Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds

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ABSTRACT

The prevalence of antimicrobial resistance (AMR) is increasing in human and animal pathogens, becoming a concern worldwide. However, prevalence and characteristics of AMR of bovine mastitis pathogens in large Chinese dairy herds are still unclear. Therefore, our objective was to determine the AMR profile of bacteria isolated from clinical mastitis in large (>500 cows) Chinese dairy herds. A total of 541 isolates of the 5 most common species, *Staphylococcus aureus* (n = 103), non-*aureus* staphylococci (NAS; n = 107), *Streptococcus* species (n = 101), *Klebsiella* species (n = 130), and *Escherichia coli* (n = 100), isolated from bovine clinical mastitis on 45 dairy farms located in 10 provinces of China were included. Presence of AMR was determined by minimum inhibitory concentrations using the microdilution method. Prevalence of multidrug resistance (resistance to >2 antimicrobials) was 27% (148/541). A very wide distribution of minimum inhibitory concentrations was screened in all isolates, including *Staph. aureus* isolates, which were resistant to penicillin (66%). In addition, NAS (30%) were more resistant than *Staph. aureus* to oxacillin (84%), penicillin (62%), tetracycline (34%), and clindamycin (33%). Prevalence of resistance to tetracycline was high (59%) in *Streptococcus* spp. Additionally, prevalence of resistance of both *E. coli* and *Klebsiella* spp. was high to amoxicillin/clavulanate potassium (81 and 38%, respectively), followed by tetracycline (only *Klebsiella* spp. 32%). A high proportion (27%) of isolates were multidrug resistant; the most frequent combinations were clindamycin-cefalexin-tetracycline or enrofloxacin-

cefalexin-penicillin patterns for *Staph. aureus*; enrofloxacin-oxacillin-penicillin-tetracycline patterns for NAS; clindamycin-enrofloxacin-tetracycline patterns for *Streptococcus* spp.; amoxicillin/clavulanate potassium-ceftiofur-polymyxin B patterns for *Klebsiella* spp.; and amoxicillin/clavulanate potassium-ceftiofur-polymyxin B patterns for *E. coli*. Resistance for 4 kinds of antimicrobials highly critical for human medicine, including daptomycin, vancomycin, imipenem, and polymyxin B, ranged from 0 to 24%. In conclusion, prevalence of AMR in mastitis pathogens was high on large Chinese dairy farms, potentially jeopardizing both antimicrobial efficacy and public health. Results of this study highlighted the need for improvements in antimicrobial stewardship and infection control programs in large Chinese dairy farms to reduce emergence of AMR.

Key words: antimicrobial resistance, minimum inhibitory counts, multidrug resistance, bovine mastitis, China

INTRODUCTION

Mastitis is a major economic burden on the dairy industry, affecting milk production and milk quality (Abebe et al., 2016; Hogeveen and Van Der Voort, 2017). Numerous microorganisms associated with cases of mastitis have been isolated (Krömker and Leimbach, 2017; Vakkamäki et al., 2017); the most frequently isolated pathogens associated with clinical mastitis (CM) in China are *Escherichia coli*, *Klebsiella* spp., NAS, *Streptococcus dysgalactiae*, and *Staphylococcus aureus* (Gao et al., 2017). Identification of pathogens causing CM enables appropriate choices for antimicrobial treatment (Pinzón-Sánchez et al., 2011) and preventive mastitis management. Antimicrobials are used in the dairy industry for prevention and control of mastitis and other bacterial diseases affecting dairy cows (Oliver and Murinda, 2012). Therefore, dependence on antimicrobials has become a widespread phenomenon on dairy farms.

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Antimicrobial resistance (AMR) occurs when microorganisms are able to overcome effects of antimicrobials that were effective in the past. Based on EARS-Net data in 2016, AMR remains a serious threat to public health in Europe (ECDC, 2017). Additionally, AMR is one of the biggest threats to global health, food security, and development (WHO, 2015).

In 2010, China had the largest share of global antimicrobial use in food animal production (23%), with projections for use of 30% by 2030 (Van Boeckel et al., 2015). Furthermore, AMR is becoming a serious healthcare problem, with high resistance rates of most common bacteria to clinically important antimicrobial agents (Shi et al., 2010; Xiao et al., 2011; Wang et al., 2015).

Prevalence of AMR in clinical bovine mastitis pathogens has been investigated numerous times recently in China, including regional results for *Staph. aureus* (Gao et al., 2012; Liu et al., 2017), *E. coli* (Liu et al., 2014), and *Strep. dysgalactiae* (Zhang et al., 2018b). However, to our knowledge a nationwide survey has apparently never been performed. The objective of the present study was to determine AMR profiles of 5 predominant bacteria isolated from CM in large Chinese dairy herds: *Staph. aureus*, NAS, *Streptococcus* spp., *E. coli*, and *Klebsiella* spp.

MATERIALS AND METHODS

Statement of Ethics

The present study was conducted according to the ethical guidelines of China Agricultural University (CAU; Beijing, China). Before start of the study, ethical approval was granted by the departmental committee of the College of Veterinary Medicine, CAU.

Herds and Sampling

Details of collected samples are presented in Table 1. One hundred ninety-eight isolates, including *Staph. aureus* (n = 77), *Streptococcus* spp. (n = 3), *Escherichia coli* (n = 47), and *Klebsiella* spp. (n = 71), used in this study were collected (March 2014 to September 2016) during a previous study on the incidence of CM in large (>500 cows) Chinese dairy herds (Gao et al., 2017); an additional 343 CM isolates, including *Staph. aureus* (n = 26), NAS (n = 107), *Streptococcus* spp. (n = 98), *E. coli* (n = 53), and *Klebsiella* spp. (n = 59), were collected from October 2016 to July 2017. In total, 45 dairy herds from herds submitting CM samples to the CAU mastitis laboratory were randomly included until a minimum of 100 isolates per bacterial group, *Staph. aureus*, *Streptococcus* spp., NAS, *Klebsiella* spp., and *E. coli*, were available for analysis. Briefly, milk samples were collected, according to National Mastitis Council (NMC, 1999) guidelines, from every udder quarter (1 sample per quarter) with CM, and diagnosed on the basis of visual abnormality and inflammation of the udder or its milk secretion. Samples were collected in sterile bottles (5–10 mL) and transported on ice to the mastitis laboratory at the CAU. Recurrent CM cases (<14 d apart), as well as repeated cases in same quarters, were not included.

Isolates

Upon arrival at the laboratory, all frozen samples were thawed at room temperature. Microbiological procedures were conducted as described by Ali et al. (2017) and Gao et al. (2017). Briefly, 10 µL of milk from each sample was plated onto a tryptic soy agar (Beijing Aoboxing Biotechnology Co. Ltd., Beijing,

Table 1. Distribution of 541 bacteria isolated from clinical mastitis in 45 large Chinese dairy herds

Province	<i>Staphylococcus aureus</i>		NAS		<i>Streptococcus</i> spp.		<i>Escherichia coli</i>		<i>Klebsiella</i> spp.	
	Herds	Isol. ¹	Herds	Isol.	Herds	Isol.	Herds	Isol.	Herds	Isol.
Beijing	3	5	2	2	2	4	3	7	3	6
Guangdong	2	6	1	8	3	6	3	9	2	4
Hebei	6	13	7	31	5	20	5	24	13	35
Heilongjiang	2	44	2	32	14	39	6	27	4	9
Henan	2	3	2	6	5	8	2	4	2	6
Liaoning	2	12	—	—	—	—	2	5	3	13
Inner Mongolia	6	13	6	22	7	15	5	18	10	32
Shandong	1	1	2	4	4	6	2	6	4	13
Shanxi	2	6	1	2	1	3	—	—	2	8
Tianjin	—	—	—	—	—	—	—	—	2	4
Total	26	103	23	107	41	101	28	100	45	130

¹Number of clinical mastitis isolates.

China) with 5% sheep blood plate (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) and then incubated (37°C) for 24 to 48 h. Samples were considered culture-positive if 1 or more colonies were observed (≥ 100 cfu/mL). Milk samples yielding 2 bacterial species were grouped as a mixed culture, whereas samples yielding >2 bacterial species were considered contaminated (Verbeke et al., 2014). Preliminary isolation and identification of bacteria was performed using procedures of Mastitis Diagnostic Laboratory of China Agriculture University (Beijing, P.R. China) as described in Gao et al. (2017). Subsequently, *E. coli*, *Streptococcus* spp., NAS, and *Klebsiella* spp. were confirmed by 16S rRNA sequencing (Frank et al., 2008). *Streptococcus* spp. included *Streptococcus agalactiae* (n = 28), *Streptococcus uberis* (n = 29), and *Streptococcus dysgalactiae* (n = 44). Finally, all isolates were stored at -80°C .

Recovery of Isolates

First, bacteria were recovered on a tryptic soy agar plate and incubated at 37°C for 24 h. A single colony was then transferred into Mueller-Hinton Broth (Beijing Aoboxing Biotechnology Co. Ltd.) medium (5% bovine serum added for *Streptococcus* spp.) and shaken at 37°C for 12 h. A bacterial turbidimeter (WGZ-2-XJ, Shanghai Xinrui Instruments and Meters Co. Ltd., Shanghai, P.R. China) was used to adjust the suspension to a density equivalent to a 0.5 McFarland standard, which was used for the MIC determination.

MIC and PCR

Phenotypic AMR was obtained using a microdilution method for antimicrobials commonly administered to dairy cattle and humans, following Clinical Laboratory and Standards Institute (CLSI) guidelines (Table 2; CLSI, 2017). Antimicrobials and concentrations evaluated for gram-positives included cephalexin (0.03–64 $\mu\text{g/mL}$), tetracycline (0.03–64 $\mu\text{g/mL}$), penicillin (0.03–64 $\mu\text{g/mL}$), and vancomycin (0.03–64 $\mu\text{g/mL}$). Additionally, for ceftiofur, cefquinome, enrofloxacin, oxacillin, clindamycin, amoxicillin/clavulanate potassium (**amoxi/clav**), and daptomycin, a concentration of 0.015 to 64 $\mu\text{g/mL}$ was used for streptococci, whereas a concentration of 0.03 to 64 $\mu\text{g/mL}$ was used for staphylococci (*Staph. aureus* and NAS). Subsequently, oxacillin-resistant NAS with MIC values for oxacillin of 0.5 to 2 mg/L were tested for presence of the *mecA* gene using PCR, as reported (CLSI, 2017). For gram-negative bacteria, MIC of 8 antimicrobials were tested:

ceftiofur (0.03–128 $\mu\text{g/mL}$), cefquinome (0.03–128 $\mu\text{g/mL}$), enrofloxacin (0.03–64 $\mu\text{g/mL}$), amoxi/clav (0.03–128 $\mu\text{g/mL}$), tetracycline (0.03–128 $\mu\text{g/mL}$), kanamycin (0.03–128 $\mu\text{g/mL}$), imipenem (0.03–64 $\mu\text{g/mL}$), and polymyxin B (0.03–128 $\mu\text{g/mL}$). Positive controls (antimicrobial-free wells) were included in all plates. Included as quality control strains were *Staph. aureus* ATCC 29213 for *Staph. aureus* and NAS, *Streptococcus* spp. ATCC 19615, *E. coli* ATCC 25922, and *Klebsiella* spp. ATCC 700603.

Cephalexin, ceftiofur, cefquinome, enrofloxacin, oxacillin, clindamycin, tetracycline, and kanamycin were obtained from Ehrenstorfer Biotechnology Co. Ltd. (Augsburg, Germany); vancomycin, polymyxin B, and amoxi/clav were obtained from National Institutes for Food and Drug Control (Beijing, China); and penicillin, daptomycin, and imipenem were obtained from Shanghai Yuanze Biotechnology Co. Ltd. (Shanghai, China). We determined values for various isolate-antimicrobial combinations representing the minimum concentration of an antimicrobial that inhibited the visible growth of ≥ 50 and $\geq 90\%$ of microorganisms (**MIC₅₀** and **MIC₉₀**, respectively).

Resistance was defined according to CLSI (2013, 2017) or EUCAST (2015) with slight modifications. If no CLSI breakpoint existed, EUCAST cutoff values were used (Table 2). Additionally, if specific cutoff values were not available for a particular species (e.g., *Klebsiella* spp. as a group), cut-value of the family (*Enterobacteriaceae*) were used. Isolates were classified as resistant on the basis of clinical breakpoints (CLSI, 2013, 2017); AMR was defined as nonsusceptibility to a given antimicrobial by combining intermediate and resistant categories into a single category. Therefore, isolates with MIC values equal or above the intermediate breakpoint were classified as resistant. Multidrug resistance (**MDR**) was defined as resistance to ≥ 3 antimicrobials (Magiorakos et al., 2012).

For each antimicrobial agent and bacterial species, **MIC₅₀**, **MIC₉₀** and resistant rate, defined as number of resistant isolates divided by total number of isolates tested, were calculated using descriptive statistics.

RESULTS

Isolates

Five hundred forty-one isolates were used: 103 *Staph. aureus* from 26 dairy herds in 9 provinces, 107 NAS from 23 dairy herds in 8 provinces, 101 *Streptococcus* spp. from 41 dairy herds in 8 provinces, 100 *E. coli* from 28 dairy herds in 8 provinces, and 130 *Klebsiella* spp. from 45 dairy herds in 10 provinces in China (Table 1).

Table 2. References of interpretative breakpoints for *Staphylococcus aureus*, *NAS*, *Streptococcus* species, *Escherichia coli*, and *Klebsiella* species used in this study

Antimicrobial	<i>Staph. aureus</i>		<i>NAS</i>		<i>Strep. species</i>		<i>E. coli</i>		<i>Klebsiella</i> species	
	Breakpoint (µg/mL)	Reference ¹	Breakpoint (µg/mL)	Reference	Breakpoint (µg/mL)	Reference	Breakpoint (µg/mL)	Reference	Breakpoint (µg/mL)	Reference
Amoxi/clav ²	≥8	CLSI HUM	≥8	CLSI HUM	≥32	CLSI HUM	≥16	CLSI HUM	≥32	CLSI HUM
Cefalexin ³	≥8	CLSI VET	≥8	CLSI VET	≥8	CLSI VET	—	—	—	—
Cefquinome	NA ⁴	—	NA	—	NA	—	≥4	CLSI HUM	≥4	CLSI HUM
Ceftiofur	≥8	CLSI VET	≥8	CLSI VET	≥8	CLSI VET	≥8	CLSI VET	≥8	CLSI VET
Clindamycin	≥4	CLSI HUM	≥4	CLSI HUM	≥1	CLSI HUM	—	—	—	—
Daptomycin	≥2	EUCAST	≥2	EUCAST	≥2	EUCAST	—	—	—	—
Enrofloxacin	≥4	CLSI VET ⁵	≥4	CLSI VET ⁵	≥2	CLSI VET ⁶	≥4	CLSI VET ⁵	≥4	CLSI VET ⁵
Imipenem	—	—	—	—	—	—	≥4	CLSI HUM	≥4	CLSI HUM
Kanamycin	—	—	—	—	—	—	≥64	CLSI HUM	≥64	CLSI HUM
Oxacillin	≥4	CLSI HUM	≥0.5	CLSI HUM	≥4	CLSI HUM	—	—	—	—
Penicillin	≥0.25	CLSI HUM	≥0.25	CLSI HUM	≥16	CLSI HUM	—	—	—	—
Polymyxin B	—	—	—	—	—	—	≥4	EUCAST	≥4	EUCAST
Tetracycline	≥16	CLSI HUM	≥16	CLSI HUM	≥8	CLSI HUM	≥16	CLSI HUM	≥16	CLSI HUM
Vancomycin	≥4	CLSI HUM	≥32	CLSI HUM	≥32	CLSI HUM	—	—	—	—

¹CLSI VET = CLSI breakpoints used in veterinary medicine (CLSI, 2013); CLSI HUM = CLSI breakpoints used in humans (CLSI, 2017); EUCAST = European antimicrobial susceptibility testing (EUCAST, 2015).

²Amoxicillin/clavulanate potassium.

³Breakpoint of cefalexin was based on cephalothin.

⁴NA = no breakpoint.

⁵Interpretative breakpoint based on canine data in CLSI-VET (CLSI, 2013).

⁶Interpretative breakpoint based on swine data in CLSI-VET (CLSI, 2013).

Staphylococcus aureus

For the 103 *Staph. aureus* isolates, MIC of antibiotics tested had a very wide distribution; MIC₉₀ values were at least 2 times dilutions of its MIC₅₀ values (Table 3). Except for penicillin (>64 µg/mL), MIC₉₀ values were less than or equal to the highest antimicrobial concentration tested. Additionally, the lowest MIC₉₀ value of *Staph. aureus* to these 11 antibiotics was for daptomycin and vancomycin (1 µg/mL). In *Staph. aureus* isolates, prevalence of resistance was highest for penicillin (66%), followed by cefalexin (32%), clindamycin (19%), oxacillin (18%), tetracycline (18%), amoxi/clav (17%), ceftiofur (16%), enrofloxacin (10%), daptomycin (8%), and vancomycin (3%). A total of 34 (33%) isolates were MDR (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>), in which 2, 4, 6, 10, and 12 isolates were resistant to 7, 6, 5, 4, and 3 antimicrobials, respectively. One isolate had an amoxi/clav-clindamycin-daptomycin-oxacillin-penicillin-vancomycin resistance pattern (Supplemental Table S1). Additionally, clindamycin-cephalexin-tetracycline and enrofloxacin-cephalexin-penicillin resistance patterns were present in top 2 isolates (both 3 isolates).

NAS

Regarding the 107 NAS isolates, MIC of all kinds of antimicrobials also had a very wide distribution (Table 4). The MIC₉₀ of clindamycin and daptomycin was higher than the highest concentration (64 µg/mL) tested. All MIC₉₀ values were ≥4 dilutions higher than its MIC₅₀ values. For these 11 antibiotics, the lowest MIC₉₀ value of NAS was for ceftiofur (4 µg/mL), whereas the highest value was for clindamycin (>64 µg/mL) or daptomycin (>64 µg/mL). Additionally, NAS were most often resistant to oxacillin (84%), followed by penicillin (62%), tetracycline (34%), clindamycin (33%), enrofloxacin (23%), daptomycin (21%), vancomycin (20%), cefalexin (15%), amoxi/clav (12%), and ceftiofur (9%). Additionally, among oxacillin-resistant strains, 67 strains carried the *mecA* gene. Strains with MIC value 0.5 to 2 µg/mL to oxacillin all carried the *mecA* gene. In total, 56% (60/107) of NAS isolates were MDR (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>). Two, 1, 5, 5, 11, 16, and 20 isolates were resistant to 9, 8, 7, 6, 5, 4, and 3 antimicrobials, respectively. Furthermore, 1 isolate exhibited a resistance pattern composed of 9 antimicrobials: amoxi/clav-clindamycin-daptomycin-ceftiofur-cephalexin-oxacillin-penicillin-tetracycline-vancomycin (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>). Five and 4 isolates had resistance patterns to nrofloxacin-oxacillin-penicillin-tetracycline and

Table 3. Distribution (%) of MIC of Staphylococcus aureus (n = 103) isolated from clinical mastitis cases¹

Item	Distribution of MIC (µg/mL)														Resistance rate (%)	MIC ₅₀ ² (µg/mL)	MIC ₉₀ ² (µg/mL)
	<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64			
Amoxi/clav ³	0	0	0	3	6	12	11	28	23	11	4	1	1	0	17	2	8
Cefalexin	0	0	0	0	3	5	13	24	23	20	5	1	2	4	32	4	16
Ceftiofur	2	0	1	1	4	22	25	17	12	4	3	4	4	1	16	1	16
Cefquinome	1	1	0	3	11	27	30	14	11	2	0	0	0	0	—	1	4
Clindamycin	1	4	9	23	19	14	6	5	0	0	5	10	2	2	19	0.25	32
Daptomycin	2	0	0	10	20	44	16	2	2	4	0	0	0	0	8	0.5	1
Enrofloxacin	4	4	10	19	23	17	9	4	4	3	3	0	0	0	10	0.25	4
Oxacillin	1	0	5	15	20	16	16	9	6	1	6	5	0	0	18	0.5	16
Penicillin	18	1	9	6	8	6	5	6	5	2	4	8	7	15	66	1	>64
Tetracycline	0	0	0	4	31	32	13	2	0	1	7	6	4	0	17	0.5	32
Vancomycin	3	0	0	0	8	62	24	0	0	0	1	0	1	1	3	0.5	1

¹Bold indicates the CLSI (2013) or EUCAST (2015) epidemiological cutoff values.

²Minimum concentration of an antimicrobial that inhibited the visible growth of ≥50 and ≥90% of microorganisms (MIC₅₀ and MIC₉₀, respectively).

³Amoxicillin/clavulanate potassium.

Table 4. Distribution (%) of MIC of non-*aureus* staphylococci (n = 107) isolated from clinical mastitis in large Chinese dairy herds¹

Item	Distribution of MIC (μg/mL)														Resistance rate (%)	MIC ₅₀ ² (μg/mL)	MIC ₉₀ ² (μg/mL)
	<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64			
Amoxi/clav ³	0	0	4	7	15	12	19	18	13	5	1	3	1	2	12	1	8
Cefalexin	0	2	0	6	8	7	7	32	22	6	2	2	2	4	15	2	8
Ceftiofur	0	0	1	0	6	20	35	21	8	1	4	1	2	1	9	1	4
Cefquinome	0	0	2	3	6	16	7	27	23	9	5	1	1	0	—	2	8
Clindamycin	0	0	4	9	8	28	10	8	8	3	1	4	2	15	33	1	>64
Daptomycin	11	0	7	14	23	14	10	2	2	1	1	0	3	12	21	0.25	>64
Enrofloxacin	1	0	3	14	10	22	16	11	5	2	5	4	5	2	23	1	32
Oxacillin	0	0	0	3	13	17	15	21	10	6	7	4	3	1	84	2	16
Penicillin	22	0	7	9	6	8	3	3	12	5	12	1	1	11	62	0.5	>64
Tetracycline	0	0	2	6	18	18	7	10	3	2	9	22	2	1	34	1	32
Vancomycin	0	0	0	2	17	36	17	2	1	2	3	12	2	6	20	0.5	32

¹Bold indicates the CLSI (2013) or EUCAST (2015) epidemiological cutoff values.²Minimum concentration of an antimicrobial that inhibited the visible growth of ≥50 and ≥90% of microorganisms (MIC₅₀ and MIC₉₀, respectively).³Amoxicillin/clavulanate potassium.

oxacillin-penicillin-tetracycline, respectively, the most frequent patterns we observed.

Streptococcus Species

In the 101 *Streptococcus* spp. isolates, the MIC₉₀ value of clindamycin was higher than the highest concentration (64 μg/mL) tested (Table 5). All MIC₉₀ values were ≥2 dilutions higher than MIC₅₀ values. The lowest MIC₉₀ value was for ceftiofur and penicillin (0.25 μg/mL), whereas the highest value was for clindamycin (>64 μg/mL). Resistance of *Streptococcus* spp. to tetracycline was most prevalent (59%), followed by clindamycin (29%), cefalexin (19%), oxacillin (16%), enrofloxacin (8%), vancomycin (8%), daptomycin (5%), amoxi/clav (3%), penicillin (2%), and ceftiofur (1%). In total, 21% (21/101) of isolates were MDR; 2, 7, and 12 isolates were resistant to 5, 4, and 3 antimicrobials, respectively (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>). Furthermore, we noted 2 resistance patterns of 5 antimicrobials, exhibiting an amoxi/clav-clindamycin-cephalexin-oxacillin-tetracycline or clindamycin-cephalexin-oxacillin-tetracycline resistance pattern (Supplemental Table S1). Clindamycin-enrofloxacin-tetracycline was the most frequent resistance pattern with 3 isolates.

Klebsiella Species

In these 130 *Klebsiella* spp. isolates, we noted differences in MIC distributions for various antimicrobials (Table 6). Except for amoxi/clav or ceftiofur, the MIC₉₀ values of other antimicrobials used in this experiment were ≥2 dilutions higher than MIC₅₀ values. The lowest MIC₉₀ value of the 8 antibiotics was for enrofloxacin and imipenem (1 μg/mL), whereas the highest value was for amoxi/clav and ceftiofur (>128 μg/mL). Resistance in *Klebsiella* spp. was most prevalent to amoxi/clav (38%), followed by tetracycline (32%), polymyxin B (24%), ceftiofur (21%), kanamycin (15%), cefquinome (10%), enrofloxacin (2%), and imipenem (1%). Furthermore, a total of 25 (19%) isolates were resistant to >2 antimicrobials carrying amoxi/clav-ceftiofur-polymyxin B, amoxi/clav-ceftiofur-ceftiofur-tetracycline-kanamycin-polymyxin B, and amoxi/clav-ceftiofur-polymyxin B resistance patterns, which were observed in 5, 3, and 3 isolates, respectively (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>).

Escherichia coli

The MIC values of 100 *E. coli* isolates from 100 individual cows on 28 dairy farms in 8 provinces of China were determined. Total MIC₉₀ values covered in this

Table 5. Distribution (%) of MIC for *Streptococcus species* (n = 101) from clinical mastitis in large Chinese dairy herds¹

Item	Distribution of MIC (μg/mL)															Resistance rate (%)	MIC ₅₀ ² (μg/mL)	MIC ₉₀ ² (μg/mL)
	<0.015	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64			
Amoxi/clav ³	0	1	4	11	12	43	5	4	10	0	6	1	2	1	0	3	0.25	8
Cefalexin	NA ⁴	NA	0	0	5	9	7	5	28	27	10	2	6	1	0	19	2	8
Ceftiofur	0	60	0	8	11	8	6	4	2	0	0	0	1	0	0	1	0.015	0.5
Cefquinome	13	13	15	31	9	9	2	5	1	1	0	0	1	0	0	-	0.06	0.25
Clindamycin	6	12	29	9	9	6	0	1	4	0	4	2	2	5	11	29	0.06	>64
Daptomycin	0	34	1	20	24	10	5	1	1	0	0	0	0	0	4	5	0.06	0.5
Enrofloxacin	0	2	1	3	15	57	14	1	0	2	2	4	0	0	0	8	0.25	0.5
Oxacillin	0	4	5	3	4	46	10	7	5	4	4	1	2	0	5	16	0.25	8
Penicillin	NA	NA	66	4	12	11	2	1	2	0	0	0	0	1	1	2	0.03	0.25
Tetracycline	NA	NA	2	0	0	9	9	9	7	5	4	10	44	1	0	59	16	32
Vancomycin	NA	NA	2	4	6	55	14	5	6	0	0	0	0	1	7	8	0.25	2

¹Bold indicates the CLSI (2013) or EUCAST (2015) epidemiological cutoff values.
²Minimum concentration of an antimicrobial that inhibited the visible growth of ≥50 and ≥90% of microorganisms (MIC₅₀ and MIC₉₀, respectively).
³Amoxicillin/clavulanate potassium.
⁴NA = untested concentrations.

Table 6. Distribution (%) of MIC for *Klebsiella* spp. (n = 130) isolated from clinical mastitis in large Chinese dairy herds¹

Item	Distribution of MIC (μg/mL)															Resistance rate (%)	MIC ₅₀ ² (μg/mL)	MIC ₉₀ ² (μg/mL)
	<0.03	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	>128			
Amoxi/clav ³	0	0	0	0	0	0	1	0	0	22	39	6	12	8	12	38	16	>128
Cefquinome	1	1	15	28	19	10	14	2	4	0	0	0	2	4	0	10	0.25	4
Ceftiofur	0	0	0	0	1	16	34	20	8	3	2	3	1	2	10	21	1	>128
Enrofloxacin	5	18	35	14	5	4	11	6	1	1	0	0	0	NA ⁴	NA	2	0.06	1
Imipenem	0	0	2	8	29	38	18	4	0	1	0	0	0	NA	NA	1	0.5	1
Kanamycin	0	0	0	0	0	0	1	8	36	21	14	5	5	6	4	15	8	128
Polymyxin B	18	4	10	12	8	8	8	8	4	6	5	4	0	5	0	24	0.25	16
Tetracycline	0	0	0	0	0	0	12	23	23	10	8	2	13	4	5	32	4	64

¹Bold indicates the CLSI (2013) or EUCAST (2015) epidemiological cutoff values.
²Minimum concentration of an antimicrobial that inhibited the visible growth of ≥50 and ≥90% of microorganisms (MIC₅₀ and MIC₉₀, respectively).
³Amoxicillin/clavulanate potassium.
⁴NA = untested concentrations.

experiment were ≥ 2 dilutions higher than its MIC₅₀ values (Table 7). Additionally, the lowest MIC₉₀ value of these 8 antibiotics was for cefquinome and enrofloxacin (0.25 µg/mL), whereas the highest value was for amoxi/clav and polymyxin B (128 µg/mL). *Escherichia coli* isolates had the highest proportion of resistance to amoxi/clav (81%), followed by polymyxin B (19%), ceftiofur (16%), tetracycline (10%), kanamycin (6%), and cefquinome (1%). No *E. coli* isolate was resistant to enrofloxacin or imipenem. Among 100 *E. coli*, 8% of isolates were resistant to ≥ 3 antimicrobials, in which isolates carried amoxi/clav-ceftiofur-polymyxin B, amoxi/clav-ceftiofur-polymyxin B-tetracycline, amoxi/clav-kanamycin-tetracycline, or cefquinome-ceftiofur-polymyxin B resistance pattern, present in 4, 2, 1, and 1 isolates, respectively (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15135>).

DISCUSSION

Bacterial infections are the predominant cause of bovine mastitis; therefore, antimicrobial therapy is commonly implemented for mastitis prevention and control (Oliver et al., 2011). Prompt treatment with effective antimicrobials is especially important to reduce the risk of a fatal outcome, especially for invasive bacterial infections (ECDC, 2017). Unfortunately, despite best possible antimicrobial treatments, failures of bacteriological cure are common, with AMR implicated in low cure rates (Barkema et al., 2006). The World Health Organization has stated that any use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacteria (WHO, 2015). In a study of 385 dairy herds in the Netherlands between 2010 and 2012, >70% of total antibiotic use was for control or treatment of (sub)clinical mastitis (van Werven et al., 2013). Additionally, antimicrobial use was positively associated with AMR in bovine mastitis pathogens (Saini et al., 2012). Therefore, over-reliance on antimicrobial use to reduce the effect of mastitis in dairy herds was not sustainable, whether from political pressure, public perception, research evidence, or other sources (Barkema et al., 2015; Kayitsinga et al., 2017). In the present study, we determined AMR in the 5 most frequently isolated major mastitis pathogens (*Staph. aureus*, *Streptococcus* spp., NAS, *E. coli*, and *Klebsiella* spp.) from milk samples of CM in large Chinese dairy herds from 2014 to 2017. We monitored a gram-positive cocci and gram-negative bacilli panel containing 11 and 8 antimicrobials, respectively, commonly used in veterinary or human medicine. This was apparently the first time that a nationwide study in China has been conducted to determine AMR in common mastitis pathogens isolated from CM samples in dairy cows.

Table 7. Distribution (%) of MIC for *Escherichia coli* (n = 100) isolated from clinical mastitis in large Chinese dairy herds¹

Item	Distribution of MIC (µg/mL)														Resistant rate (%)	MIC ₅₀ ² (µg/mL)	MIC ₉₀ ² (µg/mL)
	<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128		
Amoxi/clav ³	0	0	0	0	2	0	0	0	1	16	37	22	11	9	2	81	16
Cefquinome	2	8	35	37	12	3	1	1	0	1	0	0	0	0	0	1	0.12
Ceftiofur	0	1	0	0	12	43	20	7	1	1	5	2	2	5	1	16	0.5
Enrofloxacin	44	30	9	3	6	6	1	1	0	0	0	0	0	NA ⁴	NA	0	0.03
Imipenem	0	0	10	34	20	26	8	2	0	0	0	0	0	NA	NA	0	0.25
Kanamycin	0	0	0	0	0	0	3	6	9	42	30	4	5	1	0	6	8
Polymyxin B	10	49	6	12	1	0	2	1	1	1	1	1	1	7	7	19	0.03
Tetracycline	0	0	0	0	0	0	2	20	50	17	1	0	1	5	4	10	4

¹Bold indicates the CLSI (2013) or EUCAST (2015) epidemiological cutoff values.

² Minimum concentration of an antimicrobial that inhibited the visible growth of ≥ 50 and $\geq 90\%$ of microorganisms (MIC₅₀ and MIC₉₀, respectively).

³Amoxicillin/clavulanate potassium.

⁴NA = untested concentrations.

The majority of MIC values for various antibiotics had a very wide distribution, different from reports from Canada and 9 European countries (Saini et al., 2012; de Jong et al., 2018). For these 5 mastitis pathogens, antimicrobials with the highest MIC₉₀ values were penicillin (>64 µg/mL) for *Staph. aureus*, clindamycin and daptomycin (>64 µg/mL) for NAS, clindamycin (>64 µg/mL) for *Streptococcus* spp., amoxi/clav and ceftiofur (>128 µg/mL) for *Klebsiella* spp., and amoxi/clav and ceftiofur (128 µg/mL) for *E. coli*. Overall, 27% of isolates in our study were resistant to ≥3 antimicrobials. Our results confirmed that MDR bovine mastitis is a problem in large Chinese dairy herds.

Based on previous studies, penicillin resistance of *Staph. aureus* is the most common form of AMR among mastitis-causing bacteria (Hendriksen et al., 2008; Kalmus et al., 2011). Among various antimicrobials tested in our study, except for *Streptococcus* spp., high resistance to penicillin was most common (e.g., 66% to *Staph. aureus* and 62% to NAS). In addition, a high prevalence of the *mecA* gene was detected in oxacillin-resistant strains, especially for strains with MIC 0.5 to 2 µg/mL to oxacillin. However, in a previous study, no significant relationship was found between genotypic content and phenotypic expression represented by methicillin resistance (Al-Khafaji and Flayyih, 2015). Oxacillin is a semi-synthetic penicillin with characteristics of resistance to acids and enzymes. It is effective for gram-positive cocci producing penicillinase, but less effective than penicillin for nonenzymatic bacteria. According to our study, more isolates were oxacillin-resistant than penicillin-resistant, consistent with a previous study (Dorothy, 2004). Multidrug resistance to >2 antimicrobials was most common in *Staph. aureus* (33%), NAS (58%), and *Streptococcus* spp. (21%). Resistance of *Staph. aureus* was lower in our study than previously reported in China (Shi et al., 2010; Wang et al., 2015; Zhang et al., 2018a), whereas resistance data for *Staph. aureus* were similar to that reported by others (Rajala-Schultz et al., 2004; Güler et al., 2005). In contrast, clinical *Streptococcus* spp. mastitis was, in general, still sensitive to penicillin, which differed from reports in other countries (Saini et al., 2012). Additionally, except for NAS and *Streptococcus* spp., less resistance was detected against third- or fourth-generation cephalosporins (ceftiofur or cefquinome) compared with first-generation cephalosporins (cefalexin). We noted high resistance rates to tetracycline for these pathogens, especially for *Streptococcus* spp. (59%), NAS (34%), and *Klebsiella* spp. (32%), similar to a previous report for China (Yassin et al., 2017) and consistent with spread of tetracycline-resistance genes on a conventional dairy farm (Kyselková et al., 2015).

Antimicrobial treatment of *E. coli* and *Klebsiella* spp. has often been considered of limited value; therefore, therapy should be more targeted toward cow survival and reduction of clinical symptoms (Schukken et al., 2012). More recently, it was suggested that third-generation cephalosporin and carbapenems would likely be ineffective against a sizable proportion of *E. coli* or *Klebsiella pneumoniae* infections in most parts of the world by 2030 (Alvarez-Uria et al., 2018). In the present study, both *E. coli* and *Klebsiella* spp. were often resistant to amoxi/clav (81 and 38%, respectively), higher than reported by Schukken et al. (2012) and Alvarez-Uria et al. (2018). Additionally, our study had a lower prevalence of MDR in *E. coli* and *Klebsiella* spp. than reported by others (Schukken et al., 2012; Alvarez-Uria et al., 2018).

In the present study, 2 antibiotics critical for human use (daptomycin and vancomycin) were tested for gram-positive cocci, whereas 2 others (imipenem and polymyxin B) were tested for gram-negative bacteria. Surprisingly, in *Staph. aureus*, resistance to daptomycin and vancomycin was only 8 and 3%, respectively, whereas 21 and 20% of NAS and 5 and 9% of *Streptococcus* spp. isolated from CM were resistant to daptomycin and vancomycin, respectively; this was different from other countries, for example Canada and Uganda (Saini et al., 2012; Kateete et al., 2013; Nobrega et al., 2018). Since 1989, prevalence of vancomycin-resistant infections has increased exponentially in humans. It is recommended that vancomycin, a drug highly critical for human medicine, should only be used to treat humans infected with methicillin-resistant staphylococci (Zheng et al., 2018). The observed vancomycin-resistant isolates in bovine CM in our study were a risk to public health.

Compared with 8 European countries (Thomas et al., 2015), resistance rates were higher in our Chinese study. We found major differences in antimicrobial consumption patterns among countries (Mather et al., 2011), reflecting a combination of factors including national guidelines for proper antimicrobial use, veterinarian prescription patterns, pharmaceutical marketing strategies, animal demographics, and specific needs for antimicrobials in specific countries (ESVAC, 2012). Major differences in occurrence of AMR have also been observed among bacteria (e.g., *E. coli*) isolated from different countries (Chantziaras et al., 2014; ECDC et al., 2015). Therefore, it is very likely that our findings arise from the use or abuse of these antimicrobial agents in IMI in China.

This investigation highlighted the worrisome trend of increased AMR rates in Chinese dairy herds. However, we must highlight that results of our study cannot

be generalized to the entire Chinese dairy industry, especially because small farms were not recruited.

CONCLUSIONS

In these large Chinese dairy herds, *Staph. aureus* and *Streptococcus* spp. isolates were highly resistant to penicillin (66%) and tetracycline (59%), whereas NAS were more often resistant to oxacillin (84%) and penicillin (62%). Both *E. coli* and *Klebsiella* spp. were frequently resistant to amoxicillin-clavulanate potassium (81 and 38%); however, resistance rates against 4 antimicrobials that should be reserved for use in human medicine (daptomycin, vancomycin, imipenem, and polymyxin B), ranged from 3 to 21%. A high proportion of MDR was frequently observed, where clindamycin-cephalexin-tetracycline and enrofloxacin-cephalexin-penicillin were the most commonly observed patterns for *Staph. aureus*, and enrofloxacin-oxacillin-penicillin-tetracycline and clindamycin-enrofloxacin-tetracycline were the most frequent multi-drug resistant patterns for NAS and *Streptococcus* spp., respectively. This investigation highlighted the worrisome trend of increased prevalence of AMR in large Chinese dairy herds.

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