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NOTE

Copper Resistance and Its Relationship to Erythromycin Resistance in *Enterococcus* Isolates from Bovine Milk Samples in Korea

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Antibiotic resistance in animal isolates of enterococci is a public health concern, because of the risk of transmission of antibiotic-resistant strains or resistance genes to humans through the food chain. This study investigated copper resistance and its relationship with erythromycin resistance in 245 enterococcal isolates from bovine milk. Phenotypic and genotypic resistance to erythromycin and copper sulfate were investigated. Of the 245 enterococcal isolates, 79.2% (n=194) displayed erythromycin resistance (≥8 μg/ml). Of the erythromycin-resistant isolates, 97.4% (n=189) possessed *erm*(B), 73.7% (n=143) possessed *mef*(A), and 71.6% (n=139) possessed both genes. Of the 245 enterococcal isolates, only 4.5% (n=11) displayed copper resistance (≥28 mM) and the copper resistance gene, *tcr*(B), was detected in seven isolates that all possessed *erm*(B). This study is the first to report the *tcr*(B) gene in enterococci isolated from Korean bovine milk and its relationship to erythromycin resistance.

Keywords: antibiotic resistance, enterococci, bovine milk, erythromycin, copper

Rates of antibiotic resistance are growing in enterococci isolated from patients and dairy products in many countries (Hershberger *et al.*, 2005). The emergence of antimicrobial resistance has attracted worldwide concern in recent decades and there is increased attention to the potential public health impact of antimicrobial use in animal agriculture (WHO, 1997, 2000). This trend is partly attributable to the use of antibiotics for promoting animal growth. However, the contribution of agricultural antimicrobial use to the development and spread of resistance in human pathogens is debatable, and it is still under investigation (Sørensen *et al.*, 2001; Kelly *et al.*, 2004; Lester *et al.*, 2006). Enterococci are a complex, diverse, and important group of bacteria in terms of their interactions with humans. Some strains are used for the manufacture of foods, while others cause serious infectious diseases in humans and animals. Enterococci are normal flora found in the alimentary tracts of humans and other animals, and they are commonly found in soil, water, and food (Devriese *et al.*, 1995). Enterococci are resistant to a wide variety of antibiotics and disinfectants (Aerestrup and Hasman, 2004). This allows enterococci to survive in environments where antibiotics or disinfectants are used and provides an opportunity for the spread of resistant enterococci to human or animals (Murray, 1990; Leclercq, 1997; Barbosa *et al.*, 2009). Resistance can be intrinsic, mediated by genes located on the chromosome (Moellering and Krogstad, 1979), or acquired, mediated by genes residing on plasmids or transposons (Murray, 1990; Kühn *et al.*, 2000). Several reports show that Korean enterococcal isolates have high levels of resistance to several antibiotics, including erythromycin (Kwon *et al.*, 2007; Jeong *et al.*, 2008; Nam *et al.*, 2010). Copper and its derivatives also have antimicrobial activity and they are used as supplements to enhance animal growth. In Korea, copper sulfate is used for growth promotion at a concentration of 100–150 ppm mainly in pigs and poultry, and rarely in cows (Kim *et al.*, 2010). Recent reports show that the incidence of the copper resistance gene *tcr*(B) is linked with the erythromycin resistance gene *erm*(B) and the glycopeptide resistance gene *van*(A) (Hasman and Aarestrup, 2002). This means that copper-resistant strains might be a reservoir of *erm*(B) or *van*(A), and they could spread these resistance genes. We investigated the phenotypic and genotypic resistance profiles of *Enterococcus* species isolated from bovine milk samples against erythromycin and copper. The presence of the copper resistance gene *tcr*(B) in enterococcal isolates in Korea and its relationship to *erm*(B) were also investigated for the first time in this study. A total of 950 bovine milk samples were randomly collected from 15 stock-raising farms located in the northern area of Gyeonggi Province in Korea, between June 2008 and May 2010. Bovine milk samples were cultured to isolate *Enterococcus* species by spreading 0.1 ml of samples on mEnterococcus Medium (Difco, USA). Plates were incubated for 24–48 h at 37°C and presumed enterococcal colonies were picked for further identification. To avoid repetition, only a single colony was selected from each milk sample. Presumptive *Enterococcus* colonies were identified using the Vitek 2 System in the Sekang Medical Laboratory (Korea). Minimum inhibitory concentrations (MICs) were determined by the agar dilution method, according to the procedure of the Clinical and Laboratory Standards Institute

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Table 1. An overview of target genes and PCR primers

Drug	Gene		Sequences (5′-3′)	Tm (°C)	Length (bp)	References
EM[a]	*erm*(A)	F	TCAGTTACTGCTATAGAAATTGATGGAG	58.7	28	Jung *et al.* (2009)
		R	ATACAGAGTCTACACTTGGCTTAGG	58.8	29	
	erm(B)	F	TTGGATATTCACCGAACACTAGGG	59.1	24	
		R	ATAGACAATACTTGCTCATAAGTAACGG	59.2	28	
	erm(C)	F	GACAATTATAAGATTAAATGAACATGATAATATC	56.2	34	
		R	AAACAATTTTGCGTATTATATCCGTAC	56.8	27	
	mef(A)	F	ATTGCAGCTGGTTTACAGGC	57.3	20	Lee *et al.* (2010)
		R	CATGATACAATGCACACGCA	55.3	20	
	msr(A)	F	GCA AATGGTGTAGGTAAGACAACT	57.2	24	
		R	ATCATGTGATGTAAACAAAAT	58.7	21	
	msr(C)	F	TATAACAAACCTGCAAGTTC	59.4	20	
		R	CTTCAATTAGTCGATCCATA	58.3	20	
Cu[b]	*tcr*(B)	F	CATCACGGTAGCTTTAAGGAGATTTTC	58.2	27	Amachwadi *et al.* (2010)
		R	ATAGAGGACTCCGCCACCATTG	58.1	22	

[a] EM, erythromycin; [b] Cu,$CuSO_4 \cdot 5H_2O$

(CLIS, 2009). *Enterococcus faecalis* ATCC 29212 was used for quality control purposes, in susceptibility testing. Appropriate concentrations of erythromycin (Sigma, USA) were added to Mueller-Hinton agar to produce serial dilutions of the antimicrobial agents. Copper resistance was tested on Mueller-Hinton agar plates containing 0, 0.9, 1.75, 3.5, 7, 14, 28, and 56 mM copper sulfate ($CuSO_4$) (Sigma), which was adjusted to pH 7 with 1 M NaOH (Fard *et al.*, 2011). Overnight cultures of enterococcal isolates were diluted to adjust the inoculums size to 0.5 McFarland turbidity standards. Growth was assessed after 16–20 h of incubation at 37°C. Resistance genes were amplified by isolating total DNA from each *Enterococcus* isolate using a GeneAll Cell SV system (GeneAll, Korea). Erythromycin resistance genes *erm*(A), *erm*(B), *erm*(C), *mef*(A), *msr*(A), and *msr*(C), and the copper resistance gene *tcr*(B) were amplified by PCR using the primers listed in Table 1, and identified by electrophoresis. Amplified DNA from each gene was sequenced by Genotech (Korea) and the results were compared with GenBank data. A total of 245 enterococci were isolated from 950 milk samples. The predominant strain were *E. faecalis* (n=199, 81.2%) and *E. faecium* (n=25, 10.2%). *E. avium* (n=7, 2.9%), *E. durans* (n=6, 2.5%), *E. gallinarum* (n=4, 1.6%), and *E. raffinosus* (n=4, 1.6%) were also isolated (Table 2). Most of the enterococcal isolates were resistant to erythromycin. The MIC range for erythromycin was 0.13- >64 μg/ml, while the MIC_{50} and MIC_{90} for erythromycin was >64 μg/ml, with a resistance rate of 79.2% (Table 2). Of the erythromycin-resistant isolates, 97.4% (n=189) possessed *erm*(B), 73.7% (n=143) possessed *mef*(A), and 71.6% (n=139) possessed both genes. Other erythromycin resistance genes were not detected in this 245 isolates. The *erm*(B) gene and *mef*(A) gene were found to be the source of erythromycin resistance. *erm*(B) was present in a high proportion of isolates from all six species, whereas *mef* (A) was not detected in *E. durans*, *E. gallinarum*, and *E. raffinosus*. Resistance rates for erythromycin was 79.2% and these values were similar to those reported in previous studies of enterococci isolated from Korean livestock products, or slightly higher than other reports (Jeong *et al.*, 2008; Nam *et al.*, 2010). The investigation of copper resistance (≥28 mM) found that most enterococcal isolates were sensitive to copper. The MIC range for copper sulfate was 3.5–56 mM, while the MIC_{50} and MIC_{90} for copper sulfate was 14 mM, with a resistance rate of 4.5% (n=11) (Table 2). Seven of the 245 isolates possessed copper resistance gene, *tcr*(B) and all of these isolates also possessed the *erm*(B) gene. The copper resistance rate (4.5%) was much lower than that reported by Haseman and Aarestrup (2002) but slightly higher than that reported by Fard *et al.* (2011). However, this was the first study to focus on enterococcal isolates from bovine milk; previous studies have focused on swine. Feed containing copper sulfate tends to be given to swine or fowl in Korea. The use of copper is not common in Korean cattle feed (Kim *et al.*, 2010). Thus, it could be hypothesized that, compared to swine-derived enterococci, cattle-derived enterococci are exposed to copper compounds on fewer occasions, which makes them less likely to develop copper resistance. The lower rate of copper resistance was supported by a lower incidence of copper resistance *tcr*(B) genes. Five of seven strains with copper resistance genes

Table 2. Identification of Enterococcus spp. and their susceptibility to erythromycin and copper sulfate

Strains	No. of isolates (%)	Phenotypic characteristics								Genotypic characteristics					
		Erythromycin				Copper				Erythromycin		Copper	Cross resistance		
		Range	MIC_{50} (μg/ml)	MIC_{90}	Resistance rate (%)	Range	MIC_{50} (μg/ml)	MIC_{90}	Resistance rate (%)	*erm*(B)	*mef*(A)	*tcr*(B)	*erm*(B)+*mef*(A)	*erm*(B)+*tcr*(B)	*erm*(B)+*mef*(A)+*tcr*(B)
E. faecalis	199 (81.2)	0.13~>64	>64	>64	170 (85.4)	3.5~56	14	14	7 (3.5)	165	135	5	131	5	1
E. faecium	25 (10.2)	3.13~25	>64	>64	15 (60.6)	3.5~28	14	14	2 (8)	15	5	0	5	0	0
E. avium	7 (2.9)	4~>64	4	>64	3 (2.9)	3.5~14	7	14	0 (0)	3	3	0	3	0	0
E. durans	6 (2.5)	2~>64	4	>64	2 (33.3)	14~56	14	14	1 (16.7)	2	0	1	0	1	0
E. gallinarum	4 (1.6)	0.25~>64	>64	>64	3 (75.0)	14~56	14	28	1 (25)	3	0	1	0	1	0
E. raffinosus	4 (1.6)	0.13~32	0.25	32	1 (25.0)	3.5~7	3.5	7	0 (0)	1	0	0	0	0	0
Total	245(100)	0.13~>64	>64	>64	194 (79.2)	3.5~56	14	14	11 (4.5)	189	143	7	139	7	1

were *E. faecalis*, while the other two were *E. durans* and *E. gallinarum*. A recent study conducted in the USA (Amachawadi *et al.*, 2010) reported that all strains possessing *tcr*(B) genes were *E. faecalis* and *E. faecium*. However, the present study also detected *E. gallinarum* and *E. durans*, in addition to *E. faecalis*. This is the first report that *E. durans* has copper resistance *tcr*(B) gene. A number of *tcr*(B) genes were isolated from different species of enterococci in current study, but DNA sequence analysis showed 98% identity with GenBank reference gene [|EU869871.1| *Enterococcus faecium* strain 263 copper resistance protein (*tcr*B), (data not shown), which may suggests gene transfer from *E. faeciums* to other species. Previous studies (Hasman and Aarestrup, 2002) reported *erm*(B) and *van*(A) genes were observed in strains possessing *tcr*(B) genes. However, the current study found that *tcr*(B)-positive strains possessed *erm*(B) genes, but no *van*(A) genes, and they were sensitive to vancomycin (data not shown). The treatment of animals with antibiotics for therapeutic and growth promotion purposes may contribute to the selection of bacteria resistant to antibiotics used by humans, and bacterial resistance genes could spread to humans through the food chain (Witte *et al.*, 1999). The EU banned all subtherapeutic antibiotic growth promoters in animal feeds from late 1990s (Phillips, 2007). The ban is intended to maintain the effectiveness of antibiotics used to treat human infections. Korea has been gradually reducing the 44 types of antibiotics that were permitted as feed additives in 2005, and only 18 antibiotics were allowed as growth promoters in 2009. According to the Ministry of Food, Agriculture, Forestry and Fisheries, the Korea government is planning to strictly enforce and ban the use of antibiotic growth promoters in animal feed from July 2011 (Korea Animal Health Products Association, http://www.kahpa.or.kr/). Korea is now in a period of transition to antibiotic restriction. The primary object of this study is to show the prevalence of copper resistance gene, *tcr*(B) and its relationship to *erm*(B). It is known that some enterococci are resistant to the nutritional mineral copper, and the copper resistance gene *tcr*(B) is reported to be linked with the erythromycin resistance gene *erm*(B) (Hasman and Aarestrup, 2002). Thus, erythromycin resistance genes may be coselected with copper resistance genes when a copper compound is used as a nutrient or antimicrobial, notwithstanding prohibition against adding antibiotics to feed (Hasman *et al.*, 2006). Erythromycin-resistant enterococci are known to be resistant to copper compounds in European countries and the USA (Hasman and Aarestrup, 2002; Amachawadi *et al.*, 2010) but this has not yet been reported in Korea. As in other countries, *tcr*(B) genes were found in some cases and *tcr*(B) was genetically linked to erythromycin resistant determinant *erm*(B) in this study. The importance of this result is the potential association between copper resistance and other antibiotics (erythromycin or vancomycin) and the inclination of enterococci to transfer *tcr*(B) and antibiotic resistant determinants to other strains or other species. In the future, it will necessary to monitor periodically whether mineral supplementation selects for copper resistance and in turn co-selects for erythromycin resistance. In conclusion, results from this study confirm that most of the enterococcal isolates from Korean bovine milk samples displayed high level resistance to erythromycin. Of the enterococcal isolates only 4.5% (n=11) showed copper sulfate resistance but seven copper resistant isolates have transferable copper resistance gene, *tcr*(B). Furthermore this copper resistance gene is genetically linked to erythromycin resistance determinant, *erm*(B). In particular, of the Korean milk isolates of *Enterococcus*, not only *E. faecalis*, but also *E. durans* and *E. gallinarum* carry copper resistance gene, *tcr*(B). This is the first study to address copper resistance in enterococci isolated from bovine milk in Korea, rather than swine.

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