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Comparison of antimicrobial susceptibilities of bacterial isolates between cured and uncured cases of bovine mastitis

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ABSTRACT. To evaluate the effect of antimicrobial susceptibility on outcomes, we compared the minimum inhibitory concentrations (MICs) for *Staphylococcus*, *Streptococcus*, and the family *Enterobacteriaceae* from cured and uncured mastitis cases; milk shipment for uncured cases could not be resumed within 3 weeks after initial clinical examination. A higher MIC_{50} of ampicillin and a higher MIC_{90} of cefazolin for *Enterobacteriaceae* isolates were observed for cured rather than uncured cases with differences in ≥2 tubes. Endotoxins are generally released from *Enterobacteriaceae* upon antimicrobial treatment; their amounts are presumed to be greater in mastitis cases resulting from β-lactam antibiotic-susceptible rather than -resistant microbes. For staphylococcal and streptococcal isolates, the MIC_{50} and MIC_{90} of β-lactam antibiotics were similar for cured and uncured cases.

KEY WORDS: antimicrobial resistance, mastitis

Mastitis is considered one of the most highly incident diseases, causing high treatment costs and reduced milk shipment in the dairy industry [7]. Antimicrobial agents have been primarily administered to treat mastitis [15]. However, antimicrobial resistance is an increasing global health threat, potentially affecting the effective treatment of bacterial infections [19]. Therefore, clinical veterinarians should prudently use antimicrobials and should select antimicrobials expected to display a response on the basis of bacteriological analysis and the clinical conditions of diseased animals. However, clinical treatments for mastitis are often ineffective and cure rates are poor [6, 17]. It is important to identify mastitis cases in which antimicrobial treatment is ineffective before antimicrobial administration to reduce antimicrobial agent use. To evaluate the effect of antimicrobial resistance on resumption of milk shipment, we determined the minimum inhibitory concentrations (MICs) for causative bacterial isolates from mastitis cases and compared the MICs of isolates that were obtained from cured cases with those obtained from uncured cases.

All dairy cattle diagnosed with bacterial mastitis between November 2013 and December 2014 by clinical veterinarians at the Central Veterinary Clinical Center of Chiba Prefectural Federated Agricultural Mutual Aid Association (Chiba NOSAI) were enrolled in this study. However, three cases of combined infections, where two or more bacterial species were isolated from a quarter of milk, were excluded. Each gargety quarter was defined as a case. Thus, 209 cases of 172 heads were involved in the study.

Individual identification numbers of each animal and antimicrobial treatment (type of antimicrobial and the route and duration of administration) were confirmed through medical records. We confirmed if each cattle farmer could resume milk shipment for human consumption from a recovered gargety quarter within 3 weeks after initial clinical examination. That is, was confirmed from each farmer determined whether the milk can could be shipped [17]. In general, after antimicrobial treatment, farmers ask the dairy manufacturer to test somatic cell count, residual antimicrobials, and the quality of milk samples from treated quarters. If normal values are confirmed, the raw milk is shipped. It was confirmed that the quality of milk was within the standard of the ministerial ordinance of Ministry of Health, Labor and Welfare (MHLW) (No. 52 of 1951) based on Food Sanitation Act (No. 233 of 1947). Maximum residue levels for each antimicrobial in milk are established in a notification of MHLW (No. 499 of 2005).

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No blood contamination is also confirmed. Ninety-three cases (44.5%), wherein milk shipment was resumed within 3 weeks were defined as cured cases. Milk shipment was not resumed for 87 cases (41.6%), which were thus defined as uncured cases. Outcomes of resuming milk shipment were not confirmed for the remaining 29 cases (13.9%). Seventy-four cases (35.4%) were treated exclusively via intramammary infusion; 95 cases (45.5%) were treated via intramuscular injection and an intramammary infusion; and 21 cases (10.0%) were treated via intramuscular injection. Twelve cases (5.7%) were not treated with antimicrobials. We could not confirm the history of antimicrobial treatment for seven cases (3.3%). Intramammary infusions were administered to 169 cases (80.9%; 53 *Streptococcus* cases, 54 *Staphylococcus* cases, 38 *Enterobacteriaceae* cases, and 24 other cases), of which 154 (91.1%; 51 *Streptococcus* cases, 52 *Staphylococcus* cases, 30 *Enterobacteriaceae* cases, and 21 other cases) were administered cephalosporin antibiotics (primarily cefazolin). Other cases were treated with a combination of penicillin and aminoglycoside antibiotics (17 cases), kanamycin (8 *Enterobacteriaceae* cases) or erythromycin [one *Trueperella pyogenes* case (formerly *Arcanobacterium pyogenes*) [20]). Ten cases (5.9%) were treated with two or three types of intramammary infusions. Of the 209 cases, 116 (55.5%; 33 *Streptococcus* cases, 32 *Staphylococcus* cases, 33 *Enterobacteriaceae* cases, and 18 other cases) were treated via intramuscular injection of antimicrobials. Most cases (102 cases, 87.9%; 28 *Streptococcus* cases, 28 *Staphylococcus* cases, 30 *Enterobacteriaceae* cases, and 16 other cases) were intramuscularly administered ampicillin. Other cases were intramuscularly administered cefazolin (26 cases, 22.4%; two *Streptococcus* cases, six *Staphylococcus* cases, 14 *Enterobacteriaceae* cases, and 4 other cases), penicillin (8 cases), tetracycline (6 cases), enrofloxacin (6 cases), or tylosin (one case). Individual antimicrobials were administered to 90 cases. Two or more antimicrobials were intramuscularly administered to 26 cases.

For bacteriological examination, quarter milk samples were kept cool and transported to the Central Veterinary Clinical Center of Chiba NOSAI. The samples were tested on the day of collection. Bacterial isolates obtained from these samples were transported to the Laboratory of Veterinary Public Health, Tokyo University of Agriculture and Technology. Upon initial analysis, the isolates were Gram-stained and hemolytic activity was confirmed on Muller-Hinton agar (Oxoid Ltd., Hampshire, UK) supplemented with 5% defibrinated sheep blood. Gram-negative bacilli were assessed for oxidase activity. These biochemical characteristics were confirmed using API 20E (bioMérieux, Japan Ltd., Tokyo, Japan). Catalase-negative cocci were identified through rapid ID 32 STREP api (bioMérieux, Japan Ltd.). Catalase-positive coccal isolates were identified based on the *hsp60* sequence, as previously described [12]. Phylogenetic trees were constructed based on *hsp60* sequences and are shown in Supplementary Fig. 1. Putative *Staphylococcus aureus* was identified using PCR [14]. Gram-positive bacterial isolates with clubbed ends arranged in a V formation grew gradually and were presumed to be *T. pyogenes*, identified using PCR (specific *T. pyogenes*) [8]. The isolates did not display the aforementioned characteristics and could not be identified using identification kits and PCR, but rather based on the 16S rDNA sequence [18]. Similarly, phylogenetic trees were constructed based on the 16S rDNA sequences and are shown in Supplementary Fig. 2. Bacterial species of isolates from the 209 cases are listed in Supplementary Table 1. The major causes of mastitis were *Streptococcus* spp. (70/209, 33.5%), *Staphylococcus* spp. (64/209, 30.6%), and *Enterobacteriaceae* (44/209, 21.1%).

The minimal inhibitory concentration (MIC) was determined using the broth micro-dilution method with Dry Plate "Eiken" (Eiken Chemical Co., Ltd., Tokyo, Japan) for *Staphylococcus* spp. and *Streptococcus* spp. or Frozen Plate "Eiken" (Eiken Chemical Co., Ltd.) for the *Enterobacteriaceae*. The MIC_{50} and MIC_{90} were compared between isolates from cured and uncured cases for *Staphylococcus* spp., *Streptococcus* spp., and the *Enterobacteriaceae* (Table 1). Cases not yielding outcomes were excluded from this analysis.

For 87 cases (48.3%), milk shipment could not be resumed within 3 weeks after the initial clinical examination. Of the 209 cases, 174 (83.3%) were treated with antimicrobial agents through intramammary infusion and/or intramuscular injection. Therefore, it was presumed that antimicrobial-resistant bacteria caused mastitis in several cases and did not respond to antimicrobial treatment. However, the MIC_{50} and MIC_{90} of ampicillin and cefazolin were the same or displayed a 1-tube difference between cured and uncured cases among *Staphylococcus* spp. and *Streptococcus* spp. (Table 1). Demon *et al.* reported that *in vitro* MIC data on cephalosporin did not fully concur with *in vivo* clinical outcomes in a mouse model of mastitis and changing the excipient for intramammary application improved the antimicrobial efficacy [5]. The accessibility of antimicrobials to infection sites is expected to adequately influence the therapeutic effect. The administered antimicrobials might not have reached the site of infection in uncured cases. Antibacterial agents are classified according to their potential distribution through the udder after intramammary administration [6]. Although cefazolin that was administered to most mastitis cases herein via intramammary infusion is not included in this classification, four kinds of cephalosporin are classified into 'limited distribution'. Ampicillin that was administered to most cases via intramuscular injection is also classified into 'limited distribution' after parental administration. The effect of other factors on the outcome, e.g., dosage and administration of antimicrobials and the general status of cattle, should be evaluated.

The higher MIC_{50} of ampicillin and the higher MIC_{90} of cefazolin for the *Enterobacteriaceae* isolates were observed from cured cases rather than uncured cases with a difference of ≥2 tubes (Table 1). Moreover, the MIC_{50} of ampicillin for *Escherichia coli* isolates from cured cases (128 μg/m*l*) was higher than that from uncured cases (4 μg/m*l*), displaying a difference of 5 tubes (Table 1). These results indicate that more cases of infections with β-lactam antibiotic-resistant isolates rather than -susceptible isolates could recover after treatment with antimicrobials, primarily β-lactam antibiotics including ampicillin and cefazolin. Endotoxins are the primary virulence factors in coliform bacteria. Clinical signs in acute coliform mastitis are induced by endotoxins and the subsequent release of inflammatory mediators [9, 16]. Antimicrobials reportedly induce endotoxin release [10]. Some β-lactam antibiotics inhibit penicillin-binding protein (PBP)-3, leading to filament formation. These filaments are associated with high endotoxin release [2]. In general, β-lactam antibiotic-resistant *Enterobacteriaceae* isolates produce β-lactamases [1, 11]. Filament-inducing concentrations of ceftazidime and cefotaxime for extended-spectrum β-lactamase (ESBL)-positive isolates were higher than those for ESBL-negative isolates [3]. Therefore, the amount of endotoxin released upon antimicrobial treatment was presumed

Table 1. Comparison of minimum inhibitory concentration (μg/m*l*) for causative bacteria by outcome (cured and uncured cases)

Antimicrobials	Cured[1] (a)	Uncured (b)	Difference [$\log_2$ (b/a)]
Ampicillin			
Staphylococcus spp. (a, n=36; b, n=23)			
MIC_{50}	0.25	0.5	1
MIC_{90}	2	2	0
Streptocuccus spp. (a, n=30; b, n=25)			
MIC_{50}	0.12	0.12	0
MIC_{90}	0.5	0.5	0
Enterobacteriaceae (a, n=17; b, n=24)[2]			
MIC_{50}	128	8	–4
MIC_{90}	>128	>128	NA
Escherichia coli (a, n=14; b, n=20)			
MIC_{50}	128	4	–5
MIC_{90}	>128	>128	NA
Cefazolin			
Staphylococcus spp. (a, n=36; b, n=23)			
MIC_{50}	0.5	0.5	0
MIC_{90}	1	1	0
Streptocuccus spp. (a, n=30; b, n=25)			
MIC_{50}	0.25	0.5	1
MIC_{90}	0.5	1	1
Enterobacteriaceae (a, n=17; b, n=24)[2]			
MIC_{50}	2	1	–1
MIC_{90}	>64	4	–5
Escherichia coli (a, n=14; b, n=20)			
MIC_{50}	2	1	–1
MIC_{90}	>64	>64	NA

1) Cases wherein milk shipment was resumed within 3 weeks were defined as cured cases.
2) The data of *Enterobacteriaceae* (n=41) includes the data of *Escherichia coli* (n=34).
NA, no application; MIC, minimum inhibitory concentration. Underlined, MIC of isolates from cured cases was higher than those from uncured cases with a difference of ≥2 tubes.

Table 2. Antimicrobial resistance patterns for *Staphylococcus* spp. isolated from mastitis samples

Antimicrobial resistance pattern	Subtotal	*S. aureus*
PC-ABPC-CPDX-EM	1	
PC-ABPC-CEZ	1	1
PC-ABPC-CPDX	1	
PC-ABPC-EM	1	1
PC-ABPC-OFLX	1	1
CPDX-EM	1	
PC-ABPC	19	15
PC-CPDX	1	
ABPC	2	2
OFLX	1	1
Susceptible	35	13
Total	64	34

PC, penicillin (breakpoint, 0.25 μg/m*l*); ABPC, ampicillin (0.5 μg/m*l*); CEZ, cefazolin (32 μg/m*l*); CPDX, cefpodoxime (4 μg/m*l*); EM, erythromycin (8 μg/m*l*); OFLX, ofloxacin (4 μg/m*l*).

to be higher in cases resulting from β-lactam antibiotic-susceptible isolates rather than -resistant isolates at the same concentration of β-lactam antibiotics. Increased endotoxin release might result in intractable mastitis in cases resulting from β-lactam antibiotic-susceptible isolates. Further study needs to determine concentrations of endotoxin for cases resulting from β-lactam antibiotic-susceptible and -resistant *Enterobacteriaceae* isolates.

The breakpoints reported by the CLSI guidelines [4] and breakpoint for colistin reported by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical_breakpoints/) were applied, and antimicrobial resistance patterns were confirmed, and are summarized in Table 2 (64 *Staphylococcus* isolates), Table 3 (70 *Streptococcus* isolates), and Table 4 (44 *Enterobacteriaceae* isolates). Thirty-five of 64 *Staphylococcus* isolates (54.7%) were susceptible to all antimicrobials tested herein (Table 2). Almost all of the 10 antimicrobial resistance patterns displayed resistance to β-lactam antibiotics. To enhance the effect of treatment for staphylococcal mastitis, antimicrobials of other classes including macrolides and tetracycline may be selected based on antimicrobial susceptibilities according to the CLSI breakpoints.

Although only 10 *Streptococcus* isolates were susceptible to all tested antimicrobials (14.3%) (Table 3), the percentage of resistance to β-lactam antibiotics (22.9%) was low, concurrent with a previous report [13]. However, 25 of 55 cases (45.5%) were uncured. If clinical veterinarians select other classes of antimicrobials to treat mastitis caused by *Streptococcus* spp., they should focus on kanamycin and tetracycline resistance.

Among the *Enterobacteriaceae* isolates, 16 antimicrobial resistance patterns were confirmed (Table 4). Of the 16 patterns, 13 patterns included ampicillin resistance. Considering that, endotoxins are released upon treatment with the aforementioned antimicrobials, antimicrobial treatment should not be recommended for cases of mastitis caused by *Enterobacteriaceae*.

In conclusion, numerous mastitis cases caused by β-lactam antibiotic-resistant *Enterobacteriaceae* isolates could recover upon antimicrobial treatment rather than cases resulting from susceptible isolates, probably owing to low amounts of endotoxin released upon antimicrobial treatment for mastitis by β-lactam antibiotic-resistant isolates. The high endotoxin release induced by antimicrobial administration in cases resulting from susceptible isolates potentially inhibited resumption of milk shipment. Differences in MIC_{50} and MIC_{90} were not observed between *Streptococcus* spp. and *Staphylococcus* spp. isolated from cured cases and uncured cases. The low cure rate may not be attributed to mastitis caused by antimicrobial-resistant bacteria.

Table 3. Antimicrobial resistance patterns for *Streptococcus* spp. isolated from mastitis samples

Antimicrobial resistance pattern	Subtotal	*S. dysgalactiae*	*S. uberis*
PC-ABPC-KM-TC	1		
PC-KM-EM-TC	1		1
KM-EM-TC	2		2
PC-KM-TC	4		4
PC-EM-TC	3		3
EM-TC	6		5
KM-OFLX	1		
KM-TC	10	7	2
PC-ABPC	1	1	
PC-KM	3		2
PC-TC	1		1
KM	21	1	3
PC	2	1	1
TC	4	2	2
Susceptible	10	8	1
Total	70	20	27

PC, penicillin (breakpoint, 0.25 μg/m*l*); ABPC, ampicillin (8 μg/m*l*); CEZ, cefazolin (32 μg/m*l*); CPDX, cefpodoxime (8 μg/m*l*); EM, erythromycin (1 μg/m*l*); OFLX, ofloxacin (8 μg/m*l*).

Table 4. Antimicrobial resistance patterns for family *Enterobacteriaceae* isolated from mastitis samples

Antimicrobial resistance pattern	Subtotal	*Escherichia coli*
ABPC-CEZ-CPDX-ACV-CPDX/CVA-KM-TC-CP-ST	1	1
ABPC-CEZ-CPDX-ACV-CPDX/CVA-KM-TC-ST	1	1
ABPC-CEZ-CPDX-TC-CP-CL-NA-CPFX	1	1
ABPC-CEZ-ACV-TC-CL	1	
ABPC-CEZ-CPDX-TC-CL	1	1
ABPC-TC-NA-ST	1	1
ABPC-KM-TC-ST	1	1
ABPC-CEZ-ACV	2	
ABPC-TC-CL	2	2
ABPC-TC-CP	1	1
ABPC-TC-ST	3	3
ABPC-TC	3	3
TC-CL	1	1
ABPC	6	2
CL	2	2
TC	3	3
Susceptible	14	13
Total	44	36

ABPC, ampicillin (breakpoint, 32 μg/m*l*); CEZ, cefazolin (8 μg/m*l*); CPDX, cefpodoxime (8 μg/m*l*); ACV, amoxicillin/clavulanic acid (32/16 μg/m*l*); CPDX/CVA, cefpodoxime/clavulanic acid (32/4 μg/m*l*); KM, kanamycin (64 μg/m*l*); TC, tetracycline (16 μg/m*l*); CP, chloramphenicol (32 μg/m*l*); CL, colistin (4 μg/m*l*); NA, nalidixic acid (32 μg/m*l*); CPFX, ciprofloxacin (4 μg/m*l*).

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