

Antimicrobial resistance and virulence traits of *Enterococcus faecium* isolated from clinical bovine mastitis in Ningxia, China

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ABSTRACT

This study was conducted to determine the antimicrobial resistance and virulence traits of 32 *Enterococcus faecium* isolates from clinical bovine mastitis cases in Ningxia Province, China. In total, 32 *E. faecium* isolates were taken from subclinical bovine mastitis on the basis of morphological characterization and biochemical testing, and screened for antimicrobial susceptibility. The virulence genes of the isolates were studied using polymerase chain reaction (PCR). The disc diffusion assay revealed a high occurrence of resistance against tetracycline (78.1%) and erythromycin (68.8%) in the *E. faecium* isolates. However, all tested *E. faecium* were susceptible to linezolid and vancomycin. Moreover, all *E. faecium* isolates harbored the erythromycin-resistant genes *ermA*, *ermB* and *ermC*, as well as the tetracycline-resistant genes *tetK*, *tetL* and *tetM*. Furthermore, all *E. faecium* isolates carried more than 3 of the tested virulence genes. The presence of *agg* (100%), *cpd* (100%), *efaA* (100%), *gelE* (93.4%), and *esp* (75.0%) was found most frequently in all the tested isolates. These findings are useful for making appropriate antimicrobial choices and developing antivirulence therapies for subclinical bovine mastitis caused by *E. faecium* in Ningxia Province, China.

Key words: bovine mastitis; *Enterococcus faecium*; antimicrobial resistance; virulence genes

Introduction

Bovine mastitis is an inflammation of the mammary glands in dairy cows, which causes economic losses due to the reduction in milk production, the decline of milk quality and the increase in medical and veterinary services (GAO et al., 2019). Mastitis pathogens can be divided into contagious and environmental pathogens on the basis of their epidemiological association with a disease (ROSSITTO et al., 2002). Contagious mastitis is usually caused by *Staphylococcus*

aureus, *Streptococcus agalactiae* and *Mycoplasma bovis* (HAAPALA et al., 2018). Environmental mastitis is caused by environmental Streptococci (*Streptococcus bovis*, *Streptococcus uberis* and *Streptococcus dysgalactiae*), coliforms (*Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella oxytoca* and *Enterobacter aerogenes*) and Enterococci (*Enterococcus faecium* and *Enterococcus faecalis*) (GAO et al., 2019). Current mastitis control programs, including antibiotic therapy and teat

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disinfection, have significantly reduced contagious pathogens in recent decades. However, these control measures have little effect on environmental mastitis pathogens. Consequently, environmental pathogens, such as enterococci, have become increasingly important opportunistic pathogens of bovine mastitis (OLIVER et al., 2004; WERNER et al., 2012).

Enterococci are intrinsically resistant to commonly used antimicrobials and are characterized by their potent and unique ability to transfer antibiotic resistance to others via horizontal transfer, posing a serious threat to public health through the food chain (ASLAM et al., 2012; BESHIRU et al., 2017). Furthermore, virulence genes, such as gelatinase (*gelE*), hyaluronidase (*hyl*), aggregation substance (*agg*), and enterococcal surface protein (*esp*) s-pheromone determinant (*cpd*), the regulator of the expression of *gelE* (*frs*), accessory colonization factor (*ace*), and endocarditis-specific antigen *efaA* (*efaA*) are linked to pathogenicity and increase the virulence of enterococci (HAMMAD et al., 2014; GUERRERO-RAMOS et al., 2016). *Enterococcus faecalis* and *Enterococcus faecium* are accountable for most enterococcal infections (WANG et al., 2016). Previously, a few studies investigated the molecular characterizations of antimicrobial resistance and virulent factors of enterococci from bovine mastitis in China (GAO et al., 2019; YANG et al., 2019). However, little information is available for characterizations of *E. faecium* in Ningxia Province. The aim of this study is to report the characterization of the antimicrobial resistance and virulent traits of *E. faecium* isolated from clinical bovine mastitis cases in Ningxia, China.

Materials and methods

Sample collection and E. faecium identification.

Milk samples of cows (n=153) were collected from 12 commercial dairy farms in Ningxia Province, China from April 2018 to June 2019. Before sampling, the first streams of milk were discarded, and the teat ends were disinfected with cotton swabs soaked in 70% alcohol and allowed to dry. Then a 5 mL secretion was collected into a sterile 10 mL tube. Any samples that were not processed immediately were kept at 4 °C for microbiological

examination within 18 h of collection (PITKÄLÄ et al., 2004). *E. faecium* was presumptively identified on the basis of morphological characterization and biochemical testing, and then confirmed by PCR, as previously described (JAHANSEPAS et al., 2018).

Antimicrobial susceptibility testing. The antimicrobial susceptibility of all *E. faecium* samples against 10 antimicrobial agents was evaluated by the disc diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2015). The assay covered a panel of the following antimicrobials (Oxoid, United Kingdom): penicillin (10 units), ampicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), quinupristin/dalfopristin (15 µg), and linezolid (30 µg). *Staphylococcus aureus* ATCC 25923 was used as a quality control reference strain. Multiple drug resistance was defined as resistance to at least one antibiotic in three or more antimicrobial categories (MAGIORAKOS et al., 2012).

Detection of resistance and virulence genes. Bacterial DNA Kit (Omega Bio-Tek, USA) was used to extract bacterial DNA according to the manufacturer's instructions. The PCR assays were performed for erythromycin-resistant genes *ermA*, *ermB*, and *ermC* and tetracycline-resistant genes *tetK*, *tetL*, *tetM*, *tetO*, and *tetS*, as well as for virulence genes *gelE*, *hyl*, *agg*, *esp*, *cpd*, *frs*, *ace*, and *efaA* according to a previous study (GUERRERO-RAMOS et al., 2016).

Results

Antimicrobial susceptibility. The isolates exhibited high resistance to tetracycline (78.1%) and erythromycin (68.8%), followed by penicillin (46.9%), ciprofloxacin (34.4%), ampicillin (37.5%), nitrofurantoin (12.5%), chloramphenicol (3.1%), and quinupristin/dalfopristin (3.1%). Multiple drug resistance was observed in 16 of 32 (50.0%) isolates. However, none of the isolates was resistant against vancomycin and linezolid. In addition, 6 isolates were susceptible to all antimicrobials tested.

Resistance and virulence genes. The characterization of antimicrobial resistance and virulent genes in 32 *E. faecium* is shown in Table 1.

Table 1. Antimicrobial resistance and virulence traits of *E. faecium* from clinical bovine mastitis

Antimicrobial resistance pattern ^a	Antimicrobial resistance genes										Virulence genes								No. ^b
	ermA	ermB	ermC	ermK	tefL	tetM	tetO	tetS	gelE	hyl	agg	esp	cpd	frs	ace	efaA			
PEN, AMP, ERY, TET, CIP	+	+	+	+	+	+	-	-	+	-	+	-	+	-	-	+	4		
PEN, AMP, ERY, TET, CIP	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	3		
PEN, AMP, ERY, TET, CIP	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-	+	1		
PEN, ERY, TET, CIP, NIT	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	1		
ERY, TET, CIP, CHL, QDA	+	+	+	+	+	+	-	-	+	-	+	+	+	-	+	+	1		
PEN, AMP, ERY, TET	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	1		
PEN, AMP, ERY, TET	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	3		
PEN, ERY, TET, CIP	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	1		
ERY, TET, NIT	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	1		
PEN, TET	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	1		
ERY, TET	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	5		
TET, NIT	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	2		
ERY	+	+	+	+	+	+	-	-	+	-	+	-	+	+	-	+	1		
TET	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	+	1		
N	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	3		
N	+	+	+	+	+	+	-	-	+	-	+	+	+	-	-	+	2		
N	+	+	+	+	+	+	-	-	+	-	+	-	+	-	-	+	1		

^a PEN, penicillin; AMP, ampicillin; ERY, erythromycin; TET, tetracycline; CIP, ciprofloxacin; NIT, nitrofurantoin; CHL, chloramphenicol; QDA, quinupristin/dalfopristin; N, No resistance.

^b No., number of isolates.

All isolates carried the erythromycin-resistant genes *ermA*, *ermB* and *ermC*, as well as the tetracycline-resistant genes *tetK*, *tetL* and *tetM*. However, *tetO* and *tetS* were not observed in any of the isolates. In addition, All the *E. faecium* isolates in this study carried more than 3 of the tested virulence genes. The *agg*, *cpd* and *efaA* genes were determined in all the isolates, followed by *gelE* (93.4%), *esp* (75.0%), *frs* (15.6%), *hyl* (12.5%), and *ace* (3.1%).

Discussion

Antimicrobial resistance develops at a high frequency in bacteria in the food animal production environment (HAYES et al., 2004). *E. faecium* presents serious clinical challenges due to increasing resistance against antimicrobials (BOURDON et al., 2011), which is considered as a potential risk to humans (RÓŻAŃSKA et al., 2019). In our study, the most frequent antimicrobial resistance was to tetracycline and erythromycin, followed by penicillin, ciprofloxacin, ampicillin, nitrofurantoin, chloramphenicol, and quinupristin/dalfopristin. Our results are in accordance with previous studies showing that *E. faecium* of food or animal origin exhibited high resistance against tetracycline and erythromycin (DIARRA et al., 2010; GOUSIA et al., 2015; DE JONG et al., 2018). The high phenotypic resistance to tetracycline and erythromycin could be due to the indiscriminate use of these antimicrobials in livestock production (BEUKERS et al., 2017). Moreover, we observed that 50% of the isolates were resistant to both tetracycline and erythromycin. HIDANO et al. (2015) demonstrated that tetracycline resistance was usually accompanied by erythromycin resistance in enterococci. Similarly, NOWAKIEWICZ et al. (2017) reported that 66% of *Enterococcus* isolates from poultry were resistant to both tetracycline and erythromycin.

Food-producing animals are not always a source of enterococci for humans, but they may cause resistance gene transmission to humans (ARIASE and MURRAY, 2012). The *ermB* gene and *tetM* gene alone, or in combination with *tetL*, were the most common genes encoding resistance of erythromycin and tetracycline in enterococci,

respectively (MUS et al., 2017). As expected, our results showed that all isolates resistant to erythromycin and tetracycline carried the corresponding resistance genes *ermB* and *tetM* in combination with *tetL*. However, a high prevalence of *ermA* and *ermC* conferring erythromycin resistance and *tetK* conferring tetracycline resistance were observed in this study, which is consistent with previous studies reporting that these genes were rare in enterococci. This disparity may be attributable to different sample sizes and study locations. Moreover, 6 isolates containing resistance genes to erythromycin and tetracycline were susceptible to both of the antimicrobials. This may be due to the lack of these resistant genes' expression (HAMMAD et al., 2014). Furthermore, *tetO* and *tetS* were not observed in any of the isolates. These findings agree with other study reports that *tetO* gene is frequently found among isolates from broilers and *tetS* from humans (AARESTRUP et al., 2000).

Virulence factors are widely distributed among enterococci, and highly related to colonization and pathogenesis (MANNU et al., 2003). In our study, the *agg*, *cpd*, *efaA*, *gelE* and *esp* genes were the most predominant virulence factors observed in *E. faecium* isolates. These virulence genes were frequently detected in *E. faecium* isolated from various sources (TOGAY et al., 2010; IWERIEBOR et al., 2015). The *agg* gene is associated with adherence to eukaryotic cells, and frequency of aggregation and conjugation. The presence of *cpd* and *gelE* are involved in sex pheromone and toxin production, respectively. Furthermore, *efaA* and *esp* are reported to be associated with colonization and the persistence of enterococci infections (CHAJECKA-WIERZCHOWSKA et al., 2016). The high occurrence of these virulence genes in this study indicates their potential role in the pathogenicity of *E. faecium* in subclinical bovine mastitis in Ningxia, China.

In conclusion, our study revealed high phenotypic and genotypic resistance against erythromycin and tetracycline in *E. faecium* strains. These data suggest that particular attention should be paid to making appropriate antimicrobial choices for mastitis treatment, to prevent further

dissemination of the resistance genes. In addition, the high occurrence of *agg*, *cpd*, *efaA*, *gelE* and *esp* reveals that these virulence factors are highly associated with subclinical bovine mastitis in Ningxia Province, China.

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SAŽETAK

Istraživanje je provedeno kako bi se odredila antimikrobna rezistencija i svojstva virulencije izolata bakterije *Enterococcus faecium* uzetih u goveda s kliničkim mastitisom. U ukupno 32 izolata goveda iz pokrajine Ningxia u Kini, procijenjena je antimikrobna osjetljivost na temelju morfološke karakterizacije i biokemijskih pretraga. Geni virulencije izolata istraženi su polimeraznom lančanom reakcijom (PCR). Disk-difuzijski test je u izolatu bakterije *E. faecium* pokazao visoku pojavnost rezistencije na tetraciklin (78,1 %) i eritromicin (68,8 %). Svi su pretraženi izolati bili osjetljivi na linezolid i vankomicin i imali gene rezistentne na eritromicin *ermA*, *ermB* i *ermC*, kao i na tetraciklin, *tetK*, *tetL* i *tetM*. Osim toga svi izolati *E. faecium* nosili su više od tri istraživana gena virulencije. Najčešći geni bili *agg* (100 %), *cpd* (100 %), *efaA* (100 %), *gelE* (93,4 %) i *esp* (75,0 %). Ovi rezultati mogu u pokrajini Ningxia u Kini pridonijeti pravilnom izboru antimikrobnog lijeka i razvoju uspješne terapije za supklinički goveđi mastitis uzrokovan bakterijom *E. faecium*.

Ključne riječi: goveđi mastitis; *Enterococcus faecium*; antimikrobna rezistencija; geni virulencije
