Genetic Characteristics of CTX-M-type ESBL-Producing Enterobacteriaceae Involved in Mastitis Cases on Japanese Dairy Farms, 2007 to 2011

running title: CTX-M PRODUCERS IN BOVINE MASTITIS

(category: Bacteriology)

Short-Form Paper

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ABSTRACT

Sixty-five CTX-M-2/15/14 extended-spectrum β-lactamase–producing Enterobacteriaceae were isolated from 258,888 mastitic milk samples from Japanese dairy farms between 2007 and 2011. CTX-M-2–producing Klebsiella pneumoniae and CTX-M-15–producing Escherichia coli were the predominant strains isolated. There was no predominant clonal type and clonal diversity was found even in strains isolated from a single farm.

Keywords

CTX-M type ESBLs, bovine mastitis, RAPD-PCR, MLST, PFGE
Since 2000, *Escherichia coli* and other *Enterobacteriaceae* species producing CTX-M-type extended-spectrum β-lactamases (ESBLs) (CTX-M) have been commonly isolated from community-acquired extraintestinal infections in humans and their companion animals (1, 2, 3, 4), from food-producing animals (3, 5, 6, 7, 8), and retail meats of chicken, beef, and pork (3) worldwide. The CTX-M-type genes are assumed to have been transferred separately to plasmids including complex class 1 integrons and transposons (9) from chromosomes of different *Kluyvera* species (i.e., *K. ascorbata*, *K. georgiana*, and *K. cryocrescens*) that live in water, soil, human and animal intestinal tract; and thereby, CTX-M has been derived in five CTX-M clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) from base sequence homology (1, 9). CTX-M confers resistance against penicillins, oxyimino-cephalosporins, and monobactams (1, 4). Recently, the CTX-M-15–producing *E. coli* ST131 (O25:H4) clone has emerged as a multidrug-resistant pandemic strain affecting humans worldwide (4).

Bovine mastitis has been the most common disease affecting dairy cattle (10). Both *E. coli* and *Klebsiella pneumoniae* often cause life-threatening clinical mastitis (5, 11). The incidence of bovine mastitis has been reported to be higher in Japan (30 to 35 cases per 100 cows-years at risk) (12) than in North America, Europe, and New Zealand (10 to 30 cases per 100 cow-years) (10). Only a few classes of antimicrobials are approved for the treatment of mastitis in Japan; however, a large amount of antimicrobials are used for mastitis treatment, creating selective pressure for drug-resistant organisms (13). In our previous report, we showed that Japanese dairy cattle might be a source of CTX-M-15/2/14 and CMY-2–producing *Enterobacteriaceae* (7). However, few studies have reported the prevalence of *Enterobacteriaceae* producing CTX-M in bovine mastitis (5). The aims of this study were to determine the genetic characteristics, antimicrobial susceptibility, and genetic relatedness of ESBL- and plasmid-mediated AmpC β-lactamase-producing *Enterobacteriaceae* isolated from bovine mastitis cases.

**Screening of ESBLs.** Bacterial cultures were carried out using standard procedures on a total of 258,888 quarter milk samples obtained from 176,808 cows affected by (mainly clinical)
mastitis on 1,000 dairy farms in the Nemuro Subprefecture of Hokkaido Prefecture, Japan, between February 2007 and April 2011 (14). *Streptococcus* spp. (dominantly *S. uberis*) and *Enterococcus* spp., coagulase-negative staphylococci, *Staphylococcus aureus*, *E. coli*, and *Klebsiella* spp. were the organisms most commonly isolated from culture-positive samples.

Of the isolates, 28,900 were identified as Gram-negative bacilli and were submitted for susceptibility testing by disc diffusion according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (15); 419 isolates were identified as being cefazolin-resistant and oxidase-negative. These strains were then submitted for CLSI combination disc ESBL confirmatory tests (15) and a chromogenic oxyimino-cephalosporins hydrolysis test (Cica-β-test I; Kanto Chemical, Tokyo, Japan) to detect ESBLs, plasmidic AmpC β-lactamases and metallo-β-lactamases. Isolates with a positive ESBL confirmatory test and/or Cica-β-test were screened for metallo-β-lactamases using the sodium mercaptoacetic acid (SMA) double-disc synergy test (SMA-test) using 2 Kirby-Bauer discs containing ceftazidime and one disc containing SMA (Eiken Chemical, Tokyo, Japan). The ESBL test-positive *Enterobacteriaceae* isolates were identified using the ID 32 E API system (Sysmex bioMérieux, Tokyo, Japan).

**CTX-M genes and antimicrobial susceptibility.** The ESBL- and/or Cica-β-test–positive, and the SMA-test-negative, isolates (n=65) were analyzed by multiplex polymerase chain reaction (PCR) for the presence of *bla*CTX-M genes (16), and plasmid-mediated AmpC β-lactamase genes (i.e., CMY, ACC, FOX, MOX, DHA, CIT, and EBC groups) (17). The CTX-M types of the CTX-M-positive isolates were identified by bidirectional sequencing using group-specific PCR primers for *bla*CTX-M-1 group (18), *bla*CTX-M-2 group and *bla*CTX-M-9 group (19). AmpC-positive isolates were analyzed using type-specific PCR primers (e.g., *bla*CMY-1 and *bla*CMY-2 genes), and *bla*TEM and *bla*SHV genes were analyzed and bidirectionally sequenced using previously described primers (19) (Table S1). Comparison of nucleotide sequences and identification of each CTX-M, TEM and SHV type were carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
For these 65 isolates, the minimum inhibitory concentrations (MICs) of 23 antimicrobials were determined by the CLSI broth microdilution (15, 20) using a customer-designed microtiter panel (Opt Panel MP; Kyokuto Pharmaceutical, Tokyo, Japan). The 23 drugs were ampicillin, cefazolin, cefuroxime, cepazidime, ceftazidime/clavulanic acid, cefotaxime, cefotaxime/clavulanic acid, ceftriaxone, cefpodoxime, ceftiofur, cefquinome, cefepime, cefmetazole, moxalactam, imipenem, meropenem, aztreonam, gentamicin, amikacin, oxytetracycline, trimethoprim-sulfamethoxazole (SXT), enrofloxacin, and ciprofloxacin. Additional susceptibility test for cefoxitin, kanamycin, chloramphenicol and levofloxacin was performed by the CLSI disc diffusion method (15, 20). The breakpoints for veterinary pathogens were used for 12 antimicrobial agents (15), and the breakpoints for human Enterobacteriaceae isolates were used for the other 12 antimicrobial agents (20). *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as quality-control strains.

Sixty-five of the 419 cefazolin-resistant isolates were identified as CTX-M–producing strains. Fifty-one isolates (78.5%), which included 41 *K. pneumoniae*, 6 *Klebsiella oxytoca*, 2 *Citrobacter koseri*, 1 *E. coli*, and 1 *Enterobacter aerogenes*, harbored $bla_{CTX-M-2}$; 10 *E. coli* isolates (15.4%) harbored $bla_{CTX-M-15}$; and 4 isolates (6.2%, 2 *K. pneumoniae*, and 2 *E. coli*) harbored $bla_{CTX-M-14}$.

No isolates contained the plasmidic AmpC gene (Table 1, and Fig. 1 and 2). Thirty-seven (90.2%) of 41 CTX-M-2–producing *K. pneumoniae* isolates also harbored genes encoding SHV-1/11/28/52/83/92/98/108/148, OKP-A, or TEM-1. Four (66.6%) of 6 CTX-M-2–producing *K. oxytoca* isolates also harbored $bla_{TEM-1}$, $bla_{SHV-1}$, or $bla_{OKP-A}$. Four (40.0%) of 10 CTX-M-15–producing *E. coli* isolates also harbored $bla_{TEM-1}$, but no *E. coli* isolates harbored $bla_{SHV}$ (Table 1, and Fig. 1 and 2). The gene sequences of the CTX-M-2/1/9, TEM, and SHV groups were 99-100% homologous with those of the CTX-M-2/15/14, TEM-1, and SHV subtypes which are available on GenBank, respectively.

The 65 CTX-M-producing Enterobacteriaceae were isolated from 61 quarters of 58 mastitis
cases on 25 dairy farms in the Nemuro Subprefecture. Each of the 25 farms fed between 180 and 500 Holstein cattle with total mixed ration in free-stall barns, or with grass-silage and concentrates fed separately in tie-stall barns; almost all used sawdust bedding. Their rolling yearly herd averages for milk production were 7,800 to 9,500 kg. The 58 affected cows had either subclinical, or local to systemic clinical, mastitis. Despite antimicrobial treatment six cows were culled; and the remaining cows’ clinical signs resolved 3 to 10 weeks after onset.

The isolation rate of strains producing CTX-M-2/15/14 in bovine mastitis was 0.22% of the 28,900 Gram-negative bacilli isolates from the 258,888 quarter milk samples taken from 176,808 cows. The CTX-M-2/15–producing K. pneumoniae and E. coli strains were the most common ESBL producers causing bovine mastitis in this study. In France, CTX-M-1/14–producing E. coli and K. pneumoniae strains had an isolation rate of 0.4% (6 of 1427 E. coli and K. pneumoniae isolates) from bovine mastitis cases (5). There were no significant ($P>0.05$) differences between the isolation rates found in our study and the French study (5) by the chi-square test using StatFlex ver. 6.0 (Artech Co., Ltd., Osaka, Japan). Among human and animal isolates in Western European countries and Japan, the most common CTX-M types were the CTX-M-1 cluster (CTX-M-1/15/55) and CTX-M-9 cluster (CTX-M-9/14/27) (2, 3, 4, 5, 6). Except for the dominance of CTX-M-2, our results are similar to these previous reports. The blaTEM-1, blaSHV-1, and blaSHV-11 genes detected in the present study encode non-ESBL enzymes (21), however, it is not clear whether the SHV-28/52/83/92/98/108/148 and OKP-A are ESBLs because the kinetic parameters of their purified enzymes were not determined.

Isolates producing CTX-M exhibited high resistance to oxyimino-cephalosporins, however, they exhibited high susceptibility rates to cefmetazole, moxalactam, imipenem, meropenem, gentamicin, and amikacin. The isolates producing CTX-M-2 and CTX-M-14 showed high susceptibility rates to ceftazidime and fluoroquinolones. In contrast, the CTX-M-15–producing E. coli showed significantly higher rates of resistance to ceftazidime, aztreonam, cefepime, SXT,
oxytetracycline, ciprofloxacin, levofloxacin, cefoxitin, and kanamycin in comparison to
CTX-M-2/14–producing *Klebsiella* spp. and/or other CTX-M-2/14–producing *Enterobacteriaceae*
(P<0.05) by the chi-square tests (Table 2). Our results are consistent with previous study (1), and
the CTX-M types other than CTX-M-15, CTX-M-16 and CTX-M-27 efficiently hydrolyse
cefotaxime and ceftriaxone but not ceftazidime (1).

**Molecular subtyping profiles.** Random amplified polymorphic DNA (RAPD)-PCR analysis of
the 41 CTX-M-2–producing *K. pneumoniae* isolates was performed using oligonucleotide RAPD7
as previously described (22). Pulsed-field gel electrophoresis (PFGE) of a total of 13
CTX-M–producing *E. coli* isolates was conducted according to the PulseNet standardized
laboratory protocol (23) using *Xba I* (Roche Applied Science, Mannheim, Germany) and the
CHEF-DR III electrophoresis systems (Bio-Rad, Hercules, CA, USA). Dendrograms of RAPD
patterns and PFGE patterns were analyzed using BioNumerics software, version 5·1 (Applied
Maths, Austin, TX, USA). Four CTX-M-15–producing *E. coli* strains isolated from bovine feces
on farm M in our previous study (7) were used for comparison with the *E. coli* isolates from
mastitis cases.

Multilocus sequence typing (MLST) of the 13 CTX-M–producing *E. coli* isolates was
conducted according to standard protocols using the *E. coli* database on the MLST website.
(http://mlst.ucc.ie/mlst/dbs/Ecoli). The 13 *E. coli* isolates were serotyped according to O and H
antigens using the pathogenic *E. coli* antisera ‘SEIKEN’ Set 1 and Set 2, respectively (Denka
Seiken, Tokyo, Japan).

The 41 CTX-M-2–producing *K. pneumoniae* isolates from 15 farms revealed 32 RAPD types.
More than half of the strains were isolated from 2 farms (F and M). The 18 isolates from farm F
revealed 16 RAPD types. There was not a predominant RAPD type among the 41 isolates.
However, 2 to 3 isolates each of *K. pneumoniae* (MCK17/18/8, MCK25/26, and MCK31/32),
which were isolated from 2 different cows on same farm (F or M), showed closely related RAPD
The 13 *E. coli* isolates from 7 farms belonged to 10 STs, and showed 12 PFGE types. Two isolates each of *E. coli* (MCE1/3, MCE4/6, and MCE9/10), which were isolated from 2 different cows on same farm (D, M, or P), had the same ST and closely related PFGE types (ST23/W, ST58/V1 and V2, and ST10/Z1 and Z2, respectively). There were not any closely related strains between the 5 mastitis and the 4 fecal *E. coli* CTX-M-15–producing isolates from farm M (Fig. 2). Most of the *E. coli* isolates were O and H antigens untypable: (OUT, HUT). Neither *E. coli* clone ST131 (O25:H4) nor enterohemorrhagic *E. coli* O157, O26, O111 or other serotypes commonly isolated from human infections (8) were detected from the 13 isolates.

The genetic diversity in the 18 *K. pneumoniae* isolates obtained from bovine mastitis cases on farm F suggests that these were opportunistic infections originating from a wide variety of environmental sources (11). However, the presence of some strains of *K. pneumoniae* and *E. coli* showing the closely related genotype, which were isolated from the different cows on the same farm, suggests a contagious infection or an infection from an environmental point source (11). Similar to our results, *E. coli* clones ST10/23/58 producing CTX-M-14/1 have also been isolated from bovine mastitis in France (5). Consistent with this French study, we detected no *E. coli* clone ST131 (O25:H4) producing CTX-M-15/27. Thus, these results suggest that cattle, unlike humans, canines and felines, have little significance as a source of this clone (2, 4). In contrast, recently, the enterohemorrhagic *E. coli* O26:H11 and B1 phylogenetic group carrying p*CTX-M-9* was isolated from diarrheic cattle in France (8).

In conclusion, the genes encoding CTX-M2/15/14 are present at a low frequency in *Enterobacteriaceae* isolates causing bovine mastitis on Japanese dairy farms. The ESBL producers were dominated by CTX-M-2–producing *K. pneumoniae* and CTX-M-15–producing *E. coli* which showed multidrug-resistance to ceftazidime, aztreonam, and cefepime. There was not a predominant clonal type and even the 18 *K. pneumoniae* strains isolated from a single farm
showed the clonal diversity by molecular subtyping.

REFERENCES


23. Centers for Disease Control and Prevention (CDC). 2004. One-day (24-28 h) standardized laboratory protocol for molecular subtyping of Escherichia coli O157:H7, non-typhoidal Salmonella serotypes, and Shigella sonnei by pulsed field gel electrophoresis (PFGE). Sections 5.1, 5.2, 5.4. CDC, Atlanta, GA.
FIG. 1 RAPD-PCR of 41 *K. pneumoniae* isolates producing CTX-M-2. Cluster analysis was performed by the unweighted pair group method using arithmetic averages with a 1.0% band position tolerance window and 1.0% optimization. DNA relatedness was calculated based on the Dice coefficient. Thirty-two band patterns were typed using similarity cut-off values of approximately $\geq 85\%$.

FIG. 2 PFGE patterns and cluster analysis of 13 CTX-M-producing *E. coli* isolates (MCE1 to 13) from mastitis cases, obtained using *Xba*I enzyme. Cluster analysis was performed by the unweighted pair group method using arithmetic averages with a 1.0% band position tolerance window and 1.0% optimization. DNA relatedness was calculated based on the Dice coefficient. Twelve band patterns were typed using similarity cut-off values of $\geq 90\%$. *Salmonella* Braenderup CCUG50923 was used as a marker for assessing PFGE banding patterns. FCE3, 4, 5, and 16 were isolated from feces from cattle on farm M in our previous study (Ohnishi M, Okatani AT, et al. 2013. J. Appl. Microbiol. [7]).
TABLE 1 Origins of CTX-M-2 or CTX-M-14 ESBL-producing Enterobacteriaceae isolates other than CTX-M-2 producing K. pneumoniae and E. coli

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation date; Farm: Cow</th>
<th>Bacterial species</th>
<th>CTX-M genotypes</th>
<th>TEM, SHV genotypes</th>
</tr>
</thead>
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<tr>
<td>MCKo1</td>
<td>July 08; F: 20</td>
<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>OKP-A</td>
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<tr>
<td>MCKo2</td>
<td>Sep 08; H: 22</td>
<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>TEM-1</td>
</tr>
<tr>
<td>MCKo3</td>
<td>May 09; O: 30</td>
<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>TEM-1</td>
</tr>
<tr>
<td>MCEa1</td>
<td>Sep 09; H: 36</td>
<td>E. aerogenes</td>
<td>CTX-M-2</td>
<td>negative</td>
</tr>
<tr>
<td>MCKo4</td>
<td>Jan 10; M: 42</td>
<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>negative</td>
</tr>
<tr>
<td>MCCk1</td>
<td>Apr 10; U: 47</td>
<td>C. koseri</td>
<td>CTX-M-2</td>
<td>negative</td>
</tr>
<tr>
<td>MCCk2</td>
<td>Apr 10; V: 48</td>
<td>C. koseri</td>
<td>CTX-M-2</td>
<td>negative</td>
</tr>
<tr>
<td>MCKo5</td>
<td>Aug 10; F: 50</td>
<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>SHV-1</td>
</tr>
<tr>
<td>MCKo6</td>
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<td>K. oxytoca</td>
<td>CTX-M-2</td>
<td>negative</td>
</tr>
<tr>
<td>MCK45</td>
<td>Nov 10; Y: 57</td>
<td>K. pneumoniae</td>
<td>CTX-M-14</td>
<td>negative</td>
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<tr>
<td>MCK46</td>
<td>Nov 10; Y: 57</td>
<td>K. pneumoniae</td>
<td>CTX-M-14</td>
<td>negative</td>
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</table>
### TABLE 2 Antimicrobial susceptibilities of CTX-M-type ESBL-producing isolates from mastitic cows (41 K. pneumoniae and 6 K. oxytoca) isolates producing CTX-M-2, and 2 K. pneumoniae isolates producing CTX-M-14, 10 E. coli isolate producing CTX-M-15, and other Enterobacteriaceae isolates producing CTX-M-2 or CTX-M-14 assessed by the broth microdilution or disk diffusion method

<table>
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<tr>
<th>Antimicrobial agents</th>
<th>MIC range (μg/mL)</th>
<th>MICs (μg/mL)</th>
<th>Breakpoints† (μg/mL; mm)</th>
<th>Susceptible No. of isolates (%)</th>
<th>Intermediate No. of isolates (%)</th>
<th>(A) Resistant No. of isolates (%)</th>
<th>MIC range (μg/mL)</th>
<th>(B) Resistant No. of isolates (%)</th>
<th>MIC range (μg/mL)</th>
<th>(C) Resistant No. of isolates (%)</th>
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</thead>
<tbody>
<tr>
<td>Amoxicillin*</td>
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<td>≥12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;12 to 256</td>
<td>0</td>
<td>&gt;12 to 256</td>
<td>0</td>
</tr>
<tr>
<td>Cefazolin*</td>
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<td>≥12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;128 to 256</td>
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<td>Cefuroxime*</td>
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<td>≥12</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>≥12</td>
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<td>Ciprofloxacin*</td>
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<td>&gt;128 to 256</td>
<td>0</td>
<td>&gt;128 to 256</td>
<td>0</td>
</tr>
</tbody>
</table>

* *A: resistant to >2 mg/L Ceftriaxone, *B: resistant to >2 mg/L Ceftazidime, *C: resistant to >2 mg/L Meropenem, **B: resistant to >2 mg/L Ceftimoxone, **C: resistant to >2 mg/L SXT, ***: resistant to >2 mg/L Moxalactam, ****: resistant to >2 mg/L Enrofloxacin.
### Ciprofloxacin

<table>
<thead>
<tr>
<th>≤0.5 to 2</th>
<th>≥0.5 to ≤1</th>
<th>&gt;1</th>
<th>≥4</th>
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<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≥4</td>
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<tr>
<td>46 (93.9%)</td>
<td>3 (6.1%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Cefoxitin

<table>
<thead>
<tr>
<th>≤0.5 to 1</th>
<th>≤0.5 to &gt;2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 (10.0%)</td>
</tr>
</tbody>
</table>

### Kanamycin

<table>
<thead>
<tr>
<th>≤13mm</th>
<th>14-22mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>38 (77.6%)</td>
<td>1 (2.0%)</td>
</tr>
</tbody>
</table>

### CHL

<table>
<thead>
<tr>
<th>≤13mm</th>
<th>14-22mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>41 (83.7%)</td>
<td>0</td>
</tr>
</tbody>
</table>

### Levofloxacin

<table>
<thead>
<tr>
<th>≤13mm</th>
<th>14-22mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>41 (83.7%)</td>
<td>0</td>
</tr>
</tbody>
</table>

**Abbreviations:** CAZ/CLA, ceftazidime/clavulanic acid; CTX, cefotaxime; OTET, oxytetracycline; SXT, trimethoprim/sulfamethoxazol; CHL, chloramphenicol

- Breakpoint for resistance in accordance with CLSI document M31-A3 (2008) for veterinary pathogens
- Breakpoint for resistance in accordance with CLSI document M100-S21 (2011) for isolates from human infections with Enterobacteriaceae
- Breakpoint for resistance or intermediate is not defined in CLSI documents M31-A3 (2008) and M100-S21 (2011).
- Antimicrobial agent approved for cattle in Japan; the other 17 antimicrobials in this table are unapproved for cattle.
- Susceptibilities were tested by disc diffusion method according to CLSI documents M31-A3 (2008) and M100-S21 (2011).
- Isolates in the susceptible, intermediate, and resistant categories could not be differentiated.
- Significant difference by χ²-test: *P<0.05; **P<0.01
Isolate; Year; Farm: Cow ST (STcomplex), Serotype PFGE CTX-M TEM, SHVtype

MCE4; Dec 08; M: 24 ST58 (155), OUT: HUT V1 M-15 negative
MCE6; Mar 09; M: 27 ST58 (155), OUT: HUT V2 M-15 negative
MCE3; Oct 07; D: 12 ST23 (23), OUT: HUT W M-14 TEM-1
MCE1; May 07; D: 4 ST23 (23), OUT: HUT W M-14 negative
MCE8; Jul 09; M: 32 ST101 (101), O159: H45 X M-15 negative
FCE3; 07; M: Cow 2 ST1167 (−), OUT: H19 R M-15 negative
FCE5; 07; M: Cow 4 ST1167(−), O28ac: HUT R M-15 negative
MCE13; Dec 10; Z: 58 ST1126 (−), OUT: HUT AD M-15 TEM-1
MCE5; Dec 08; M: 25 ST648 (−), O74: HUT AC M-15 negative
FCE16; 09; M: Calf 11 ST2325 (−), OUT: HUT U M-15 negative
MCE11; Nov 09; T: 40 ST1415(−), OUT: HUT Y M-15 TEM-1
MCE2; Aug 07; E: 6 ST1284 (−), OUT: HUT O M-15 negative
MCE9; Aug 09; P: 34 ST10 (10), OUT: H5 Z1 M-15 TEM-1
MCE10; Sep 09; P: 37 ST10 (10), OUT: H5 Z2 M-15 TEM-1
FCE4; 07; M: Cow 3 ST540 (−), OUT: HUT D M-15 negative
MCE12; Oct 10; M: 56 ST88(23), OUT: H6 AA M-2 TEM-1

MCE7; Apr 09; N: 29 ST3499 (−), OUT: HUT AB M-15 negative