Short communication: Antimicrobial resistance and virulence genes of *Enterococcus faecalis* isolated from subclinical bovine mastitis cases in China

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ABSTRACT

This study aimed to investigate the antimicrobial resistance and virulence genes of *Enterococcus faecalis* isolated from subclinical bovine mastitis cases in China. *Enterococcus faecalis* isolates were identified by 16S rRNA amplification and sequencing. Antimicrobial susceptibility was determined by the disc diffusion method. Antimicrobial resistance and virulence genes were tested by PCR. Overall, *E. faecalis* was recovered from 81 of 1,787 (4.5%) mastitic milk samples. The isolates showed high resistance against tetracycline (87.7%) and erythromycin (79.0%). The most prevalent resistance genes found in the *E. faecalis* were *tetK* (96.3%), *tetL* (79.0%), and *tetM* (87.7%) for tetracycline and *ermC* (97.5%) for erythromycin. Moreover, *gelE* (70.4%), *esp* (85.2%), *efaA* (91.4%) were the most common virulence genes. This is the first report to characterize *E. faecalis* recovered from subclinical bovine mastitis cases in China.

Key words: *Enterococcus faecalis*, bovine mastitis, antimicrobial resistance, virulence genes

Short Communication

Bovine mastitis is the most common and costly disease affecting dairy cattle throughout the world (Yang et al., 2016). Although mastitis control programs such as improved milking hygiene have led to considerable progress in controlling contagious mastitis pathogens, these procedures are less effective against environmental pathogens such as *Enterococcus faecalis*, which represents a major environmental mastitis-causing pathogen (Oliver and Pighetti, 2002; Elhadidy and Elsayyad, 2013).

Antimicrobials are commonly implemented for mastitis prevention and control. Unfortunately, the therapeutic result is limited due to the antimicrobial resistance of pathogens (Saini et al., 2012). Recently, phenotypic and genotypic resistance to several antimicrobials, including erythromycin (*ermA*, *ermB*, and *ermC*) and tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, and *tetS*), have been widely documented in *E. faecalis* isolated from food-producing animals (Diarra et al., 2010; Šeputienė et al., 2012; Klibi et al., 2015). Moreover, several virulence factors have been described in *E. faecalis*, including gelatinase, hyaluronidase, aggregation substance, enterococcal surface protein, cytolysin, endocarditis-specific antigen, and collagen-binding cell wall protein (Martín-Platero et al., 2009; Perin et al., 2014). However, little is known about the *E. faecalis* isolates from bovine mastitis cases. The purpose of the current study was to investigate the antimicrobial resistance and virulence genes of *E. faecalis* isolated from subclinical bovine mastitis in China.

Mastitic milk samples of cows ($n = 1787$) were collected from 62 commercial farms located in 15 provinces in China from April 2015 to November 2017 (Figure 1). Subclinical bovine mastitis was confirmed by the California mastitis test. After delivery to the laboratory, milk samples were inoculated onto 5% sheep blood agar plates and cultivated at 37°C for 24 to 48 h. Presumptive colonies based on morphology and Gram strain were further identified by PCR and sequencing. Briefly, the bacterial genomic DNA was extracted using the Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's recommendation (<http://omegabiotek.com/store/product/bacterial-dna-kit/>). The 16S rRNA gene was amplified by PCR with the Bacterial 16S rDNA PCR Kit Fast (800; Takara, Shiga, Japan) in accordance with the manufacturer's instructions (http://catalog.takara-bio.co.jp/PDFS/rr182a_j.pdf). Subsequently, DNA sequence data for the first 500 bp of the 16S rRNA amplified gene were identified by the GenBank database comparison using the

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Figure 1. Sampling regions (provinces in China) involved in this study.

BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences with 98 to 100% identity to sequences deposited in public domain databases were considered to be positive identification.

Antimicrobial susceptibility was performed by disc diffusion method on Mueller-Hinton agar (Oxoid, Basingstoke, Hampshire, United Kingdom) according to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2013). The panel of antimicrobial agents (Oxoid) included penicillin (10 U), vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), rifampicin (5 µg), chloramphenicol (30 µg), quinupristin/dalfopristin (15 µg), and linezolid (30 µg). *Staphylococcus aureus* ATCC 25923 was used as quality control strain. Multidrug-resistant *E. faecalis* was defined as an isolate that was resistant to at least 3 antimicrobial agents belonging to different antimicrobial classes (Magiorakos et al., 2012).

Resistance genes of erythromycin (*ermA*, *ermB*, and *ermC*) and tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, and *tetS*), as well as virulence genes *gelE* (gelatinase), *hyl* (hyaluronidase), *asaI* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (en-

docarditis-specific antigen), and *ace* (collagen binding cell wall protein) were detected by PCR amplification with specific primers as described previously (Petersen and Dalsgaard, 2003; Amaral et al., 2017). Overall, 81 (4.5%) *E. faecalis* isolates were detected from 1,787 mastitic milk samples. The antimicrobial susceptibility of the isolates against 10 antimicrobial agents is shown in Table 1. The *E. faecalis* isolates showed resistance against erythromycin (79.0%), tetracycline (87.7%), quinupristin/dalfopristin (23.5%), rifampicin (18.5%), chloramphenicol (14.8%), and ciprofloxacin (11.1%). Multidrug resistance was found in 15 (18.5%) isolates. However, all the isolates were susceptible to vancomycin, nitrofurantoin, and linezolid. Moreover, 4 isolates were susceptible to all tested antimicrobial agents.

The most common antimicrobial resistance found in our study was to tetracycline and erythromycin. Thus, the resistance-associated genes of tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, and *tetS*) and erythromycin (*ermA*, *ermB*, and *ermC*) were detected and results are summarized in Table 1. Genes *tetK*, *tetL*, *tetM*, and *tetS* were found in 78 (96.3%), 64 (79.0%), 71 (87.7%), and 14 (17.3%) of the *E. faecalis* isolates, respectively. Likewise, 21 (25.9%) and 79 (97.5%) of the isolates

Antimicrobial resistance pattern¹[illegible]

ERY = erythromycin; TET = tetracycline; CIP = ciprofloxacin; RIF = rifampicin; CHL = chloramphenicol; QDA = quinupristin/dalfopristin; N = no resistance.

contained *ermB* and *ermC*, respectively. Notably, all 71 (87.7%) tetracycline-resistant *E. faecalis* isolates carried resistance genes *tetK* (97.2%), *tetL* (85.9%), *tetM* (97.2%), and *tetS* (19.7%) alone or in combination. Moreover, out of the 64 (79.0%) erythromycin-resistant isolates, 18 (28.1%) and 62 (96.9%) of the isolates harbored *ermB* and *ermC* alone or in combination. However, *ermA* and *tetO* were not found in any of the isolates.

We also detected the virulence genes of the *E. faecalis* isolates (Table 1). The results showed that the *gelE*, *hyl*, *asa*, *esp*, *efaA*, and *ace* were detected in 57 (70.4%), 2 (2.5%), 20 (24.7%), 69 (85.2%), 74 (91.4%), and 16 (19.8%) of the isolates, respectively. However, *cylA* was not observed in any of the isolates. Moreover, 77 (95.1%) of the isolates carried at least 2 virulence genes. Four isolates were negative for all tested virulence genes.

Enterococcus faecalis is an important environmental pathogen causing bovine mastitis. In this study, *E. faecalis* was detected in 4.5% of the mastitic milk samples. The occurrence was lower compared with previous studies, which reported the prevalence of *E. faecalis* in bovine mastitis cases to be 20.0% in Belgium (Devriese et al., 1999), 20.9% in Czech Republic (Rysanek et al., 2009), and 19.5% in Egypt (Elhadidy and Elsayyad, 2013), but higher than the 2.2% reported in Korea (Nam et al., 2010) and 0.2% in both Germany (Tenhagen et al., 2006) and the United States (Petersson-Wolfe et al., 2008). Variations in findings may be attributable to geographical discrepancies (Kasimoglu-Dogru et al., 2010).

Although *Enterococcus* spp. have low-level intrinsic resistance to aminoglycosides, lincosamides, and trimethoprim, emergence and prevalence of antimicrobial resistance is closely related to the use of antimicrobial agents (Klibi et al., 2015; Beukers et al., 2017). A variety of antimicrobials is available for therapeutic use in veterinary medicine in China (MOA, 2013). The high resistance against tetracycline and erythromycin found in our study could be due to long-term and widespread use of these antimicrobials on dairy farms. Previous studies have also reported high tetracycline and erythromycin resistance in *E. faecalis* isolated from food-producing animals (Diarra et al., 2010; Šeputienė et al., 2012).

Genetic determinants that may enhance the virulence of *E. faecalis* include antimicrobial resistance genes and a variety of virulence factors (Tejedor Junco et al., 2015). The antimicrobial resistance genes and virulence factors can be transferred to other bacteria, which pose a serious threat to public health through the food chain (Aslam et al., 2012; Iweriebor et al., 2015). In our study, the most common resistance genes found in

E. faecalis were *tetK*, *tetL*, and *tetM* for tetracycline and *ermC* for erythromycin. Similarly, tetracycline genes were frequently found in tetracycline-resistant *E. faecalis* isolates from animals (Petersen and Dalsgaard, 2003; Diarra et al., 2010; Klibi et al., 2015). However, previous studies revealed that *ermB* was the most frequent gene found in enterococci isolated from human and animals (Petersen and Dalsgaard, 2003). The disparity could be due to the different origins of the erythromycin-resistant *E. faecalis* isolates. Notably, 4 isolates that were susceptible to tetracycline and erythromycin carried at least 1 of the *tet* and *erm* genes. This discrepancy can be attributed to the lack of expression of those resistance genes (Hammad et al., 2014). Moreover, in our study, *gelE*, *esp*, and *efaA* were the predominant virulence genes detected in the *E. faecalis* isolates. These findings agree with other recent studies that reported these virulence genes were frequently observed in *E. faecalis* isolates from different sources (Aslam et al., 2012; Iweriebor et al., 2015; Nateghian et al., 2016). The *gelE* gene is responsible for production of gelatinase, which is a metalloproteinase hydrolyzing casein, hemoglobin, insulin, fibrinogen, collagen, gelatin, as well as various proteins or peptides. The *esp* and *efaA* genes contribute to the colonization and persistence of enterococci in infections (Chajęcka-Wierzchowska et al., 2016). Furthermore, the presence of *esp* gene is reported to be highly associated with biofilm formation in enterococci (Diarra et al., 2010). The high prevalence of *gelE*, *esp*, and *efaA* in our study may indicate their potential virulence of *E. faecalis* in subclinical bovine mastitis in China.

In summary, although the occurrence of *E. faecalis* is relatively low in subclinical bovine mastitis cases in China, high-level phenotypic and genotypic resistance to tetracycline and erythromycin require government attention by way of comprehensive surveillance for the use of antimicrobial agents in dairy cattle. In addition, the frequent prevalence of *gelE*, *esp*, and *efaA* indicates that these virulence genes may play important roles in subclinical bovine mastitis pathogenesis in China.

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