FULL PAPER *Bacteriology*

Prevalence of virulence and resistance to antibiotics in pathogenic enterococci isolated from mastitic cows

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ABSTRACT. The prevalence of enterococci was examined in 280 milk samples collected from dairy cattle diagnosed with mastitis in three provinces of western China. Sixty strains of enterococci were isolated, and the species were determined based on their biochemical characters and 16S rRNA sequences. Resistance to seven antibiotic agents, frequency of seven virulence genes and pathogenicity in *Kunming* mice were tested to evaluate biological risks. The correlation between the number of virulence genes and pathogenicity in *Kunming* mice was also evaluated. The 60 isolates were allocated to *Enterococcus hirae* (68.3%), *E. faecium* (25.0%), *E. mundtii* (3.3%) and *E. durans* (3.3%). A total of 83.3% of the isolates were resistant to penicillin, whereas 15.0% were resistant to ampicillin, 15.0% to vancomycin, 6.7% to tetracycline and 25.0% to ciprofloxacin. Moreover, isolates exhibited 50.0% and 21.7% resistance to high levels of gentamycin and streptomycin, respectively. The gene *asa1* was detected in all enterococcal isolates, whereas 66.7% of strains harbored three or more virulence factors and 56.7% were asa1-ccf-gelE-positive. In pathogenicity tests, isolates harboring numerous virulence factors did not show greater invasiveness than isolates harboring fewer virulence traits against *Kunming* mice. In conclusion, the number of virulence factors does not appear to predict the risk of enterococcal infection. Isolates were commonly resistant to penicillin and sporadically to ampicillin and vancomycin. These results suggest that the use of gentamycin, streptomycin and ciprofloxacin against enterococci should be avoided in mastitic cows. Additionally, the results demonstrate that the majority of isolates are sensitive to tetracycline.

KEY WORDS: antibiotic resistance, pathogenic enterococci, prevalence, virulence

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Multidrug-resistant enterococci have become increasingly common in human and veterinary medicine and are causing a worldwide health crisis. Enterococci are commonly found in animal intestines and their feces [10, 11] and were considered commensal organisms until the late 1970s when antibiotics were widely used in both humans and animals [4]. It is thought that oral administration of antibiotic agents eliminated sensitive enterococci and other commensal bacteria, allowing minority strains that survived to become the dominant flora. The dominance of resistant enterococci facilitated their contact with cow teats, increasing the chance of their entry into milk ducts, particularly in cows reared in captivity. Enterococci surviving mucosal immunity can cause clinical or subclinical mastitis [32]. Enterococcus species gained notoriety, because of their dramatic increase in infectious diseases, such as endocarditis, dental surgical infection and urinary tract infection, as well as for their antibiotic resistance and ability to use novel routes of evasion [38, 39]. These organisms are also been widely present in the food processing industry [20, 25, 29]. Because enterococci from various origins facilitate the evolution of resistance, their survival tactics, virulence profile, antibiotic resistance and multiple avenues of evading antibiotic treatment should be closely examined. The prevalence and mechanisms of antibiotic resistance and virulence profiles on enterococci from human medicine have been widely examined [9, 23, 30, 33], whereas reports on enterococci in animal disease are limited, particularly reports concerning enterococcal dairy cow mastitis in China. How an environmental species of bacteria becomes a mastitis-causing pathogen should be further analyzed. Although production of milk and the diversity of milk products continue to increase, the virulence profile of enterococci and their toxins remain unclear. The control of pathogens during dairy production and processing is very important. In the present study, we investigated the virulence profile, antibiotic patterns and pathogenicity of enterococci isolated from mastitic milk and evaluated their effects on dairy cows and public health.

MATERIALS AND METHODS

Bacterial isolates and growth conditions: A total of 280 mastitic milk samples were collected from dairy cows with clinical or subclinical mastitis on six large farms distributed in Gansu province (161), Qinghai province (30) and Ningxia Hui Autonomous Region (89) between November 2011 and

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Table 1. Primers for virulence gene screening and 16S rDNA sequencing

Virulence factors	Primer (5´-3´)	Product (bp) length
CylA	ATGGATGGGACAGATGGAAA AGCTGCGCTTACTTCTGGAG	519
GelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	419
Ccf	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543
Agg	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	1553
Asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	378
Esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510
Ace	6GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616
16S rDNA	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGACTT	around 1492

May 2012. Briefly, 5–8 m*l* of milk were collected from each mastitic cow (clinically confirmed mastitis or suspected mastitis with positive Lanzhou mastitis test results) by trained workers immediately after teats were cleaned using towels and disinfected with 2% iodine tincture and 75% ethanol. After sampling, the cows were treated according to individual protocols. All samples were maintained on ice and transported to the laboratory within 6 hr.

Biochemical tests: Based on enterococci's unique characteristics, 100 μl of each sample was first applied on a Todd-Hewitt selective plate (THBSP) [14, 22, 34], in which isolates able to hydrolyze esculin appeared as black colonies. Plates were incubated at 37°C for 24–48 hr for bacterial enrichment, and then, microbiological procedures were conducted according to standard guidelines [17, 18]. Briefly, 68 gram-positive strains survived THBSP screening, appearing singly, in pairs or as short chains, and were subjected to cAMP and hemolysis reactions before cultivation on plates containing esculin, hippurate salt, 4% bile salt, 6.5% NaCl, plus sorbitol, lactose, trehalose and inulin. From the previous step, 60 isolates showing negative results in the cAMP test, which survived in 6.5% NaCl broth, were further tested for their utilization of pyruvate, L-arabinose, D-raffinose and survival at 45°C and in glucose broth containing 1% potassium tellurite. All suspected "enterococcal" strains were stored at −70°C in Todd-Hewitt broth containing 15% (v/v) glycerol.

Virulence gene analysis and 16S ribosomal DNA sequencing: From 2.5 m*l* of samples cultivated overnight in Todd-Hewitt broth, genomic DNA was prepared using a commercial DNA isolation kit obtained from Forgene Co., Ltd. (Forgene, Chengdu, China), according to the manufacturer's protocol. The PCR mixture contained 25 μl 2× PCR master mix (Promega, Madison, WI, U.S.A.), 2 μl (400 nM) of each primer, 2 μl DNA template and 21 μl deionized water, and amplification was conducted in a DNA thermal cycler (Bio-Rad, Hercules, CA, U.S.A.).

All suspected enterococcal isolates were screened for their 16S rRNA and seven virulence genes via PCR with the primers shown in Table 1. The seven virulence genes examined encode enterococcal surface protein (*esp*), gelatinase (*gelE*), sex pheromone (*ccf*), cytolysin activator component A (*CylA*), aggregation substance (*asa1*), collagen binding protein (*ace*) and aggregation protein (*agg*). Primers were synthesized as previously described and were as follows: CylA [12], gelE, ccf, agg [13], asa1 and esp [35], ace [6] and 16S rRNA [37]. *Enterococcus faecalis* ATCC 51299 and ATCC 29212 (obtained from Nanjing Bianzhen Biotechnology Ltd., Nanjing, China) were adopted for quality control. Oligo synthesis and PCR product sequencing were conducted by Sangon Co., Ltd. (Sangon, Shanghai, China). Homology of the 16S rRNA and virulence sequences of these isolates were queried on the NCBI nucleotide database using the basic local alignment search tool [24] between September 17, 2014 and September 25, 2014. Enterococci characterization was confirmed according to the biochemical characteristics and 16S rRNA sequences [21, 37].

Pathogenicity test on Kunming *mice*: *Kunming* mice were used for pathogenicity tests. These mice were derived from a pair of Swiss mice from Hoffline Institution in 1944 and show strong disease resistance and adaptability as well as high reproduction and survival rates [40]. Isolates showing positive reactions to ≥3 virulence factors were designated as genetically virulent isolates (GVI), whereas those positive to ≤2 virulence factors were designated as genetically non-virulent isolates (NGVI). As shown in Table 2, five GVI (MS 38, MS 39, MS 42, MS 46 and MS 62) harboring four virulence genes and five NGVI isolates (MS 7, MS 10, MS 14, MS 17 and MS 50) harboring no more than two virulence genes were chosen for pathogenicity tests on *Kunming* mice (detailed information of the virulence screening results are shown in Supplement 1). To confirm the pathogenic, yet non-lethal, dose of overnight enterococcal culture for *Kunming* mice, an overnight culture of an isolate harboring esp-gelE-asa1 (MS 4) was graded as 0.1 m*l*, 0.2 m*l*, 0.3 m*l*, 0.4 m*l* and 0.5 m*l* and inoculated in 15 mouse subjects (three mice in each group). After the

Table 2. Virulence profile of the inoculated isolates

	Esp	*GelE*	*Ccf*	*CylA*	*Asa1*	*Ace*	*Agg*	Virulence count	Identification	
MS7	-	+	-	-	+	-	-	2	*E. hirae*	NGVI
MS10	-	-	-	-	+	-	-	1	*E. hirae*	NGVI
MS14	-	+	-	-	+	-	-	2	*E. hirae*	NGVI
MS17	-	-	-	-	+	-	-	1	*E. hirae*	NGVI
MS50	-	-	+	-	+	-	-	2	*E. faecium*	NGVI
MS38	-	+	+	-	+	+	-	4	*E. hirae*	GVI
MS39	-	+	+	-	+	+	-	4	*E. hirae*	GVI
MS42	-	+	+	+	+	-	-	4	*E. hirae*	GVI
MS46	+	+	-	-	+	+	-	4	*E. durans*	GVI
MS62	+	+	+	-	+	-	-	4	*E. faecium*	GVI

"+++", indicates isolates that were positive to asa1, ccf and gelE. GVI, genetically virulent isolates. NGVI, non-genetically virulent isolates.

pre-experiment, 0.3 m*l* overnight culture from the five GVI and five NGVI groups was inoculated peritoneally into 30 healthy female mice (three mice in each group, all mice were aged 6–8 weeks). Additional three mice were injected with only 0.3 m*l* physiological saline and served as controls. The day of inoculation was defined as day 0, after which the mice were observed for 15 days. All experimental mice were obtained from a guaranteed animal experimental center (Gansu University of Chinese Medicine, Gansu, China) and strictly housed and handled ethically in the Animal Center of Gansu Agriculture University, according to the guidelines of the Animal Ethical and Welfare Committee of the College of Veterinary Medicine at Gansu Agricultural University. After 15 days of incubation, living mice were humanely sacrificed according to the center's guidelines. All livers, hearts and kidneys were anatomically and microbiologically examined; contaminated materials and corpses were handled according to the guidelines of the Animal Ethical and Welfare Committee at Gansu Agricultural University.

Antibiotic resistance tests: Antibiotic resistance tests were performed using the microdilution method according to the National Committee on Clinical Laboratory Standards (NCCLS) [3]. The evaluated antibiotics included penicillin (pen), ampicillin (amp), vancomycin (van), tetracycline (tet), ciprofloxacin (cip), high-level gentamycin (gen) and streptomycin (str). Antibiotics were obtained from Sangon Co., Ltd. (Sangon, Shanghai, China), and all antibiotics were used at United States Pharmacopeia Grade. Supplement 2 shows the explanations of the results, standards and antibiotic gradients. Overnight bacterial culture was adjusted to 0.5 McFarland turbidity with the medium used to dilute the antibiotics, inoculated into gradient antibiotics plates and incubated at 35 ± 2°C for 18–24 hr. The results were assessed as previously described [3], and isolates resistant to ≥3 classes of the antibiotic drugs applied were categorized as multiple-drug-resistant isolates [1].

RESULTS

Biochemical results: Initially, 280 mastitic milk samples were enrolled for this experiment, of which 212 samples were excluded based on the following criteria: (1) gram-negative stained, (2) negative for the THBSP screen, (3) rod bacteria or (4) combined infection. (These excluded strains not described in this study, but subjected to further identification, including 45 *Staphylococcus aureus* strains, 13 *Escherichia coli* strains and nine *Hafnia alvei* strains; data not published.) Eight isolates were identified as *Streptococcus* spp. based on their biochemical characteristics. The other 60 gram-positive cocci that survived THBSP, tested negative in the cAMP test and survived in 6.5% NaCl were initially suspected to be *Enterococcus* spp., but their biochemical characteristics could not be used to identify the isolates at the species level. Thus, 16S rRNA and representative virulence gene sequencing were adopted for further characterization [8, 37].

Table 3. Frequencies of virulence factor of the 60 enterococcal isolates

Species	*Esp*	*GelE*	*Ccf*	*CylA*	*Asa1*	*Ace*	*Agg*
E. hirae	5	31	28	5	41	9	0
E. faecium	2	11	9	0	15	1	0
E. mundtii	0	2	2	0	2	0	0
E. durans	1	1	0	0	2	1	0
Total	8	45	39	5	60	11	0
Positive rate	13.30%	75.00%	65.00%	8.30%	100.00%	18.30%	0

16S rRNA sequencing and virulence factors: In combined biochemical tests, 16S rRNA and virulence gene sequencing confirmed the survival of 60 gram-positive strains under THBSP selection, comprising *Enterococcus hirae* (n=41), *E. faecium* (n=15), *E. mundtii* (n=2) and *E. durans* (n=2). Table 3 shows the six virulence genes detected in each species, whereas Fig. 1 shows the frequency of positive and negative enterococcal isolate ratios to the six virulence genes.

All 60 isolates were positive for asa1, whereas the rates of positive reaction for the other virulence genes were as follows: gelE (45, 75%), ccf (39, 65%), ace (11, 18.3%), esp (8, 13.3%) and CylA (5, 8.3%). Agg was not detected in our isolates. Of the 60 (66.7%) isolates, 40 were GVI [1], and 34 of the GVI were *asa1-gelE-ccf*-positive. The other 20 (33.3%) isolates were NGVI.

Pathogenicity: The health status of GVI- and NGVI-inoculated mice declined rapidly in the 24 hr after inocula-

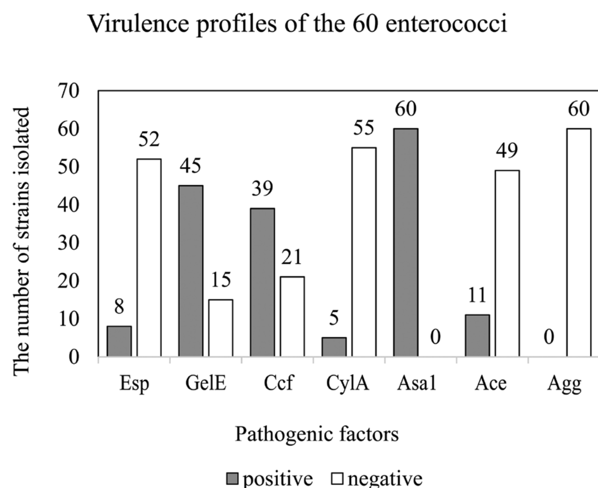

Fig. 1. Virulence patterns of the 60 enterococci isolates from mastitic dairy cows in Gansu province and Ningxia Hui autonomous region. Esp, enterococcus surface protein; gelE, gelatinase; ccf, sex pheromone; CylA, cytolysin activator component A; asa1, aggregation substance; ace, collagen binding protein; agg, aggregation protein.

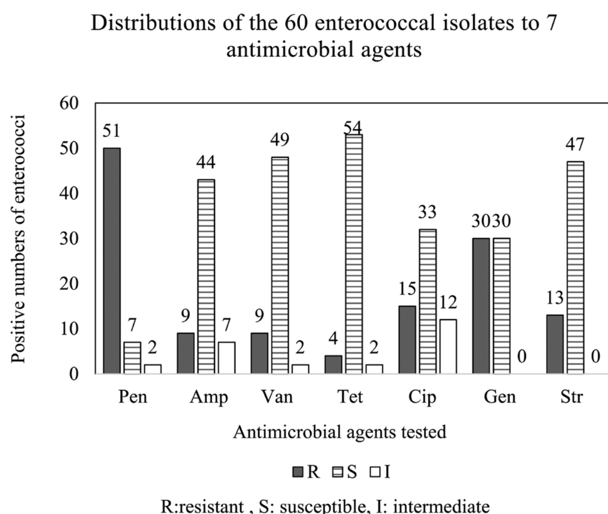

Fig. 2. Frequency of the 60 enterococcal isolates to seven antibiotic agents. Pen: penicillin. Amp: ampicillin. Van: vancomycin. Tet: tetracycline. Cip: ciprofloxacin. Gen: gentamicin. Str: streptomycin.

tion, although most mice gradually recovered in 72 hr. The five mice in the quality control group remained healthy throughout the experiment. The peak of loss among subject mice occurred on days 4 and 5. On day 4, one mouse died in the GVI group, and seven died in the NGVI group. On day 5, one mouse died in the GVI group, and two mice died in the NGVI group. On day 8, one mouse died in the NGVI group. There were no further losses on days 0–3, 6, 7 and 9–14. Under examination, gram-positive cocci of common morphology with the inoculated enterococcal isolates were detected in the blood, kidney and liver samples of the deceased subjects. No combined infections were detected. For the virulence genes, five NGVI were all negative to esp, ace and agg (Table 2). Isolates in the GVI group harbored all virulence factors that were positive in the NGVI isolates (asa1, gelE and ccf), but GVI did not cause a greater loss of *Kunming* mice than NGVI. In contrast, NGVI caused a greater loss of *Kunming* mice than GVI. However, the clinical signs and pathological changes of the two groups were similar.

Antibiotic resistance tests: Antibiotic results were evaluated according to the NCCLS [3]. As shown in Fig. 2 and Table 4, the most prevalent resistance was to β-lactams, with 51 of 60 (85.0%) isolates resistant to penicillin and nine of 60 (15.0%) to ampicillin. Considerable resistance to glycosides was also observed, with nine of 60 (15.0%) isolates resistant to vancomycin, which is considered a drug of last resort for enterococcal infection. Among the isolates, 15 of 60 (25.0%) were resistant to ciprofloxacin in the quinolone class, and four of 60 (6.7%) isolates were resistant to tetracycline. For aminoglycosides, ten isolates were resistant to both high-level gentamycin and streptomycin. In addition, 30 (50.0%) and 13 (21.7%) of 60 isolates were respectively resistant to

Table 4. Resistance of the four enterococcal species (n=60) to seven antibiotic agents

	E. hirae		*E. faecium*		*E. mundtii*		*E. durans*		Total R
	n=45		n=15		n=2		n=2		
	I	R	I	R	I	R	I	R	
Pen	2	38	0	11	0	1	0	1	51 (85%)
Amp	6	6	1	2	0	0	0	1	9 (15%)
Van	2	7	0	2	0	0	0	0	9 (15%)
Tet	2	3	0	1	0	0	0	0	4 (6.7%)
Cip	10	11	2	3	0	0	0	1	15 (25%)
Gen[a)]	0	25	0	4	0	0	0	1	30 (50%)
Str[b)]	0	12	0	1	0	0	0	0	13 (21.7%)

a) high-level gentamycin resistance (500 μg/m*l*). b) high-level streptomycin resistance (1,000 μg/ m*l*).

gentamycin and streptomycin. In analysis of antibiotic, the susceptibility results revealed 17 multiple-drug-resistant isolates.

DISCUSSIONS

In human clinics, *E. faecalis* is the most commonly reported species and is present in many mammals at similar frequency [19]. However, *E. hirae* has rarely been reported as one of the main bacterial species in clinical dairy cow mastitis or contaminated milk products. How this species evolved as to become a dominant pathogen causing bovine mastitis has remained unclear.

Asa1 and *ccf*, both coded by sex-pheromone plasmid pAD1, were originally reported early only in connection with *E. faecalis* [15], whereas later studies confirmed that *E. faecium* also harbors the *asa1* sequence [26, 35], suggesting that pAD1 and the sex pheromone-plasmid-exchanging

system is not an exclusive trait of *E. faecalis*; other enterococcal species may also function in this manner. The g*elE* gene is necessary, but not sufficient for gelatinase activity. Gelatinase activity was reported to be co-controlled by the genes *gelE* and *fsr*, and a lack of *fsr* was inadequate to produce gelatinase [16, 28, 36]. Additionally, the high level of gelatinase detected in milk samples in cows with mastitis supports the hypothesis that gelatinase promotes *Enterococcus* survival and invasion in host tissues by facilitating *Enterococcus* migration. Because 40 of the 60 enterococcal isolates harbor more than three virulence genes, we propose that multiple virulence properties confer enterococci with greater survival probability against the innate bovine immune system.

The seven virulence genes tested in this study were reported to contribute to enterococci virulence, and the NGVI were positive for esp (0), gelE (2), ccf (1), cylA (0) and ace (0). The pathogenicity to *Kunming* mice showed that the NGVI still caused a greater loss of *Kunming* mice than the GVI, which were positive for esp (2), gelE (4), ccf (4), cylA (1) and ace (3). Asa1 was positive in all in these isolates, whereas agg was negative in all isolates. The described virulence factors are insufficient for determining the prognosis of an enterococcal infection in our mouse model, suggesting that unknown mechanisms or virulence factors participated in their pathogenicity in *Kunming* mice. No obvious differences were found in hemolysis properties; there was one strain with β-hemolysis and two stains with α and γ in both the GVI and NGVI, respectively. However, these enterococcal strains were all isolated from mastitic cows, indicating that the strains had become well adapted to survive in the mammary tissue of dairy cows. The lethal effect in mice appeared to occur through different mechanisms.

Enterococcal isolates in this study showed a high frequency of resistance to penicillin, as well as a relatively high ratio of resistance to large doses of gentamycin and streptomycin. Resistance to penicillin (85.0%) was greater than 64.8% (68/105), vancomycin resistance was 15.0% to 0, ampicillin 15.0% to 0.9% (1/105), and high-level gentamycin resistance was 50.0 to 30.5% (32/105) as reported by Cortés, *et al.* [5]. Notably, vancomycin had never been used at the source farms, yet resistant strains were detected. Vancomycin-resistant enterococci were also reported in dust from pig breeding facilities (15%, 26/171) [36], wild Eurasian otter (17.2%, 5/29) [37], and poultry feed and feed ingredients (1.9%, 8/414) [7]. These results agree with our findings. Resistance to tetracycline was 6.7% (4/60), which was lower than that reported by Cortés *et al.* and da Costa *et al.*, who respectively reported 60% (63/105) and 18% (31/171) resistance [5, 7]. Resistance to ciprofloxacin was 25% (15/60), higher than that found in broiler feed at 3.9% (16/414) [7]. Ciprofloxacin is considered to have only modest antibacterial activity against enterococci [27] and thus is not the first choice for treating enterococcal infection. Schaberg also reported that an increasing number of ciprofloxacin-resistant cases were accompanied by high-level gentamycin resistance [31], which is similar to our findings. High-level aminoglycoside resistance should be further examined, as aminoglycoside resistance reduces the synergetic action of combined penicillin-aminoglycoside treatment, which would result in a selective elimination of anaerobic bacteria parallel to an increase in enterococci [2].

Although virulence genes and antibiotic resistance have been detected in milk samples, *Enterococcus* spp. have been safely present in milk products for centuries. However, when considering the safety of enterococci in food or probiotic use, strict monitoring mechanisms are necessary to guarantee consistent safety for consumers.

In conclusion, enterococci are emerging as one of the main species causing dairy cow mastitis in Gansu province and the surrounding region. This is the first report of enterococcal mastitis in western China and the first report of a virulence factor combination, *asa1-ccf-gelE*, found in mastitis originating from enterococcal strains. Based on our results, the seven examined virulence genes (*asa1*, *ccf*, *gelE*, *esp*, *CylA*, *ace* and *agg*) are inadequate for determining the prognosis of enterococcal infection. However, more work is needed to reveal the mechanism of enterococci pathogenesis. Tetracycline remains one of the most effective drugs for treating these isolates, followed by glycopeptides and new generations of β-lactams. Although few reports have demonstrated the threat of enterococcal to public health, strict and guaranteed levels of enterococci in the food and probiotic industries are necessary.

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