td>*Oxetetracycline</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>≥16</td>
</tr>
<tr>
<td>*SXT(^c)</td>
<td>0.5/9.5</td>
<td>1/19</td>
<td>≥4/76</td>
</tr>
<tr>
<td>*Enrofloxacin</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≥2</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≥4(^b)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>S</td>
<td>S</td>
<td>≤14 mm(^e)</td>
</tr>
<tr>
<td><em>Kanamycin</em></td>
<td>R</td>
<td>R</td>
<td>≤13 mm</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>R</td>
<td>R</td>
<td>≤13 mm</td>
</tr>
</tbody>
</table>

\(^{a}\)Abbreviations : CAZ/CLA, ceftazidime/clavulanic acid ; CTX, cefotaxime ; SXT, trimethoprim/sulfamethoxazole

\(^{b}\)Breakpoint in accordance with CLSI document M31-A2 for isolates from animals

\(^{c}\)Breakpoint for Enterobacteriaceae in accordance with CLSI document M100-S16 for human isolates

\(^{d}\)The category is not approved by the CLSI guidelines.

\(^{e}\)* Antimicrobial agent approved for cattle in Japan ; the other 14 antimicrobials in this table are unapproved for cattle.

References


CTX-M-2 型 β-ラクタマーゼ産生 Klebsiella pneumoniae による
牛乳房炎の発生

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要旨
本研究の目的は日本の乳牛における乳房炎に関与するCTX-M型 β-ラクタマーゼ（CTX-M）産生 Enterobacteriaceae の存在を明らかにすることと，その乳房炎罹患牛の臨床経過を調査することである。2006 年の 8 月から 2007 年 1 月に北海道根室支庁の 1,000 頭酪農場で発生した 20,194 頭の乳房炎牛の 30,237 検体の分房乳から分離した 51 株のセファゾリン耐性のオキシダーゼ陰性・グラム陰性桿菌株を Clinical Laboratory Standards Institute (CLSI) 標準のコンビネーションディスク法による基質拡張型 β-ラクタマーゼ (ESBLs) 確認テストを用いてスクリーニングした。ESBLs 確認テスト陽性株は PCR と DNA シーケンスにより CTX-M-，TEM-，SHV- 型 β-ラクタマーゼの遺伝子型別を行った。また 21 の抗菌薬の最小発育阻止濃度 (MIC) を測定した。2 酪農場における 2 頭の乳房炎罹患牛の 3 つの分房から 3 株の CTX-M-2 産生 Klebsiella pneumoniae を分離同定した。1 頭の乳牛は全身症状のない軽症の急性臨床型乳房炎（プツを含む軽症の水様乳汁，分房の軽度の腫脹と熱感・硬結）を表し，診断後 4 週間で軽快した。他 1 頭の乳牛は全身症状を伴う重症の急性臨床型乳房炎を表し，診断後 10 週間で軽快した。これらの分離株はアンピシリン，セファゾリン，セフロキシン，セフタキシム，セフトリアキシン，セフポドキシン，セフチオフル，セフキノム，カナマイシン，オキシテトラサイクリンには耐性を示した。一方，セフタジム，セフメタゾール，モクサラクタム，イミペンネム，アズトレオナム，ゲンタマイシン，トリメトプリム/スルファメトキサゾール，エノロフロキサシンには感性であった。本研究は日本における牛乳房炎に関与する CTX-M 産生 K. pneumoniae 分離株についての初報告である。

キーワード：CTX-M 型基質拡張型 β-ラクタマーゼ (ESBLs)，牛乳房炎，Klebsiella pneumoniae，薬剤感受性

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