Erythromycin resistance and virulence genes in *Enterococcus faecalis* from swine in China

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**INTRODUCTION**

*Enterococcus* spp. are natural inhabitants of the gastrointestinal tract of humans and animals (Creti et al., 2004; De Marques and Suzart 2004), but can be also found in soil, water, and vegetables (Burgos et al., 2009). The two most important species, *Enterococcus faecium* and *E. faecalis*, are most frequently implicated in human and animal infections (Aakra et al., 2005). *E. faecalis* is an opportunist pathogen known to cause serious infections, such as bacteraemia, septicaemia, urinary tract infections, wound infections, meningitis, and endocarditis (Giacometti et al., 2000; De Marques and Suzart 2004; Hällgren et al., 2009).

Although the virulence determinants of enterococci are largely unknown, the presence of gene encoding virulence factors, including collagen-binding protein (*ace*), aggregation substance (*asa1*), cytolysin (*cylA*), endocarditis antigen (*efaA*), enterococcal surface protein (*esp*), gelatinase (*gelE*), and hyaluronidase (*hyl*), have been analysed (Dupre et al., 2003; Creti et al., 2004; Vankerckhoven et al., 2004; Hebert et al., 2007; Billström et al., 2008; Hällgren et al., 2009). Enterococci readily acquire antibiotic resistance determinants, which spread rapidly among pop-
ulations (Aakra et al., 2005). Because of the general use of macrolides, macrolide-resistant Enterococcus spp. has been isolated in humans and animals (Portillo et al., 2000; Jackson et al., 2004; Barbosa et al., 2009). Two major resistance mechanisms that cause macrolide resistance in enterococci isolates are target modification due to the ribosomal methylase encoded by \( \text{erm} \) genes (MLS\(_B\) phenotype) and an efflux pump system mediated by the membrane-bound efflux protein encoded by \( \text{mef}(A/E) \) and \( \text{msr} \) genes (M phenotype) (Sutcliffe et al., 1996; Portillo et al., 2000; Singh et al., 2001; Schwaiger and Bauer 2008).

Although much has been learned about the epidemiology of nosocomial enterococci infections, the distribution of virulence genes in Enterococcus sp. of food animals in China is poorly documented. Macrolides, especially tylosin, are used in swine as therapeutic and prophylactic agents to treat bacterial infections or as growth promotants. Antimicrobial resistance has increased among isolates of animal origin on pig farms in recent years (Chen et al., 2007; Guo-Bao et al., 2009). Therefore, the objective of this study is to describe the erythromycin resistance phenotypes and genotypes, and the prevalence of virulence genes of \( \text{E. faecalis} \) isolated from swine in China.

**MATERIALS AND METHODS**

**Bacteria isolation and identification**

Samples, including lungs, lymph nodes, livers, hearts, spleens, kidneys, intestines, and blood from pigs with clinical signs of digestive and respiratory disorders, taken between 2007 and 2009, were used for the isolation of \( \text{E. faecalis} \). A total of 502 pigs were sampled from 81 different pig farms in China. Bacteria were isolated as previously described (Jackson et al., 2004) and routinely grown in trypticase soy broth or agar at 37°C. They were purified by standard methods and identified to the species level by the conventional biochemical identification scheme of De Marques and Suzart (2004). In addition, the 16S rDNA gene was amplified (Hong-Zhou et al., 2002), and the amplicons were sequenced by Shanghai Sangon Bioengineering Co., Ltd. The nucleotide sequences were analysed using the BLAST algorithm available from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Confirmed isolates were stored in trypticase soy broth containing 20% glycerol at -80°C until further characterisation could be performed.

**Antimicrobial susceptibility testing**

The minimum inhibitory concentrations (MICs) of ciprofloxacin, clindamycin, tylosin, kitasamycin, and erythromycin were determined using the broth microdilution method, and susceptibility to vancomycin was performed according to the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI 2009). The concentration range used to determine the MICs of all antibiotics was 0.06-128 \( \mu \text{g/ml} \). Staphylococcus aureus ATCC 25923 and \( \text{E. faecalis} \) ATCC 29212 were used for quality control.

**Detection of virulence and resistance genes**

Template DNA was prepared, as previously described (Vankerckhoven et al., 2004). Five microlitres of templates were used in amplification reactions, with seven oligonucleotide primers for \( \text{ace} \) (Creti et al., 2004), \( \text{asaI} \) (Billström et al., 2008), \( \text{cyl}A \) (Creti et al., 2004), \( \text{efaA} \) (Dupre et al., 2003), \( \text{esp}, \text{gelE}, \) and \( \text{hyl} \) (Billström et al., 2008), as previously described. The amplified PCR products of the virulence genes were analysed on 2% (w/v) agarose gels. To detect the erythromycin resistance genes, the DNA was amplified with primers specific for \( \text{erm}(A) \), \( \text{erm}(B) \), \( \text{erm}(C) \), \( \text{mef}(A/E) \) (Sutcliffe et al., 1996), and \( \text{msr}(C) \) (Sutcliffe et al., 1996; Singh et al., 2001) genes. The amplified PCR products of these genes were analysed on 1% (w/v) agarose gels.

The positive and negative controls for amplification were \( \text{E. faecalis} \) clinical isolates, which were confirmed by PCR and sequencing. The nucleotide sequences were aligned with ClustalX 2.0 and analysed using the BLAST algorithm. All tests were repeated three times, in parallel with the positive and negative controls. The 12 oligonucleotide primer pairs used to amplify both the virulence and resistance genes are shown in Table 1.

**Statistical analysis**

The correlation between the occurrence of antibiotic-resistant and virulence genes was calculated using the 2 or Fisher’s exact test. The tests
were performed using the SPSS statistical package. A p-value of <0.05 was considered significant.

RESULTS

Antimicrobial susceptibility
A total of 117 non-duplicate *E. faecalis* isolates, obtained from 502 pigs from different pig farms in China between 2007 and 2009, were studied, providing an isolation frequency of 23.31%. Table 2 shows the MICs of six antimicrobial agents tested against the *E. faecalis* isolates. A high incidence of antibiotic resistance was detected among the *E. faecalis* isolates. The results show that the majority of the *E. faecalis* isolates were resistant to erythromycin (66.67%; n=78), tylosin (66.67%; n=78), and ciprofloxacin (64.10%; n=75) (Table 2). No vancomycin-resistant enterococci were isolated. Unexpectedly, erythromycin-resistant isolates showed high-level resistance to the three macrolides, erythromycin, kitasamycin, and tylosin. Among 78 erythromycin-resistant isolates, 75 were found highly resistant (MIC, ≥126 μg/ml) to tylosin, which is only used in animals.

Distribution of virulence genes
The *E. faecalis* isolates were tested for the presence of seven virulence factors. The cylA and hyl genes were not detected in any of the isolates. The frequency of the other five virulence genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>erm(A)</td>
<td>Ribosomal methylase</td>
<td>TCTAAAAAGCATGTAAAAAGA CTTCGATAGTTTTATTATATTAGT</td>
<td>645</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>erm(B)</td>
<td>Ribosomal methylase</td>
<td>GAAAGTTACTCAACACAAATA AGTAACGCATCCAAATTGTTTAC</td>
<td>639</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>erm(C)</td>
<td>Ribosomal methylase</td>
<td>TCAAAACATAATATAGATAAA GCTATATGTGTTTTAATCGTCAAT</td>
<td>642</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>mef(A/E)</td>
<td>Efflux protein</td>
<td>AGTATCATTATACATAGTGTC TTCTGTTGATAAATTTG</td>
<td>348</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>msr(C)</td>
<td>Efflux protein</td>
<td>GCAAATGTGTTAGGTAAGCAACT ATCAGTGAAGTAAACAAAT</td>
<td>399</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td>ace</td>
<td>Collagen-binding protein</td>
<td>GGAATGACCGAAGACGTGCG GCTTGTGTGGCGCTGCTCCG</td>
<td>616</td>
<td>Creti et al., 2004</td>
</tr>
<tr>
<td>asa1</td>
<td>Aggregation substance</td>
<td>CACGCTATAGCACTAGTA TAAGAAAGACATACACCAGA</td>
<td>375</td>
<td>Billström et al., 2008</td>
</tr>
<tr>
<td>cylA</td>
<td>Cytolysin</td>
<td>ACTCAGGAAGTATACGCTGCCTGCCGATCCGTCATAG</td>
<td>688</td>
<td>Creti et al., 2004</td>
</tr>
<tr>
<td>efaA</td>
<td>Endocarditis antigen</td>
<td>CGTGAAGAAGAAGATGAGGAGA CTACTAAGAGCTCAGG</td>
<td>499</td>
<td>Dupre et al., 2003</td>
</tr>
<tr>
<td>esp</td>
<td>Enterococcal surface protein</td>
<td>AGATTTCATCTTTGATCTCTGTGA GATTCATATGTCATCG</td>
<td>510</td>
<td>Billström et al., 2008</td>
</tr>
<tr>
<td>gelE</td>
<td>Gelatinase</td>
<td>TATGACAATGGTTGGGTGGGAT AGATGCACCCGAATAATATA</td>
<td>213</td>
<td>Billström et al., 2008</td>
</tr>
<tr>
<td>hyl</td>
<td>Hyaluronidase</td>
<td>ACAGAAGGCTGAGGAATGAGCAGTCACAGTCAAGGTGTTCAATATA</td>
<td>276</td>
<td>Billström et al., 2008</td>
</tr>
</tbody>
</table>
ranged in prevalence from 6.84\% (esp) to 69.23\% (gelE). The gelE gene was the most widespread virulence determinant, found in 69.23\% of the isolates (n=81). The second most frequently occurring virulence gene, ace, was found in 48.72\% of the isolates (n=57). Furthermore, multiple virulence genes co-existed in the *E. faecalis* isolates (Table 3).
TABLE 4 - Distribution of the resistance genotypes in erythromycin-resistant isolates.

<table>
<thead>
<tr>
<th>No. of the isolate</th>
<th>MIC Range (µg/ml)</th>
<th>Erythromycin resistance genotype</th>
<th>No. of virulence genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>erm(A)</strong></td>
<td><strong>erm(B)</strong></td>
</tr>
<tr>
<td>16</td>
<td>≥128</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>64 to ≥128</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>≥128</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8 to 16</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>≥128</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>≥128</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>≥128</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>≥128</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>78</td>
<td>37</td>
</tr>
</tbody>
</table>

* = erythromycin; + = positive.

FIGURE 1 - Incidence of virulence genes in 78 erythromycin-resistant (ER) and 39 erythromycin-susceptible (ES) E. faecalis isolates.
Distribution of erythromycin resistance genes

All the 78 erythromycin-resistant isolates were tested for the presence of \(\text{erm}(A)\), \(\text{erm}(B)\), \(\text{erm}(C)\), \(\text{mef}(A/E)\), and \(\text{msr}(C)\) genes. The distribution of erythromycin resistance genes according to the phenotypes of macrolide resistance is presented in Table 4. Among the erythromycin-resistant isolates, \(\text{erm}(B)\) (n=54) was the most prevalent resistance gene, followed by \(\text{erm}(A)\) (n=37). Overall, 34 isolates had \(\text{erm}(B)\), 20 carried both \(\text{erm}(A)\) and \(\text{erm}(B)\), and 16 had the \(\text{erm}(A)\) genes. Only three isolates had \(\text{msr}(C)\), two had both \(\text{erm}(B)\) and \(\text{erm}(C)\), one carried \(\text{erm}(C)\), one had both \(\text{erm}(B)\) and \(\text{mef}(A/E)\), and one carried the \(\text{erm}(A)\), \(\text{erm}(B)\), and \(\text{msr}(C)\) genes.

Correlation between erythromycin and virulence resistance genes

Of all the 81 \(gelE\)-positive isolates, the majority (n=75) were resistant to erythromycin (Table 4). However, only three isolates were resistant to erythromycin among the 36 \(gelE\)-negative isolates.

Of all the 78 erythromycin-resistant isolates, 42.32% (n=33) harboured the \(gelE\) gene separately. 34.62% (n=27) carried both \(gelE\) and \(ace\) genes. 3.42% (n=4) had the \(gelE\), \(ace\), and \(asa1\) genes. 3.42% (n=4) harboured \(gelE\), \(ace\), and \(efaA\), 3.42% (n=4) carried \(gelE\) and \(efaA\), 1.72% (n=2) had \(gelE\) and \(asa1\), and 0.85% (n=1) harboured the \(gelE\), \(ace\), \(asa1\), and \(esp\) genes. The three other erythromycin-resistant isolates did not carry the \(gelE\) gene. Nevertheless, in the 39 erythromycin-susceptible isolates, only six isolates harboured the \(gelE\) gene. The correlation between the presence of the \(gelE\) virulence gene and erythromycin resistance was statistically significant. Details of detected virulence determinants in erythromycin-resistant and susceptible isolates are shown in Figure 1.

**DISCUSSION**

Enterococci infections have become increasingly common because of their intrinsic resistance to several antimicrobial agents and their propensity to acquire resistance from the environment (Laewis et al., 2006). Approximately 80% to 90% of all enterococcal infections are attributed to \(E. faecalis\), whereas \(E. faecium\) is responsible for about 5%-10% of these infections (Simjee et al., 2002; Dupre et al., 2003). The zoonotic dissemination of antibiotic-resistant and virulent strains of enterococci has become a significant public health concern. Jensen et al. (1999) reported on \(E. faecium\) strains isolated from pigs and a hospitalised patient in Denmark, suggestive of a food-borne zoonotic route of transmission of VRE. Hong-Zhou et al. (2002) also reported an outbreak of \(E. faecium\) in China, in which thousands of pigs died and 40 farmers were hospitalised due to severe illness after contact with sick pigs, strongly suggesting the spread of a virulent enterococcal strain from pigs to humans.

As shown in Table 2, a high frequency of resistance to ciprofloxacin (64.10%) and erythromycin (66.7%) was observed. The clinical \(E. faecalis\) isolates were also resistant to ciprofloxacin as reported by Hershberger et al. (2005) and Barbosa et al. (2009). Our study shows that \(E. faecalis\) clinical isolates exhibited high resistance to macrolides. The high level of resistance to erythromycin in these isolates is likely related to the wide use of these classes of antibiotics in husbandry activities, especially the widespread use of tylosin for growth promotion and treatment of disease. The same results were obtained by other authors (Jackson et al., 2004; Poeta et al., 2006; Barbosa et al., 2009; Valenzuela et al., 2009).

Of the 78 erythromycin-resistant \(E. faecalis\) clinical isolates, \(\text{erm}(B)\) was the most common resistance gene (n=58) detected. This gene plays a predominant role in the development of high-level resistance to macrolides, lincosamides, and streptogramin B (MLS\(_B\) phenotype) in \(Enterococcus\) spp. (Del Grosso et al., 2007). Out of the 78, 37 erythromycin-resistant \(E. faecalis\) clinical isolates possessed \(\text{erm}(A)\) and displayed higher MICs to erythromycin (≥128 \(\mu\)g/ml). However, there are few reports on the presence of \(\text{erm}(A)\) in \(E. faecalis\) (Schwaiger and Bauer 2008). The \(\text{msr}(C)\) gene encodes a putative efflux pump of the ABC transporter for macrolide and streptogramin B antibiotics (Portillo et al. 2000; Graham et al., 2009). The level of resistance resulting from the expression of the efflux pump is generally lower than that produced by target modification due to the ribosomal methylase encoded by \(\text{erm}\) genes. Accordingly, the strain carrying \(\text{msr}(C)\) showed a low level of resistance.
Hällgren (1996). The E. faecalis isolates were further screened for potential virulence genes. The virulence genes ace, asa1, cyaA, efaA, esp, gelE, and hyl were detected at different levels in E. faecalis. According to previous studies (Creti et al., 2004; Billström et al., 2008), the prevalence of virulence genes in E. faecalis isolated from swine is lower than that reported for human strains. The gelE gene was present in 69.2% of the 117 isolates and was the most widespread virulence determinant. The gelE positive isolates were significantly more frequent among clinical and food animal isolates - a result also in accord with the reports by Dupre et al. (2003), Creti et al. (2004), Martín-Platero et al. (2009), and Belgacem et al. (2010). A relatively high number of isolates carried ace (n=57; 48.72%), also distributed with high frequency in isolates from meat (Cariolato et al. 2008; Belgacem, 2010). However, the low prevalence of other virulence genes efa (n=18; 15.38%), esp (n=9; 7.64%), and asa1 (n=8; 6.84%) is in contrast with the data reported by other researchers on clinical and food animal isolates (Creti et al., 2004; De Marques and Suzart 2004; Martin et al., 2005; Hällgren et al., 2009; Martín-Platero et al., 2009; Valenzuela et al., 2009). The cyaA and hyl genes were not detected in all isolates as reported in a previous study on food animal isolates (Martin et al., 2005). In addition, the clustering of virulence genes in E. faecalis was more frequent in erythromycin-resistant than in erythromycin-susceptible isolates. Therefore, our results confirm a different distribution of virulence genes in clinical E. faecalis isolates from swine. In this study, the correlation between erythromycin resistance and the gelE virulence gene was statistically significant - a result that has not been observed in previous studies (Billström et al., 2008; Cariolato et al., 2008). Virulent genes and antimicrobial resistance may be transferred together to food strains, although few foodborne enterococcal infections have been reported (Valenzuela et al., 2009). In conclusion, erythromycin resistance in E. faecalis clinical isolates from swine is associated primarily with the presence of erm(B) and erm(A) genes. Our results also confirm a distribution of virulence genes in clinical E. faecalis isolates from swine in China, gelE gene being the most common virulence determinant. A strong correlation between the presence of the gelE virulence gene and erythromycin resistance was observed. The results from the present study suggest that the studied enterococci from swine should be regarded with caution because they may constitute a reservoir for antimicrobial resistance and virulence genes, and that effective measures should be taken to control antibiotic use in pig farms.

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