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Simplified–agar disk diffusion method for epidemiological breakpoint determination and characterization of antibiograms of bovine clinical mastitis–causing bacteria in Japan

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Abstract

Bovine mastitis has a high morbidity rate and causes significant economic losses due to milk wastage and antimicrobial use. Mastitis is treated with antimicrobial agents, but criteria for disk diffusion tests for pathogens that cause bovine mastitis in Japan are lacking. In this study, we used a simplified agar disk diffusion (ADD) method that is easy to implement in clinical practice to develop breakpoints for bovine mastitis–associated organisms and generate a corresponding antibiogram. We established new breakpoints for a simplified ADD method for *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS), *Streptococcus uberis*, *Streptococcus* spp. (except for *S. uberis*), and *Klebsiella* spp. We also revalidated previously reported breakpoints against *Streptococcus* spp. and *E. coli*. The antibiogram showed that the susceptibility rates of *Streptococcus* spp. (excluding *S. uberis*) to marbofloxacin in eastern Japan were significantly lower than those in Hokkaido and western Japan ($P < 0.01$). The susceptibility rate of *E. coli* to oxytetracycline (OTC) in eastern Japan was significantly lower than that in Hokkaido ($P < 0.01$). Low susceptibility rates were observed for ampicillin (ABPC) and erythromycin among CNS strains, OTC among *S. uberis* strains, and cefazolin, cefuroxime, and ABPC among *Klebsiella* spp. strains. The use of breakpoints in the simplified ADD method in clinical practice should promote the prudent use of antimicrobial agents. In cases of acute mastitis with systemic symptoms in which antimicrobial agents must be administered prior to susceptibility testing, the antibiogram can be used as a criterion for determining the drug of choice.

Key Words: antibiogram, bovine clinical mastitis, breakpoints, simplified agar disk diffusion method

Introduction

Antimicrobial resistance (AMR) is a global health problem linked to various human, animal, and environmental factors²¹⁾. The World Health Organization Global Action Plan on AMR

stresses the importance of surveillance of AMR in relation to antimicrobial use and of optimization of antimicrobial agent use^{21,36)}. To prevent the emergence of resistant organisms and control the diseases they cause, these organisms must be identified and their spread monitored over

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time³²⁾. In veterinary medicine, understanding the current status of resistant organisms through antimicrobial susceptibility surveillance is essential. This surveillance often employs antibiograms, which provide periodic summaries of the antimicrobial susceptibility of clinical isolates and are used to develop guidelines for empirical antimicrobial therapy for anticipated pathogens, monitor changes in resistance rates over time, and promote the prudent use of antimicrobials^{13,24,28)}. Although antibiograms for bovine mastitis have been reported^{12,31,35)}, there are only a few reports regarding the drug susceptibility of bovine clinical mastitis-causing organisms commonly collected in Japan, and the number of causative organisms and strains investigated is limited¹⁵⁾. In addition, there are no reports clarifying drug susceptibility rates by region in Japan and their regional differences.

Bovine mastitis is characterized by high morbidity and significant economic losses due to milk wastage and antimicrobial use. Antimicrobial agents are the primary treatment for bovine mastitis. Proper selection and use of antimicrobial agents to treat bovine mastitis based on identification of the causative organism and the results of antimicrobial susceptibility testing are critical in order to avoid the unnecessary use of antimicrobial agents^{19,27)}. Several challenges have been reported in regard to the interpretation of *in vitro* antimicrobial susceptibility testing results for treatment selection in clinical cases. For example, many of the antimicrobials used to treat bovine mastitis are typically selected based only on medical criteria. As such, the susceptibility patterns of bovine mastitis-causing isolates, the pharmacokinetic characteristics of the antimicrobials present in mastitis milk, and the relationship between these factors and clinical outcomes have not been adequately studied^{2,9,23)}. However, antimicrobial susceptibility testing can provide important basic data for guiding treatment in clinical cases when the results are interpreted comprehensively in conjunction with data pertaining to factors such as husbandry practices, milking hygiene, treatment history, and clinical symptoms. The agar disk diffusion (ADD) method, based on the internationally standardized

guidelines of the Clinical and Laboratory Standards Institute (CLSI)⁶⁾, is widely used in clinical laboratories to determine the antimicrobial sensitivity of strains isolated from cases of bovine mastitis. In addition to the standardized-ADD method based on the CLSI guidelines, the Japanese Guidelines for Clinical Pathology Tests in Livestock Mutual Aid list a simplified-ADD method for testing the antimicrobial susceptibility of bovine mastitis isolates in clinical examination²²⁾. The simplified-ADD involves a less-complex inoculum preparation procedure and uses standardized culture conditions for each bacterial species, prioritizing shorter testing times and reductions in cost. Currently, few regions of Japan have laboratory centers or other systems for centralized and rapid clinical bacteriological testing in cattle, and veterinarians in most regions of Japan therefore have no choice but to perform clinical testing at the single-clinic level. As clinics that primarily provide veterinary medical care have limited manpower, time, equipment, and money to devote to clinical testing, a more economical and simpler method is required. The simplified-ADD is widely used in clinical practice in Japan due to its affordability and simplicity. However, few antimicrobial agents have breakpoints suitable for ADD testing of bovine mastitis isolates, which has led to confusion in the interpretation of results. The diameter of the inhibition zone (DIZ) of the simplified-ADD is reportedly correlated with DIZ values of the standardized-ADD, although the DIZ values tend to be slightly smaller with the simplified-ADD¹⁷⁾. To date, simplified-ADD epidemiological breakpoints have been established for clinical mastitis-causing organisms with regard to penicillin (PCG), kanamycin (KM), cefazolin (CEZ), oxytetracycline (OTC), pirlimycin (PLM), enrofloxacin (ERFX), and marbofloxacin (MBFX)¹⁵⁾. However, breakpoints are not available for all antimicrobials used in clinical practice to treat mastitis.

In this study, we revalidated previously determined breakpoints¹⁵⁾ by incorporating additional strains (except for PLM) and epidemiological breakpoints for four antimicrobials (ampicillin [ABPC], gentamicin [GM], cefuroxime

[CXM], and erythromycin [EM]) using the simplified-ADD. We also generated an antibiogram of the pathogens that cause bovine clinical mastitis in Japan and discuss their characteristics.

Materials and Methods

This study was conducted in accordance with the regulations of the Azabu University Animal Experiment Committee (Sagamihara, Japan).

Milk sample collection and identification of mastitis-causing bacteria

Between 2015 and 2023, a total of 2,636 milk samples were collected from dairy cows at the onset of clinical mastitis in 17 prefectures of Japan (Hokkaido, Aomori, Iwate, Miyagi, Tochigi, Gunma, Saitama, Hyogo, Okayama, Hiroshima, Kagawa, Ehime, Kochi, Fukuoka, Oita, Kumamoto, Kagoshima). The study examined 1,349 samples collected by Kawai *et al.*¹⁵⁾ for which the causative organism had not yet been identified and 1,287 samples newly collected in this study. Milk samples were frozen at -20°C after collection and sent to the university within 1 week. Bacterial culture tests were conducted immediately after sample arrival. Specifically, bacteria were isolated and identified, and the identified strains were frozen and stored at -80°C until minimum inhibitory concentration (MIC) determination and disc diffusion testing. Milk samples (10 μL each) were individually added to 5% sheep blood agar and incubated aerobically at 37°C for 24 h according to National Mastitis Council methods¹⁰⁾. The species of the isolates was determined using 16S rRNA gene sequencing. Bacterial genomic DNA was extracted using InstaGene Matrix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The following primers were used for PCR analysis: 27F sense primer (5'-AGAGAGTTTGATCCTGGCTCAG-3') and 1492R antisense primer (5'-GGTTACCTTGTTACGACTT-3')³³⁾. PCR conditions were as follows: denaturation at 95°C

for 30 s, annealing at 58°C for 30 s, extension at 72°C for 2 min, for a total of 32 cycles. The amplified DNA was subjected to agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen N.V., Venlo, The Netherlands). The purified product was used as a template for sequencing reactions using Big Dye Terminator v3.1 (Applied Biosystems, Waltham, MA, USA) and analyzed using a PRISM 3730xl Genetic Analyzer (Applied Biosystems). Identification was performed using the Basic Local Alignment Search Tool (BLAST)¹⁾ by assessing the homology between known sequences and the experimentally determined DNA sequence. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for the identification of some strains. Colonies of target strains were pretreated by cell smearing or 70% formic acid extraction, and a Microflex-LT (Bruker Daltonics, Billerica, MA, USA) instrument was used for mass spectrometry. Byotyper RTC software was used to identify bacterial species based on the resulting mass spectra.

Antimicrobial susceptibility testing

Additional strains (Table 1) were tested for antimicrobial susceptibility to PCG (gram-positive bacteria only), KM, OTC, CEZ, ERFX, and MBFX, and the susceptibility of all strains was evaluated in relation to ABPC, GM, EM, and CXM.

MIC determination (broth microdilution method): For gram-positive bacteria, MIC values were determined for PCG, KM, ERFX, MBFX, ABPC, GM, EM (Fujifilm Wako Pure Chemicals Corp., Osaka, Japan), CEZ (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), OTC (Nacalai Tesque, Inc., Kyoto, Japan), and CXM (Merck

Table 1. Breakdown of strains from Kawai *et al.* and additional strains

Pathogens	Number of strains from Kawai <i>et al.</i> *	Additional number of strains	Total
SA	84	22	106
CNS	79	29	108
<i>Streptococcus</i> spp.(except for <i>S. uberis</i>)	61	108	169
<i>Streptococcus uberis</i>	83	31	114
<i>Trueperella pyogenes</i>	31	25	56
<i>Escherichia coli</i>	79	26	105
<i>Klebsiella</i> spp.	21	45	66

SA: *Staphylococcus aureus*, CNS: Coagulase negative staphylococci

*: The strains from Kawai *et al.* that could be retested were included in this study.

KGaA, Darmstadt, Germany). The same antimicrobials were used to determine MIC values for gram-negative bacteria, except for PCG. MICs were determined using the broth microdilution method according to the CLSI⁵⁾. *Staphylococcus aureus* (SA) ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality control strains.

Disk diffusion method: Disk diffusion testing was performed using the simplified-ADD²²⁾ method, which is widely used in clinical practice in Japan. In the simplified-ADD method, instead of preparing each inoculum solution as in the CLSI method protocol, a specific number of colonies of each bacterial species were collected with sterile swabs and applied directly and uniformly to the test plate. In practice, 2~3 colonies were used for *Staphylococcus* spp., 20~30 for *Streptococcus* spp., 50~100 for *Trueperella pyogenes*, and 1/8~1/4 of the volume of one colony for gram-negative bacteria. Only two types of agar media were used for testing: Müller-Hinton agar for *Staphylococcus* spp. and gram-negative bacteria, and 5% sheep blood agar for *Streptococcus* spp. and *T. pyogenes*. Antibiotic susceptibility test disks were placed on the surface of the agar within 15 min of inoculation of the plates according to the CLSI method. All bacteria were incubated under the same conditions (aerobic conditions, 24 h at 37°C). After incubation, the DIZ for each antimicrobial was measured using a caliper. In the simplified-ADD method, 10 different antibiotic susceptibility disks were tested against gram-positive bacteria: PCG, OTC, KM, CEZ, ABPC, GM, CXM, EM (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan), ERFX (Eiken Chemical Co., Ltd., Tokyo, Japan), and MBFX (Vetoquinol, Lure, France). The same antimicrobials were used for gram-negative bacteria, except for PCG. However, quality control standards for the simplified-ADD have not been established. In this study, for PCG, OTC, KM, CEZ, ERFX, and MBFX, the results of the simplified-ADD for *E. coli* ATCC25922 and SA ATCC25923 were confirmed to be within the respective reference quality control range¹⁷⁾. For ABPC, GM, CXM, and

Table 2. The alternative ranges of quality control in the simplified-ADD.

	The alternative ranges of quality control (mm)			
	ABPC	GM	CXM	EM
<i>S. aureus</i> ATCC25923	26.8 ± 3.8	21.8 ± 2.6	26.3 ± 3.4	22.6 ± 5.3
<i>E. coli</i> ATCC25922	14.6 ± 2.6	22.7 ± 1.8	20.2 ± 2.4	-

ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime, EM: erythromycin

-: not tested

Values were shown as the mean zone diameter ± 2SD.

EM, preliminary tests were conducted according to previously published methods to confirm reproducibility¹⁷⁾, and the mean DIZ ± 2 SD was set as the quality control range for this study (Table 2).

Determination of criteria for the simplified-ADD method: To determine cutoff values, MIC and DIZ values were first plotted on a scatter plot; if the MIC susceptibility distribution was unimodal, for convenience, the lower limit of the DIZ plot (excluding outliers) was used as the cutoff value. When the distribution of MICs was bimodal, a receiver-operating characteristic curve was generated using EZR¹⁴⁾ (ver. 1.68, Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), in order to determine the DIZ with maximum sensitivity and specificity³⁴⁾. The calculated value of the DIZ was rounded to the first decimal place and considered a new cutoff value for the simplified-ADD. Breakpoints for PCG, KM, CEZ, OTC, MBFX, and ERFX in the simplified-ADD method previously formulated by Kawai et al.¹⁵⁾ were used as a basis for revalidating the breakpoints with additional strains. In addition, new breakpoints were determined for ABPC, GM, CXM, and EM.

Antibiogram generation

SA, coagulase-negative *Staphylococcus* (CNS), *Streptococcus uberis*, *Streptococcus* spp. (excluding *S. uberis*), *Streptococcus* spp., *E. coli*, and *Klebsiella* spp. were identified as susceptible or resistant based on the newly established criteria of the simplified-ADD. Antibiograms were generated according to the guidelines for preparing antibiograms of the Consortium for Education on Infectious Diseases¹¹⁾. The antibiograms were calculated as the susceptibility rate (%) in

Table 3. Minimum inhibitory concentration (MIC) distribution among *Staphylococcus* spp. isolated from cases of bovine mastitis *Staphylococcus aureus* (n=106)

Staphylococcus aureus (n=108)															
Antimicrobial		MIC(μg/mL)												MIC ₅₀	MIC ₉₀
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256		
PCG	102	1			2	1								≤0.125	≤0.125
KM			7	34	48	14	2		1					2	4
CEZ		13	17	75	1									1	1
OTC	5	39	31	27	3						1			0.5	1
MBFX	10	79	17											0.25	0.5
ERFX	91	15												≤0.125	0.25
ABPC	86	13	2	1			2		1					≤0.125	0.25
GM	1	7	67	26	5									0.5	1
CXM	1		4	23	72	5	1							2	2
EM	1	42	56	2	1	1	1		1				1	0.5	0.5

Coagulase negative Staphylococci(n=108)															
Antimicrobial		MIC(μg/mL)												MIC ₅₀	MIC ₉₀
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256		
PCG	83	16	5	1	1	1		1						≤0.125	0.25
KM	27	39	21	11	5	5								0.25	1
CEZ	1	5	14	21	33	28	4		1				1	2	4
OTC	8	30	49	12				1			6	1		0.5	1
MBFX	11	42	52	3										0.5	0.5
ERFX	66	34	7	1										≤0.125	0.25
ABPC	72	14	13	5	1		2		1					≤0.125	0.5
GM	91	10	7											≤0.125	0.25
CXM		9	27	43	24	4							1	1	2
EM	5	35	39	2	1	3	8	1	7	4	1		2	0.5	32

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime, EM: Erythromycin. The vertical line indicates the MIC breakpoint used to calculate the simplified ADD breakpoint.

Hokkaido Prefecture and eastern and western Japan for each bacterial species. Eastern Japan included Aomori, Iwate, Miyagi, Tochigi, Gunma, and Saitama Prefectures, whereas western Japan included Hyogo, Okayama, Hiroshima, Kagawa, Ehime, Kochi, Fukuoka, Oita, Kumamoto, and Kagoshima Prefectures.

Statistical analyses

Statistical analyses were performed using EZR¹⁴⁾ (ver. 1.68, Saitama Medical Center, Jichi Medical University, Saitama City, Saitama Prefecture), a graphical user interface for R (The R Foundation for Statistical Computing). In the antibiogram, Bonferroni multiple comparisons were performed to determine regional differences in susceptibility rates, and $P < 0.05$ was considered statistically significant.

Results

Bacterial strains

The following bovine clinical mastitis-causing strains were added to the report by Kawai et al.¹⁵⁾: 22 SA, 29 CNS (4 *S. arlettae*, 2 *S. auricularis*, 13 *S. chromogenes*, 1 *S. cohnii*, 2 *S. devriesei*, 1 *S.*

haemolyticus, 2 *S. kloosii*, 1 *S. piscifermentans*, 2 *S. sciuri*, and 1 *S. felis*), 120 *Streptococcus* spp. (31 *S. uberis*, 57 *S. dysgalactiae*, 16 *S. lutetiensis*, 10 *S. equinus*, 5 *S. agalactiae*, and 1 *S. infantarius*), 26 *E. coli*, 45 *Klebsiella* spp. (28 *K. pneumoniae*, 1 *K. variicola*), and 25 *T. pyogenes*. A total of 19 strains of *Streptococcus* spp. and 16 strains of *Klebsiella* spp. could not be identified by 16S rRNA gene sequencing and were thus included among *Streptococcus* spp. and *Klebsiella* spp., respectively. In addition to these strains, the strains from Kawai et al.¹⁵⁾ that could be retested were included in this study, resulting in the number of strains shown in Table 2.

MICs of antimicrobials used to treat mastitis pathogens

MIC determinations for SA strains revealed one strain (0.9%) with an MIC > 256 μ g/ml for EM but no high MICs for ABPC, CXM, or GM. Among CNS, one strain (0.9%) exhibited an MIC > 256 μ g/ml for CXM, and high MICs were observed for EM (4 strains with an MIC of 64 μ g/ml [3.7%], 1 strain with an MIC of 128 μ g/ml [0.9%], 2 strains with an MIC > 256 μ g/ml [1.9%]) (Table 3). For *Streptococcus* spp., 99.6% of strains exhibited an MIC ≥ 4 μ g/ml for GM, and all strains exhibited

Table 4. Minimum inhibitory concentration (MIC) distribution among *Streptococcus* spp. and *Trueperella pyogenes* isolated from cases of bovine mastitis

<i>Streptococcus uberis</i> (n=114)															
ntimicrobia															
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC ₅₀	MIC ₉₀
PCG	59	53	2											≤0.125	0.25
KM	1					5	3	22	37	39	6		1	32	64
CEZ	4	8	34	66	2									1	1
OTC	6	25	34	10			6	6	12	4	11			0.5	64
MBFX	1	7	23	71	12									1	1
ERFX	5	21	71	15	2									0.5	1
ABPC	77	29	8											≤0.125	0.25
GM							10	36	62	6				32	32
CXM	13	23	66	12										0.5	0.5
EM	92				1	1							20	≤0.125	≤0.125
<i>Streptococcus</i> spp. (except for <i>S. uberis</i>) (n=169)															
ntimicrobia															
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC ₅₀	MIC ₉₀
PCG	168		1											≤0.125	≤0.125
KM						1	1	2	51	58	29	10	17	64	>256
CEZ	59	13	65	26	5			1						0.5	1
OTC		2	34	28	14	58	20			7	6			4	8
MBFX			6	90	52	19	2							1	4
ERFX		2	57	62	45	2	1							1	2
ABPC	169													≤0.125	≤0.125
GM			1			21	75	49	22	1				8	32
CXM	168	1												≤0.125	≤0.125
EM	167		1					1						≤0.125	≤0.125
<i>Streptococcus</i> spp. (n=283)															
ntimicrobia															
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC ₅₀	MIC ₉₀
PCG	227	53	3											≤0.125	0.25
KM	1					6	4	24	88	97	35	10	18	64	128
CEZ	63	21	99	92	7			1						0.5	1
OTC	6	27	68	38	14	58	26	6	12	11	17			2	32
MBFX	1	7	29	161	64	19	2							1	2
ERFX	5	23	128	77	47	2	1							0.5	2
ABPC	246	29	8											≤0.125	0.25
GM			1			21	85	85	84	7				16	32
CXM	181	24	66	12										≤0.125	0.5
EM	259		1		1	1		1					20	≤0.125	≤0.125
<i>Trueperella pyogenes</i> (n=56)															
ntimicrobia															
agents	≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	MIC ₅₀	MIC ₉₀
PCG	56													≤0.125	≤0.125
KM		2	2	12	26	12	1			1				2	4
CEZ	49	5	2											≤0.125	0.25
OTC	1	8	5	12	7			3	15	5				2	32
MBFX			1	40	12	2	1							1	2
ERFX			10	32	8	5	1							1	4
ABPC	56													≤0.125	≤0.125
GM	1	2	28	24						1				0.5	1
CXM	56													≤0.125	≤0.125
EM	54	1		1										≤0.125	≤0.125

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime, EM: Erythromycin

The vertical line indicates the MIC breakpoint used to calculate the simplified ADD breakpoint.

low MICs for ABPC and CXM. For *S. uberis*, 17.5% of the strains showed an MIC > 256 µg/ml for EM. By contrast, all strains of *Streptococcus* spp., except *S. uberis*, exhibited MIC values ≤ 16 µg/ml for EM. One strain of *T. pyogenes* exhibited an MIC of 64 µg/ml for GM, but all other strains

exhibited low MICs for other antimicrobial agents (Table 4). Three *E. coli* strains (2.9%) exhibited an MIC > 256 µg/ml for CXM. Three (2.9%) *E. coli* strains exhibited an MIC ≥ 64 µg/ml for GM. Against ABPC, 14.3% of *E. coli* isolates exhibited high MIC values (MIC of 128 µg/ml for 1 isolate,

Table 5. Minimum inhibitory concentration (MIC) distribution among gram-negative bacteria isolated from cases of bovine mastitis *Escherichia coli* (n=105)

MIC(μ g/mL)																MIC ₅₀	MIC ₉₀
agents	≤ 0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256				
KM					39	50	7	3					6	4	8		
CEZ				22	65	13	1				1		3	2	4		
OTC				5	20	26	16	1	6	1		13	17	8	>256		
MBFX	97	1	3	1			2	1						≤ 0.125	≤ 0.125		
ERFX	95	4	3					1	1		1			≤ 0.125	≤ 0.125		
ABPC				8	37	38	7				1	1	13	4	>256		
GM		1	12	72	14	1	2			1	1	1		1	2		
CXM				1	21	70	9	1					3	4	8		
EM [*]							1	2	7	42	42	10	1	128	256		

Klebsiella spp. (n=66)

MIC(μ g/mL)															MIC ₅₀	MIC ₉₀
agents	≤ 0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256			
KM			3	35	20	2				1		1	4	1	4	
OTC				11	27	17	2	1			2	3	2	2	128	
CEZ				13	15	14	6	1				1	16	4	>256	
MBFX	59	1	3	2			1							≤ 0.125	0.25	
ERFX	58	1	3	3				1						≤ 0.125	0.5	
ABPC							3	6	20	15	3	1	18	64	>256	
GM	1	28	30	5	1					1				0.5	1	
CXM	1			9	23	11	4	3					15	>256	>256	
EM [*]							1		1	28	29	6	1	128	256	

KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime, EM: Erythromycin

The vertical line indicates the MIC breakpoint used to calculate the simplified ADD breakpoint.

*:MIC breakpoints were not calculated due to natural resistance

Table 6. Breakpoints for the simplified–agar disc diffusion method against bovine mastitis–derived strains (inhibition zone diameter: mm)

Pathogens	PCG		KM		CEZ		OTC		MBFX		ERFX	
	S	R	S	R	S	R	S	R	S	R	S	R
<i>Staphylococcus aureus</i>	≥ 24	≤ 23	≥ 13	≤ 12	≥ 25	≤ 24	≥ 12	≤ 11	≥ 15	≤ 14	≥ 15	≤ 14
CNS	≥ 12	≤ 11	≥ 16	≤ 15	≥ 17	≤ 16	≥ 17	≤ 16	≥ 17	≤ 16	≥ 19	≤ 18
<i>Streptococcus</i> spp.	≥ 14	≤ 13	≥ 26	≤ 25	≥ 19	≤ 18	≥ 13	≤ 12	≥ 15	≤ 14	≥ 13	≤ 12
<i>Streptococcus</i> spp. (except for <i>S. uberis</i>)	≥ 14	≤ 13	≥ 26	≤ 25	≥ 19	≤ 18	≥ 13	≤ 12	≥ 16	≤ 15	≥ 13	≤ 12
<i>Streptococcus uberis</i>	≥ 18	≤ 17	≥ 22	≤ 21	≥ 19	≤ 18	≥ 16	≤ 15	≥ 14	≤ 13	≥ 14	≤ 13
<i>Trueperella pyogenes</i> *	≥ 20	≤ 19	≥ 15	≤ 14	≥ 18	≤ 17	≥ 20	≤ 19	≥ 15	≤ 14	≥ 15	≤ 14
<i>Escherichia coli</i>	-	-	≥ 15	≤ 14	≥ 13	≤ 12	≥ 16	≤ 15	≥ 16	≤ 15	≥ 16	≤ 15
<i>Klebsiella</i> spp.*	-	-	≥ 15	≤ 14	≥ 15	≤ 14	≥ 15	≤ 14	≥ 16	≤ 15	≥ 19	≤ 18

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, ERFX: enrofloxacin, MBFX: marbofloxacin

S: susceptibility, R: resistance

CNS: Coagulase negative staphylococci

*: reference criteria (*Klebsiella* spp.: n=66, *Trueperella pyogenes* : n=56)

MIC of 256 μ g/ml for 1 isolate, and MIC > 256 μ g/ml for 13 isolates). The distribution of MICs for *Klebsiella* spp. revealed low values for GM (except for one strain) and high MICs for EM and ABPC for many strains. The MIC for CXM was > 256 μ g/ml in 22.1% of the isolates (Table 5).

Breakpoint determination in the simplified-ADD method

DIZ breakpoints were established by evaluating the additional bacterial strains and further classification of bacterial species, resulting in the following differences compared with a previous report¹⁵⁾. The DIZ breakpoints for SA, CNS, *Streptococcus* spp. (except for *S. uberis*), *S.*

uberis, and *Klebsiella* spp. were newly established in this study. The DIZ breakpoints for PCG, CEZ, OTC, MBFX, and ERFX against SA differed by 3–13 mm from those reported previously for *Staphylococcus* spp.¹⁵⁾. The breakpoints for KM, OTC, and MBFX against CNS differed by 2–3 mm from those reported previously for *Staphylococcus* spp.¹⁵⁾. The breakpoints for PCG, CEZ, OTC, and MBFX against *Streptococcus* spp. differed by 2–3 mm. The breakpoints for KM and CEZ against *S. uberis* were 2 mm and 4 mm different, respectively, from those reported previously for *Streptococcus* spp.¹⁵⁾. The breakpoints for PCG and KM against *T. pyogenes* were 3 mm and 5 mm different, respectively. The breakpoints for

Table 7. Breakpoints for the simplified–agar disc diffusion method against bovine mastitis–derived strains (inhibition zone diameter: mm)

Pathogens	ABPC		GM		CXM		EM	
	S	R	S	R	S	R	S	R
<i>Staphylococcus aureus</i>	≥ 25	≤ 24	≥ 12	≤ 11	≥ 16	≤ 15	≥ 15	≤ 14
CNS	≥ 19	≤ 18	≥ 13	≤ 12	≥ 17	≤ 16	≥ 15	≤ 14
<i>Streptococcus</i> spp.	≥ 17	≤ 16	≥ 16	≤ 15	≥ 15	≤ 14	≥ 14	≤ 13
<i>Streptococcus</i> spp.(except for <i>S. uberis</i>)	≥ 17	≤ 16	≥ 16	≤ 15	≥ 17	≤ 16	≥ 12	≤ 11
<i>Streptococcus uberis</i>	≥ 17	≤ 16	≥ 18	≤ 17	≥ 15	≤ 14	≥ 14	≤ 13
<i>Trueperella pyogenes</i> *	≥ 17	≤ 16	≥ 15	≤ 14	≥ 12	≤ 11	≥ 18	≤ 17
<i>Escherichia coli</i>	≥ 11	≤ 10	≥ 12	≤ 11	≥ 14	≤ 13	-	-
<i>Klebsiella</i> spp.*	≥ 11	≤ 10	≥ 15	≤ 14	≥ 15	≤ 14	-	-

ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime, EM: erythromycin

CNS: Coagulase negative staphylococci

Table 8. Antibigrams of bovine mastitis–derived *Staphylococcus* spp. in relation to criteria of the simplified–agar disc diffusion method
Staphylococcus aureus

		Susceptibility rates (%)									
	Number of strains	PCG	KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	EM
Whole country	106	97.2	96.2	99.1	98.1	100.0	100.0	96.2	100.0	100.0	94.3
Hokkaido	66	98.5	97.0	98.5	97.0	100.0	100.0	97.0	100.0	100.0	97.0
Eastern Japan*	19	89.5	94.7	100.0	100.0	100.0	100.0	89.5	100.0	100.0	89.5
Western Japan*	21	100.0	95.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	90.5

Coagulase negative Staphylococci

		Susceptibility rates (%)									
	Number of strains	PCG	KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	EM
Whole country	108	97.2	97.2	96.3	90.7	99.1	100.0	75.9	100.0	96.3	76.9
Hokkaido	70	95.7	97.1	94.3	88.6	98.6	100.0	80.0	100.0	95.7	81.4
Eastern Japan*	20	100.0	100.0	100.0	100.0	100.0	100.0	60.0	100.0	100.0	55.0
Western Japan*	17	100.0	94.1	100.0	88.2	100.0	100.0	76.5	100.0	94.1	82.4
No regional data*	1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin,

GM: gentamicin, CXM: cefuroxime, EM: erythromycin

KM, CEZ, and OTC against *E. coli* were 2–5 mm different, respectively (Table 6). In addition, new breakpoints were determined for ABPC, GM, CXM, and EM (Table 7). The breakpoints for *T. pyogenes* and *Klebsiella* spp. were considered reference values because the number of samples was 56 and 66, respectively.

Antibiogram

Among SA strains, ≥ 90% were susceptible to all tested antimicrobial agents in all regions. CNS strains were less susceptible to ABPC and EM. No significant regional differences in susceptibility rates to SA and CNS were observed (Table 8). *Streptococcus* spp. were less susceptible to KM and GM. The susceptibility of *Streptococcus* spp. excluding *S. uberis* to MBFX was 63.9% nationwide. Regionally, the susceptibility was 43.8% in eastern Japan, 78.3% in Hokkaido, and

83.3% in western Japan. The value in eastern Japan was significantly lower than the values in Hokkaido and western Japan ($P < 0.01$) (Table 9). The susceptibility of *S. uberis* and *E. coli* (except Hokkaido) to OTC ranged from 50 to 70% (Tables 9 and 10), and the susceptibility of OTC to *E. coli* in eastern Japan (50.9%) was significantly lower than that in Hokkaido (83.3%; $P < 0.01$; Table 10). The susceptibility rates of *Klebsiella* spp. to CEZ and CXM were slightly lower, with ABPC showing a susceptibility rate of only approximately 10% (Table 10).

Discussion

Determinations of breakpoints for antimicrobial susceptibility testing should consider MIC data for mastitis-causing

Table 9. Antibigrams of *Streptococcus* spp. isolated from cases of bovine mastitis in relation to criteria of the simplified–agar disc diffusion method

<i>Streptococcus uberis</i>											
	Number of strains	Susceptibility rates (%)									
		PCG	KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	EM
Whole country	114	98.2	2.6	99.1	64.9	97.4	98.2	99.1	1.8	99.1	81.6
Hokkaido	63	96.8	1.6	100	63.5	98.4	98.4	98.4	1.6	98.4	79.4
Eastern Japan	41	100	0	97.6	65.9	97.6	100	100	2.4	100	87.8
Western Japan*	10	100	20	100	70	90	90	100	0	100	70

<i>Streptococcus</i> spp. (except for <i>S. uberis</i>)											
	Number of strains	Susceptibility rates (%)									
		PCG	KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	EM
Whole country	169	98.8	0.6	97.6	92.2	63.9	98.2	99.4	1.2	98.2	98.8
Hokkaido	69	100.0	0.0	98.6	87.0	78.3 ^a	97.1	98.6	1.4	97.1	100.0
Eastern Japan	73	98.6	0.0	97.3	94.5	43.8 ^b	98.6	100.0	1.4	98.6	100.0
Western Japan*	24	95.8	4.2	95.8	100.0	83.3 ^a	100.0	100.0	0.0	100.0	91.7
No regional data*	3	100.0	0.0	100.0	100.0	33.3	100.0	100.0	0.0	66.7	100.0

<i>Streptococcus</i> spp.											
	Number of strains	Susceptibility rates (%)									
		PCG	KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	EM
Whole country	283	98.9	0.4	98.2	81.4	85.7	98.9	99.3	2.5	99.6	91.4
Hokkaido	132	98.5	0.0	99.2	76.5	90.2	98.5	98.5	2.3	99.2	89.4
Eastern Japan	114	100.0	0.0	97.4	84.2	79.8	99.1	100.0	3.5	100.0	95.6
Western Japan	34	97.1	2.9	97.1	91.2	88.2	100.0	100.0	0.0	100.0	85.3
No regional data*	3	100.0	0.0	100.0	100.0	33.3	100.0	100.0	0.0	100.0	100.0

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime EM: erythromycin

Table 10. Antibigrams of gram-negative bacteria isolated from cases of bovine mastitis in relation to the criteria of the simplified–agar disc diffusion method

<i>Escherichia coli</i>										
	Number of strains	Susceptibility rates (%)								
		KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	
Whole country	105	94.3	96.2	63.8	97.1	97.1	85.7	97.1	96.2	
Hokkaido	42	92.9	97.6	83.3 ^a	100	100	88.1	97.6	95.2	
Eastern Japan	53	96.2	94.3	50.9 ^b	94.3	94.3	86.8	96.2	96.2	
Western Japan*	10	90.0	100.0	50.0	100.0	100.0	70.0	100.0	100.0	

<i>Klebsiella</i> spp.										
	Number of strains	Susceptibility rates (%)								
		KM	CEZ	OTC	MBFX	ERFX	ABPC	GM	CXM	
Whole country	66	92.4	77.3	89.4	97.0	98.5	10.6	98.5	75.8	
Hokkaido*	20	90.0	70.0	90.0	95.0	100.0	5.0	100.0	70.0	
Eastern Japan*	16	100.0	93.8	93.8	100.0	100.0	6.3	100.0	87.5	
Western Japan	30	90.0	73.3	86.7	96.7	96.7	16.7	96.7	73.3	

PCG: penicillin G, KM: kanamycin, CEZ: cefazolin, OTC: oxytetracycline, MBFX: marbofloxacin, ERFX: enrofloxacin, ABPC: ampicillin, GM: gentamicin, CXM: cefuroxime

organisms, pharmacokinetic/pharmacodynamic data for lactating dairy cows, and clinical and bacteriological cure rate results, although these data in lactating cows are insufficient⁷⁾. However, the choice of antimicrobial agent for the treatment of mastitis should be evidence based. The epidemiological breakpoints generated in this study can serve as a basis for evaluating

antimicrobial susceptibility test results in the treatment of bovine mastitis in Japan. In this study, new breakpoints were established for SA, CNS, *S. uberis*, *Streptococcus* spp. (except for *S. uberis*), and *Klebsiella* spp. The breakpoints for DIZ differed from those previously reported¹⁵⁾ due to the addition of strains and subdivision of the species, which was presumably due to differences

in MIC and DIZ distribution among the strains. With the addition of ABPC, GM, CXM, and EM, breakpoints that can be utilized in clinical practice were formulated. In this study, no resistant strains were found among the following causative species and antimicrobial combinations, suggesting the need for future revalidation including resistant strains: SA: CEZ, CXM, GM, ERFX, MBFX; CNS: KM, GM, ERFX, MBFX; *S. uberis*: PCG, ABPC, CEZ, CXM, ERFX, MBFX; *Streptococcus* spp. (except for *S. uberis*): PCG, ABPC, CXM; *T. pyogenes*: PCG, ABPC, CEZ, CXM, EM.

This is the first report of an antibiogram of causative organisms of bovine clinical mastitis in Japan based on breakpoints of the simplified-ADD method. However, the disk diffusion method is a qualitative drug susceptibility test, and its lower accuracy compared to quantitative methods is considered to be a limitation of this study³⁾. The antibiogram revealed various region- and species-specific characteristics of antimicrobial susceptibility. In Japan, SA isolates exhibited high susceptibility to all antimicrobial agents studied. By contrast, slightly lower susceptibility to ABPC and EM was observed among CNS. Twenty-six CNS strains exhibited resistance to ABPC. *Staphylococcus arlettae* accounted for 12 of these isolates, and all 12 *S. arlettae* isolates examined in this study were ABPC resistant. EM resistance was observed among 25 isolates, of which 11 were *S. arlettae* and 5 *S. equorum*. The low susceptibility to ABPC and EM among CNS may reflect certain characteristics of these species. *Streptococcus* spp. are known to exhibit low levels of spontaneous resistance to aminoglycoside antimicrobial agents²⁰⁾, and rates of susceptibility to these agents are low in Japan. OTC-resistant strains were widely distributed among Japanese isolates of *E. coli* (excluding Hokkaido) and *S. uberis*. A slightly reduced rate of susceptibility to MBFX was observed among *Streptococcus* spp., excluding *S. uberis*. In eastern Japan, the susceptibility rate was significantly lower than the rates in Hokkaido and western Japan. In western Japan, there were 24 *Streptococcus* spp. (excluding *S. uberis*), so the susceptibility rate is considered a reference value¹¹⁾. However, the possibility of

nationwide MBFX resistance to *Streptococcus* spp. (excluding *S. uberis*) is suggested. Although CEZ is widely used in Japan as a first-line antimicrobial agent in the treatment of bovine mastitis^{16,30)}, high susceptibility rates were exhibited by all bacteria except *Klebsiella* spp. By contrast, low susceptibility rates were observed among *Klebsiella* spp. with regard to CEZ, CXM, and ABPC. *Klebsiella pneumoniae* reportedly serves as a reservoir of AMR genes, and this bacterium is thought to impart AMR genes to other strains and species through plasmids that confer multidrug resistance^{4,26)}. The “One Health” perspective stresses the importance of close monitoring of AMR trends in veterinary medicine.

In selecting an antimicrobial therapy, factors such as the type and severity of infection, causative organism(s), and medical history and past use of antimicrobial agents in the affected animal must be considered; it is not possible to select an optimal empirical antimicrobial therapy for an individual animal based on a local antibiogram alone¹³⁾. The development of resistance cannot be detected based solely on an increased MIC or decreased DIZ when they remain below the susceptibility breakpoint¹³⁾. Furthermore, antibiograms only tabulate the percentage of isolates that are susceptible to a given microorganism–antimicrobial agent combination and are not suitable for determining cross-reactivity with other antibiotics¹³⁾. These limitations should be taken into account when utilizing antibiograms.

Yeasts and *Prototheca* can also cause bovine mastitis, and mastitis caused either by these organisms or certain gram-negative bacteria does not require treatment with antimicrobials²⁵⁾. Although some recent reports have suggested that lactoferrin⁸⁾, intramammary irrigation²⁹⁾, and glycyrrhizin¹⁸⁾ should be considered as adjuvant therapies for mastitis, any consideration of antimicrobial administration should be based on identification of the causative microorganism and results of subsequent antimicrobial susceptibility testing. The epidemiological breakpoints established in this study for Japan may help ensure that the most appropriate treatment is

selected based on the results of antimicrobial susceptibility testing. In addition, the antibiogram generated in this study revealed region- and species-specific drug susceptibility characteristics. Thus, an antibiogram should be considered when choosing to treat acute mastitis with systemic symptoms using antibacterial drugs before drug susceptibility test results are available, avoiding antibacterial drugs associated with low microbial susceptibility.

Only a limited number of studies have investigated the distribution of antimicrobial susceptibility among bovine mastitis-causing microorganisms over a wide area of Japan. Leveraging the results of this study as baseline data, along with monitoring over time, expanded specimen collection, and evaluations of antimicrobial use in clinical settings, future studies should periodically generate new antibiograms and conduct systematic surveillance of AMR.

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