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Antimicrobial resistance patterns of *Streptococcus uberis* isolates from bovine milk in Chiba prefecture, Japan: association between multidrug resistance and clonal complex 996

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ABSTRACT. *Streptococcus uberis* is one of major pathogens causing bovine mastitis. However, there is poor information on antimicrobial resistance (AMR) among the Japanese isolates. To provide treatment information for the mastitis caused by *S. uberis* in Japan, we aimed to clarify AMR patterns of the isolates from bovine milk mainly in Chiba. AMR phenotyping/genotyping [*blaZ*–*erm*(A)–*erm*(B)–*mef*(A)–*linB*–*lnuD*–*tet*(M)–*tet*(O)–*tet*(K)–*tet*(L)–*tet*(S)] and multilocus sequence typing were performed to analyze relationships between AMR patterns and clonal complexes (CCs). Resistance to tetracycline-, macrolide-, and lincosamide-classes was mainly associated with possession of *tet*(O), *tet*(S), *erm*(B), *linB*, and *lnuD* genes. CC996 was significantly associated with multidrug resistance ($P<0.0001$). These findings will aid Chiba farm animal clinics in treating bovine mastitis.

KEYWORDS: antimicrobial resistance, bovine milk, Chiba, clonal complex, *Streptococcus uberis*

Bovine clinical mastitis in dairy cattle can affect udder health, milk quality, and milk production, leading to economic losses in dairy farms. Major causative pathogens of clinical mastitis include *Escherichia coli*, *Staphylococcus aureus* [20], coagulase-negative staphylococci, and contagious or environmental streptococci. Contagious *Streptococcus* is *S. agalactiae* (Lancefield group B *Streptococcus*), whereas environmental streptococci include *S. dysgalactiae*, *S. canis*, and *S. uberis* [21]. Based on economic calculations using stochastic bio-economic model of bovine intramammary infection (IMI) [6], the average total annual net costs of clinical and subclinical IMIs due to *S. uberis* were € 484 and € 306, respectively.

S. uberis is isolated from environmental sources (including soil, pasture, bedding materials, and bovine feces) and is also present on the skin of dairy cows [21]. It can cause bovine clinical mastitis during lactating and non-lactating periods after direct contact with the teat apex [10]. Some cows infected with *S. uberis* may develop repetitive and refractory mastitis. Molecular epidemiological studies of clinical mastitis caused by *S. uberis* in dairy herds [3] have reported that either predominant or a limited number of isolates probably cause IMIs or transmission among cows (including potential transmission of the isolates via milking machine or environment).

Coffey *et al.* [2] constructed a multilocus sequence typing (MLST) system of *S. uberis*. This system provides further insights into population biology and epidemiology of *S. uberis*. Furthermore, PubMLST (https://pubmlst.org/organisms/streptococcus-uberis/) reveals latest sequence type (ST) 2015 [as of January 10th, 2024], suggesting that population structure of *S. uberis* isolates is highly diverse. On the contrary, several *S. uberis* isolates from different geographic regions can be clustered within limited clonal complexes (CCs) [i.e., global clonal complexes (GCCs)] on MLST-based minimal spanning tree [18]. Thirty percent of the twenty-seven isolates from bovine mastitis in Victoria and Queensland of Australia were clustered within GCC86 and GCC143. In Japan, Watanabe *et*

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al. [19] have performed MLST using *S. uberis* from bovine mastitis. Two ST996 isolates were from Hokkaido and Okayama. Two ST1003 isolates belonging to both CC996 and GCC143 were from Hokkaido, five ST1001 isolates belonging to CC996 were from Hokkaido and Tochigi, and one ST997 isolate belonging to CC996 was from Hokkaido.

Various groups of antimicrobial classes (macrolide-, lincosamide-, and beta-lactam-classes) are administered for treatment of *S. uberis*-associated mastitis [22]. The excessive usage of antimicrobials in dairy herds may lead to increased antimicrobial resistance (AMR) among mastitis pathogens [14]. Zhang *et al.* [22] have described AMR profiles and genes in *S. uberis* isolates associated with bovine mastitis in Thailand. The isolates were resistant to tetracycline (82.0%), ceftiofur (19.3%), and erythromycin (8.3%). Prevalent AMR genes were *tet*(M) (87.3%), *erm*(B) (66.2%), and *blaZ* (6.6%). Thus, AMR monitoring of bovine mastitis-associated *S. uberis* can support an antimicrobial stewardship program for dairy farms. However, there are a few reports on AMR patterns and genes among Japanese isolates.

We aimed to clarify AMR phenotypes/genotypes of the isolates from bovine milk in Chiba and other prefectures. We also analyzed whether isolates clustered into limited CCs were associated with multidrug resistance (MDR).

Sanritsu Zelkova Veterinary Laboratory approved our study design (approval no. SZ20220324) to ensure the privacy of diseased cows. We collected the Chiba isolates in collaboration with five farm animal clinics from March to October 2022 (hereafter Chiba isolates). Additionally, Obihiro Clinical Laboratory with Sapporo Clinical Laboratory kindly provided the Hokkaido or Okinawa isolates from August to September 2023 (hereafter Hokkaido or Okinawa isolates) to compare AMR patterns in Chiba isolates with those in Hokkaido or Okinawa isolates. Species identification was based on the results of mass spectrometry. One isolate per host was stored at −70°C to −80°C in these commercial laboratories. American Type Culture Collection (ATCC) 700407 was used as a reference strain. We performed polymerase chain reaction (PCR) methods for differentiation of *S. uberis* and *S. parauberis* according to the fragments of 16S/23S rRNA genes [7], to decide the isolates as analytical strains.

One hundred one *S. uberis* isolates were obtained from four farm animal clinics in Chiba (mapped on Supplementary Fig. 1). Additionally, the Hokkaido (*n*=18) or Okinawa (*n*=2) isolates were from seven farm animal clinics in Hokkaido and one farm animal clinic in Okinawa.

Minimum inhibitory concentrations (MICs, μg/mL) of 14 antimicrobials (penicillin G, ampicillin, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, chloramphenicol, cefotaxime, ceftriaxone, cefepime, cefozopran, meropenem, and vancomycin) were determined using broth microdilution method (MICroFAST Panel Types 7J for *Streptococcus* spp.; Beckman Coulter Inc., Tokyo, Japan) recommended in Clinical and Laboratory Standards Institute (CLSI) guidelines for alpha-hemolytic streptococci [1]. Susceptibility quality control was performed using ATCC 29212/49619 strains. To determine susceptibility or resistance to minocycline, we used tetracycline breakpoints in accordance with the CLSI guidelines [1].

Supplementary Table 1 reveals MICs of each isolate against 14 antimicrobial agents. Table 1 shows MIC_{50} and MIC_{90} values with AMR rates. The rates of resistance to tetracycline-, macrolide-, and lincosamide-class in Chiba were 48.5%, 33.7%, and 54.5%, respectively. Supplementary Fig. 2 shows MIC distributions of the 101 isolates against 14 antimicrobial agents. We found penicillin G-intermediate susceptibility (MIC=0.25 μg/mL) in six Chiba isolates and three Hokkaido or Okinawa isolates.

Supplementary Table 2 shows oligonucleotide primer sequences to amplify AMR genes [*blaZ*–*erm*(A)–*erm*(B)–*mef*(A)–*linB*–*lnuD*–*tet*(M)–*tet*(O)–*tet*(K)–*tet*(L)–*tet*(S)], melting temperatures, and amplicon sizes by PCR [5, 11, 16]. We included 16S rRNA gene (amplicon size 1,459 bp) as an internal control [4, 17]. The correct nucleotide sequences of several positive isolates were confirmed

Table 1. Antimicrobial activities of oral and parenteral antibiotics against *Streptococcus uberis* isolates from bovine milk in Chiba and other prefectures

Antibiotic	Chiba isolates (*n*=101)			Hokkaido or Okinawa isolates (*n*=20)			Percent resistance rate
	MIC range (μg/mL)[a]	MIC_{50}[a]	MIC_{90}[a]	MIC range (μg/mL)[a]	MIC_{50}[a]	MIC_{90}[a]	Chiba (*n*) and Hokkaido or Okinawa (*n*) isolates
Penicillin G[b]	≤0.03–0.25	0.12	0.12	0.06–0.25	0.12	0.25	
Ampicillin	≤0.06–0.25	0.12	0.25	0.12–0.25	0.25	0.25	
Minocycline	≤0.5– >4	4	>4	≤0.5– >4	≤0.5	>4	48.5 (49) and 40.0 (8)
Erythromycin	≤0.12– >2	≤0.12	>2	≤0.12– >2	≤0.12	>2	33.7 (34) and 35.0 (7)
Chloramphenicol	≤4	≤4	≤4	≤4	≤4	≤4	
Cefotaxime	≤0.12–1	0.5	0.5	0.25–1	0.5	1	
Cefozopran	≤0.12–0.5	0.25	0.5	≤0.12–1	0.25	0.5	
Cefepime	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	
Clindamycin	≤0.12– >1	>1	>1	≤0.12– >1	≤0.12	>1	54.5 (55) and 50.0 (10)
Levofloxacin	≤0.25–1	0.5	0.5	≤0.25–1	0.5	1	
Meropenem	≤0.12–0.5	0.25	0.25	≤0.12–0.5	0.25	0.25	
Vancomycin	0.5–1	0.5	0.5	0.5	0.5	0.5	
Ceftriaxone	≤0.12–1	0.5	0.5	≤0.12–1	0.5	1	
Azithromycin	≤0.25– >4	≤0.25	>4	≤0.25– >4	≤0.25	>4	33.7 (34) and 35.0 (7)

MIC, minimum inhibitory concentration. [a]MICs of 14 antimicrobials were examined using broth microdilution method (MICroFAST Panel Type 7J, Beckman Coulter Inc., Tokyo, Japan), according to Clinical and Laboratory Standards Institute Document M100-S26 for alpha-hemolytic streptococci. [b]We found penicillin G-intermediate susceptibility (MIC=0.25 μg/mL) of six Chiba and three Hokkaido or Okinawa isolates.

using direct sequencing as previously described [4, 17].

Supplementary Table 1 reveals AMR genotype of each isolate. Table 2 shows detection rates of AMR genes. Prevalence of *tet*(O), *tet*(S), *erm*(B), *linB*, *lnuD*, and *blaZ* in Chiba were 35.6%, 16.8%, 35.6%, 11.9%, 30.7%, and 4.0%, respectively. We observed the isolates that possessed both *tet*(O) and *erm*(B) from Chiba (n=36) and other prefectures (n=6). The major AMR genotypes were *tet*(S)–*linB*–*lnuD* (n=9), *tet*(S)–*lnuD* (n=7), and *tet*(O)–*erm*(B)–*blaZ* (n=3).

PCR was conducted for amplification seven housekeeping genes (*arcC-ddl-gki-recP-tdk-tpi-yqiL*), and direct sequencing after amplicon purification was performed. The obtained sequences were submitted to the following website: https://pubmlst.org/bigsdb?db=pubmlst_suberis_seqdef. Allele numbers of the housekeeping genes were profiled to identify STs [8]. We constructed CCs from main STs and their corresponding single-locus/double-locus/triple-locus variants, which exhibited differences in one, two, and three housekeeping gene(s), respectively. Novel allele combinations were registered into PubMLST Isolates database (https://pubmlst.org/bigsdb?db=pubmlst_suberis_isolates) to identify novel STs. An expansion of the goeBURST program implemented in PHYLOViZ was used to produce a minimum-spanning tree representing possible relationships among STs [12]. We evaluated the significant associations (using a two-sided Fisher's exact probability test) between determined AMR phenotypes/genotypes and CCs. Statistical significance was set at P value <0.05.

Table 3 shows the STs, MICs, and AMR phenotypes/genotypes of the 31 *S. uberis* strains analyzed in this study. Eighteen Chiba isolates with *tet*(O)–*erm*(B) in addition to three Chiba isolates and three Hokkaido or Okinawa susceptible isolates were selected based on random sampling numbers using Excel application. We registered six novel ST1979–ST1984 into PubMLST Isolate database. We observed the clustering distributions of ST996 (allelic profile, 1-1-37-3-17-2-3), ST995 (1-1-37-4-17-2-3, single-locus variant), ST997 (1-1-3-3-3-2-3, double-locus variant), ST1001 (1-1-37-3-101-2-5, double-locus variant), ST1003 (1-1-37-2-17-2-3, single-locus variant), ST1053 (1-15-2-2-17-2-3, triple-locus variant), ST1980 (1-1-37-3-101-2-3, single-locus variant), and ST1982 (3-1-37-2-17-18-3, triple-locus variant), which constituted CC996. There was a significant association (P<0.0001) between CC996 (n=24) and MDR strains harboring *tet*(O)–*erm*(B) (Table 2). Supplementary Fig. 3 shows the goeBURST diagram of antimicrobial susceptibility patterns (MDR/susceptibility) by the strains. ST143 (18-1-2-2-17-4-3) and ST86 (3-2-3-3-13-1-3) constitute GCC143 and GCC86. ST1003, ST1053, and ST1982 belonged to GCC143 [triple-locus variant], whereas ST1023 (3-2-3-2-5-2-3) belonged to GCC86 [triple-locus]. We found CC996 population including three MDR GCC143 isolates and a GCC143 isolate susceptible to the 14 antibiotics, whereas a GCC86 isolate was susceptible to all the antibiotics and apart from CC996 population on the goeBURST diagram (Supplementary Fig. 3).

CC996 *S. uberis* can be distributed popularly nationwide because isolation of this clone has been already reported in Japan [19]. We found that some CC996 isolates belonged to GCC143, suggesting that some CC996 isolates diverging from GCC143 may be spreading in Japan (Table 3/Supplementary Fig. 3).

We identified a CC996 population structure, which is unique to Japan. Molecular typing and AMR profiles of *S. uberis* isolates from sheep milk in Italy [13] have been described. Seventeen isolates (13.7%) showed MDR: ten were resistant to three different classes, six to four different classes, and one to five different classes. No specific correlation was observed between the GCCs (containing GCC143) and resistance to aminoglycosides or other antimicrobials. Of the 17 isolates, only four belonged to GCC143: one and one to GCC86 and GCC5. Small numbers of these GCC143/86/5 isolates may lead to the no significant correlation. Therefore, future studies monitoring variations in AMR patterns and CCs/GCCs among populations similar with this study are required.

We confirmed penicillin-intermediate susceptibility (MIC=0.25 µg/mL) in 6 Chiba isolates and 3 Hokkaido or Okinawa isolates, and 4 Chiba isolates harbored *blaZ* gene. AMR profiles of the isolates from bovine mastitis in Switzerland [9] revealed that penicillin MIC was slightly increased (0.25 µg/mL) for two (1.3%) of the isolates. *blaZ* detection rate among isolates in Thailand was 6.6% [22]. Therefore, AMR patterns in some countries highlight the importance of monitoring and antimicrobial stewardship programs (particularly penicillin usage) in farm animal clinics and hospitals.

We found that the two isolates harboring both *tet*(O) and *erm*(B) were resistant to tetracycline-class, but susceptible to macrolide-class antibiotics. Korean clonal spread of clindamycin-resistant erythromycin-susceptible *S. agalactiae* was reported based on whole genome sequences [15]. This *erm*(B) sequence (738 bp) contained C222T (N74N), T224C (I75T), and A299G (N100S) nucleotide

Table 2. Detection rates of antimicrobial resistance (AMR) genes among *Streptococcus uberis* isolates from bovine milk in Chiba and other prefectures

AMR gene	Chiba isolates (n=101)		Hokkaido or Okinawa isolates (n=20)		CC996 isolates (n=24)		Non-CC996 isolates (n=7)		P value
	No. of isolates with AMR gene	Detection rate (%)	No. of isolates with AMR gene	Detection rate (%)	No. of isolates with AMR gene	Detection rate (%)	No. of isolates with AMR gene	Detection rate (%)	CC996 vs. non-CC996 isolates
blaZ	4	4	0	0	0	0	0	0	
tet(M)	0	0	1	5	0	0	1	14.3	0.2258
tet(O)[a]	36[a]	35.6	6[a]	30	23	95.8	1	14.3	<0.0001
tet(S)	17	16.8	2	10	0	0	0	0	
erm(B)[a]	36[a]	35.6	6[a]	30	23	95.8	1	14.3	<0.0001
mef(A)	0	0	1	5	0	0	0	0	
linB	12	11.9	4	20	0	0	0	0	
lnuD	31	30.7	2	10	0	0	0	0	

[a]We observed the combined detection of *tet*(O)–*erm*(B) of the identical isolates (n=36 and n=6) from Chiba and other prefectures.

Table 3. Sequence types (STs) among *Streptococcus uberis* strains with multidrug resistance or with susceptibility

Strain	ST[a] (*arcC-ddl-gki-recP-tdk-tpi-yqiL*)	Clonal complex[b]	Global clonal complex[b]	Minimum inhibitory concentration μg/mL (susceptible–intermediate–resistant)														AMR genotype
		(CC) [variant]	(GCC) [variant]	PCG	ABPC	MINO	EM	CP	CTX	CZOP	CFPM	CLDM	LVFX	MEPM	VCM	CTRX	AZM	
UB2	ST996 (1-1-37-3-17-2-3)	**CC996**		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	1 (S)	0.25 (S)	0.5 (S)	0.25 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB7	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.25 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB25	ST995 (1-1-37-4-17-2-3)	**CC996** [single-locus]		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB36	Novel ST1980 (1-1-37-3-101-2-3)[a]	**CC996** [single-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB37	ST996 (1-1-37-3-17-2-3)	**CC996**		0.25 (I)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB38	ST996 (1-1-37-3-17-2-3)	**CC996**		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB41	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB56	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	≤0.12 (S)	0.5 (S)	0.25 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB58	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB63	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.25 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB68	ST1053 (1-15-2-2-17-2-3)	**CC996** [triple-locus]	**GCC143** [triple-locus]	0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB80	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB82	ST1001 (1-1-37-3-101-2-5)	**CC996** [double-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB85	ST1003 (1-1-37-2-17-2-3)	**CC996** [single-locus]	**GCC143** [triple-locus]	0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB92	ST1053 (1-15-2-2-17-2-3)	**CC996** [triple-locus]	**GCC143** [triple-locus]	0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB97	ST996 (1-1-37-3-17-2-3)	**CC996**		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB99	ST996 (1-1-37-3-17-2-3)	**CC996**		0.25 (I)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
UB100	ST996 (1-1-37-3-17-2-3)	**CC996**		0.25 (I)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	1 (S)	>4 (R)	*tet*(O)-*erm*(B)
HK1	ST996 (1-1-37-3-17-2-3)	**CC996**		0.25 (I)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	1 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	1 (S)	>4 (R)	*tet*(O)-*erm*(B)
HK6	ST996 (1-1-37-3-17-2-3)	**CC996**		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	1 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	1 (S)	>4 (R)	*tet*(O)-*erm*(B)
HK7	ST994 (1-1-5-2-100-11-7)			0.06 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	0.25 (S)	≤0.12 (S)	≤0.5 (S)	>1 (R)	≤0.25 (S)	≤0.12 (S)	0.5 (S)	≤0.12 (S)	>4 (R)	*tet*(M)-*tet*(O)-*erm*(B)
HK14	ST995 (1-1-37-4-17-2-3)	**CC996** [single-locus]		0.12 (S)	0.12 (S)	>4 (R)	>2 (R)	≤4 (S)	1 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	≤0.12 (S)	0.5 (S)	1 (S)	>4 (R)	*tet*(O)-*erm*(B)

Table 3. Continued.

Strain	ST[a] (*arcC-ddl-gki-recP-tdk-tpi-yqiL*)	Clonal complex[b]	Global clonal complex[b]	Minimum inhibitory concentration μg/mL (susceptible–intermediate–resistant)														AMR genotype
		(CC) [variant]	(GCC) [variant]	PCG	ABPC	MINO	EM	CP	CTX	CZOP	CFPM	CLDM	LVFX	MEPM	VCM	CTRX	AZM	
HK16	ST997 (1-1-3-3-3-2-3)	**CC996** [double-locus]		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.5 (S)	0.5 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
HK20	ST996 (1-1-37-3-17-2-3)	**CC996**		0.12 (S)	0.25 (S)	>4 (R)	>2 (R)	≤4 (S)	0.25 (S)	0.25 (S)	≤0.5 (S)	>1 (R)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	>4 (R)	*tet*(O)-*erm*(B)
ATCC 700407	ST154 (23-1-5-2-26-1-17)			≤0.03 (S)	≤0.06 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	≤0.12 (S)	≤0.12 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	≤0.12 (S)	0.5 (S)	≤0.12 (S)	≤0.25 (S)	Not detected
UB30	Novel ST1979 (3-1-3-4-9-2-3)[a]			0.12 (S)	0.12 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	1 (S)	0.5 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	≤0.12 (S)	1 (S)	0.5 (S)	≤0.25 (S)	Not detected
UB96	Novel ST1982 (3-1-37-2-17-18-3)[a]	**CC996** [triple-locus]	**GCC143** [triple-locus]	0.06 (S)	0.12 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	0.25 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	≤0.12 (S)	0.5 (S)	0.5 (S)	≤0.25 (S)	Not detected
UB98	ST1023 (3-2-3-2-5-2-3)		GCC86 [triple-locus]	0.12 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	1 (S)	0.5 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	0.25 (S)	0.5 (S)	1 (S)	≤0.25 (S)	Not detected
HK3	Novel ST 1984 (3-2-3-2-5-2-10)[a]			0.12 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	1 (S)	0.5 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	0.25 (S)	0.5 (S)	1 (S)	≤0.25 (S)	Not detected
HK5	Novel ST1983 (2-1-6-4-101-28-3)[a]			0.12 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	1 (S)	≤0.12 (S)	0.5 (S)	0.5 (S)	≤0.25 (S)	Not detected
HK12	Novel ST1981 (6-1-3-1-17-1-71)[a]			0.12 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	≤4 (S)	0.5 (S)	0.25 (S)	≤0.5 (S)	≤0.12 (S)	0.5 (S)	0.25 (S)	0.5 (S)	0.5 (S)	≤0.25 (S)	Not detected

PCG, penicillin G; ABPC, ampicillin; MINO, minocycline; EM, erythromycin; CP, chloramphenicol; CTX, cefotaxime; CZOP, cefozopran; CFPM, cefepime; CLDM, clindamycin; LVFX, levofloxacin; MEPM, meropenem; VCM, vancomycin; CTRX, ceftriaxone; AZM, azithromycin; AMR, antimicrobial resistance; ATCC, American Type Culture Collection. [a]We registered novel STs into PubMLST Isolate database. CC included single-locus, double-locus, and triple-locus variants. Allelic profiles of ST143 and ST86 are 18-1-2-2-17-4-3 and 3-2-3-3-13-1-3, respectively. [b]CC996 and GCC143 isolates are underlined in bold letters. A CC996 [triple-locus/single-locus variants] isolate was shown to belong to the GCC143 [triple-locus variant] isolate.

(amino acid) substitutions, in addition to insertion of an IS1216E element at nucleotide position 642, which resulted in deletion of a segment spanning nucleotides 642–738 (97 bp). This immature ErmB protein could not confer resistance to erythromycin. We also observed the clindamycin-susceptible isolates that possessed *lnuD* and penicillin G-intermediate isolates that didn't carry *blaZ*. Therefore, the discrepancies between AMR phenotypes and genotypes should be assessed.

We obtained very limited host demographics (prefecture and collection date) for these isolates (Supplementary Table 1). More detailed information (i.e., therapeutic strategies including antimicrobial dosing and duration) is needed to clarify relationships between AMR patterns and their clinical implications, because it was unclear what usage of antimicrobial agents were associated with MDR in CC996. Moreover, we could not collect the information on dairy farms, which requested the examination on milk pathogens to the farm clinics. To trace the sources, the detailed information on the farms should be obtained in future.

In conclusion, our data indicated AMR patterns in the isolates from bovine milk in Chiba and other prefectures as well as spread of CC996 MDR isolates in Chiba. This is the updated study to describe AMR patterns and genes among the Chiba isolates. These data will aid Chiba farm animal clinics in treating bovine clinical mastitis, because rates of resistance to tetracycline-, macrolide-, and lincosamide-classes among our collected isolates in Chiba were 48.5%, 33.7%, and 54.5%, respectively.

CONFLICT OF INTEREST. The authors declare that they have no conflicts of interest.

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