

Full Length Research Paper

# Isolation and identification of *Enterococcus faecalis* and detection of its virulence factor genes in lambs presenting with encephalitis in Xinjiang province, China

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Accepted 19 April, 2013

In order to investigate the reasons of lamb death, 11 bacteria were isolated from the brain, liver and other tissues of natural infectious lambs presenting neurological symptoms and septicaemia as the main clinical feature from different farm of two regions in Xinjiang, China in recent years. They were identified as *Enterococcus faecalis* according to morphological, cultural and biochemical characterization. The infecting strains were identified as belonging to serotype D, G or to an unidentified serotype with Lancefield Group A to G Latex Diagnostic Kit. All 11 *E. faecalis* pathogens were resistant to norfloxacin, penicillin, tetracycline, streptomycin, gentamicin and erythromycin at varying degrees. PCR screening for nine common virulence factor genes associated with *Enterococcus* species revealed the presence of eight virulence factor genes (*esp*, *cylA*, *asa1*, *ace*, *efa*, *gelE*, *ef0591* and *ef3314*) in 11 of the isolates, five genes were present in all strains examined and *gelE* was present in 1 pathogenic strain examined. Nine virulence factor genes were not present in two strains examined. Virulence gene analysis revealed a 96% homology to Enterococcal genes from pathogenic *E. faecalis* sequences deposited in GenBank. Some genus from *Enterococcus* can also lead to the infection and death of lambs.

**Key words:** Lamb, encephalitis, identification of *Enterococcus faecalis*, virulence factor gene.

## INTRODUCTION

*Enterococcus* species are Gram-positive bacteria that comprise part of the natural gastrointestinal tract flora of both humans and animals (Lance et al., 2009). *Enterococcus* strains are also commonly found in soil, sewage, water and food samples resulting from fecal contamination (Giard et al., 2001; Franz et al., 2003). *Enterococcus* are opportunistic pathogens and are the causative agents of infective endocarditis, meningitis, pneumonia, surgical wound, burn, skin and soft tissue, urinary tract, bone and joint infections (Kavindra et al., 2010). It includes 16 species in which *Enterococcus faecalis* (*E. faecalis*) had the highest isolation rate (Li, 2006). Natural and acquired resistance of *Enterococcus*

species to many antimicrobial agents has led to the emergence of significant cases of nosocomial infections in recent decades (Mannua et al., 2003; Moro et al., 2004; Rice et al., 2003). One of the reasons that *enterococcus* infections is becoming more serious is its special resistance, but virulence factor is also another very important factor, more than 10 kinds of virulence factors have been found in clinical isolates currently, it includes haemolysin activator (*cylA*), gelatinase (*gelE*), *E. faecalis* surface proteins (*esp*), endocarditis antigen (*efaA*), collagen-binding protein (*ace*) and 2 new surface proteins (*ef0591* and *ef3314*) etc. (Creti et al., 2004).

Respective *Enterococcus* clones can acquire several

genetic elements encoding potential virulence factors, antibiotic resistance genes and genes that facilitate adaptation to harsh environments thought to enhance pathogen survival in hospitalized patients. *Enterococcus* infections are also a threat to animals in veterinary clinics (Mannua et al., 2003; Stalker et al., 2003), but there were few reports about the infection of sheeps. Lambs (20 to 40 days old) from different sheep farms in the northern Xinjiang province, China, presented with disease that is, sepsis and neurological manifestations that resulted in 20 to 30% morbidity and significant economic losses in recent years, this report describes that 11 bacteria were isolated from above natural infected lambs, the infecting strains were identified as *E. faecalis* according to morphological, cultural and biochemical characterization and belonged to serotype D, G or to an unidentified serotype with Lancefield Group A to G Latex Diagnostic Kit. These isolates were then carried out antibiotic resistance test and detection of 9 virulence factor genes by PCR respectively.

This result can also provide experimental datas that can be used in the diagnosis and prevention of lamb *E. faecalis* species infections in the future.

## MATERIALS AND METHODS

### Animals

Natural infections lambs with neurological manifestations were obtained from sheep farms in the northern region of Xinjiang province, China.

### *E. faecalis* culture and identification

Tissues or organs (brain, liver, spleen, blood from heart) collected from natural infectious and dead lambs were cultured in Todd-Hewitt broth (THB) at 37°C in 5% CO<sub>2</sub> without agitation (MacInnes et al., 1999). Isolated bacteria were identified using the VITEK-AMS60 (BioMérieux Corporate, French) bacteria automatic identification system. Haemolysin production was evaluated on Columbia agar base supplemented with 5% (v/v) fresh sheep blood. Zones of hemolysis surrounding respective colonies after 24 h at 37°C indicated haemolysin production (Creti et al., 2004). Meanwhile, virus was also cultured from each tissues or organs.

### Observation of pathological changes

Tissues or organs (brain, liver, spleen, kidney, lung and heart) were collected from the natural infectious and dead lambs and fixed with formaldehyde, embedded in wax, sectioned and H&E stained to observe pathological changes.

### Antibiotic susceptibility testing

Antibiotic resistance was determined using the disk diffusion Kirby-Bauer method recommended by the WHO. Antibiotic resistance to gentamicin, tetracycline, torfloxacin, streptomycin 2000, penicillin G, vancomycin, nitrofurantoin, chloramphenicol, rifampicin and ery-

thromycin were tested (Kit of Gram-positive bacterial antibiotic susceptibility, Tianhe Bio Reagent Co., Ltd. Hangzhou, China).

### PCR amplification

Detection by PCR of the following genes encoding virulence factors included: haemolysin activator (*cytA*), gelatinase (*gelE*), *E. faecalis* surface proteins (*esp*), endocarditis antigen (*efaA*), collagen-binding protein (*ace*), aggregation substances (*asa373* and *asa1*) and 2 genes encoding new proteins *ef0591* and *ef3314*. *E. faecalis* DNA was prepared by taking an inoculation loop equivalent of an overnight colony and adding it to 500 µl sterile distilled water, boiling for 10 min and then centrifuging at 14,000 xg for 5 min. 5 µl were used as template DNA. PCR reactions of 25 µl were carried out and contained 1 µl PCR buffer, 2 mM MgCl<sub>2</sub>, 200 µM each dNTP, 400 nM each primer and 0.25 U Taq DNA polymerase (Sangon Bio-Engineering Company, Shanghai, China). Product amplification was carried out on a DNA thermal cycler under the following conditions: 95.8°C for 5 min, followed by 30 cycles of 95.8°C for 60 s, 58.8°C for 60 s (52.8°C for *gelE* and 63.8°C for *esp*) and 72.8°C for 60 s followed by a final step at 72.8°C for 10 min.

PCR products were analyzed by gel electrophoresis using 0.8% (w/v) agarose gels (Creti et al., 2004; Chang et al., 2002). Oligonucleotides synthesized by Sangon Bio-Engineering Company are described in Table 1.

### Virulence factor genes homology analysis

Fragments of those virulence factor genes from the isolates were cloned into the pMD-19 vector, and sequenced by Sangon Bio-Engineering Company. Sequences were analyzed using the CLUSTALW software.

## RESULTS

### Isolate morphology and culture characteristics

Eleven (11) bacterial isolates were cultured and isolated from different sheeps in distinct farms, but no viruses were isolated. All isolates microscopically presented as single Gram-positive cocci or in chains, however, Gram-negative bacteria presenting in long chains could also be observed in aged media (data not shown). Colonies were colorless, transparent, spherical, slightly smooth with neat edges and had a wet surface when grown in THA for *Streptococcus*. Respective isolates grew poorly in common agar. When grown in Martin broth media with 5% serum, bacterial cultures were turbid and presented with small amounts of flocculent precipitate.

All 11 strains grew in media (pH 9.6) containing 6.5% NaCl or 2.0% NaCl, also grew at 45°C, 10°C, and in 10% CO<sub>2</sub> or the pre-sence of O<sub>2</sub>, respectively. The 11 isolates were identified as *E. faecalis* according to the characteristic of biochemical reactions from VITEK-AMS60 systematic analysis system. Eight isolates were hemolysin positive and eight belonged to serotype D, 1 to serotype G and two strains could not be identified.

**Table 1.** PCR primers used to amplify *E. faecalis* virulence factor genes.

Gene	Sequence 5'→3'	GenBank accession No.	Product size
<i>esp</i>	TTG CTA ATG CTA GTC CAC GAC C GCC TCA ACA CTT GCA TTG CCG A	AF034779	932
<i>geE</i>	ACC CCG TAT CAT TGG TTT CAG CAT TGC TTT TCC ATC	M37185	405
<i>cylA</i>	GAC TCG GGG ATT GAT AGG C GCT GCT AAA GCT GCC CTT AC	AD1CLYL	688
<i>asa1</i>	CCA GCC AAC TAT GGC GGA ATC CCT GTC GCA AGA TCG ACT GTA	SFPASA1	529
<i>asa373</i>	GGA CGC ACG TAC ACA AAG CTA C CTG GGT GTG ATT CCG CTG TTA	AJ132039	619
<i>ace</i>	GGA ATG ACC GAG AAC GAT GGC GCT TGA TGT TGG CCT GCT TCC G	AF159247	616
<i>efaA</i>	GCC AAT TGG GAC AGA CCC TC CGC CTT CTG TTC CTT CTT TGG C	EFU03756	688
<i>ef0591</i>	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C	NC_004668	844
<i>ef3314</i>	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTTC	NC_004668	566

### Characterization of antibiotic resistance

All 11 isolates were highly sensitive to nitrofurantoin and moderately sensitive to chloramphenicol, rifampicin and vancomycin but resistant to norfloxacin, penicillin G, tetracycline, streptomycin 2000, gentamicin and Erythromycin in various degrees.

### Pathological changes

Some coccobacteria were observed microscopically in brain, cerebrospinal fluid, liver and spleen sections (Figure 1). The main pathological changes were observed in brain, lung and heart tissues followed by involvement of the liver and kidneys. Tissues presented with edema, thickening and hyperemia of the cerebellum meninges and thrombosis in micro-capillaries. Edema was also observed in the cerebrum and thrombosis in micro-capillaries. In addition, myocardial fiber space was increased and presented with moderate levels of lymphocyte infiltration, alveolar walls presented with capillary congestion and telangiectasia, liver cells were granular with degeneration and necrosis, and glomerular capillaries were dilated and congested [part of pathological changes

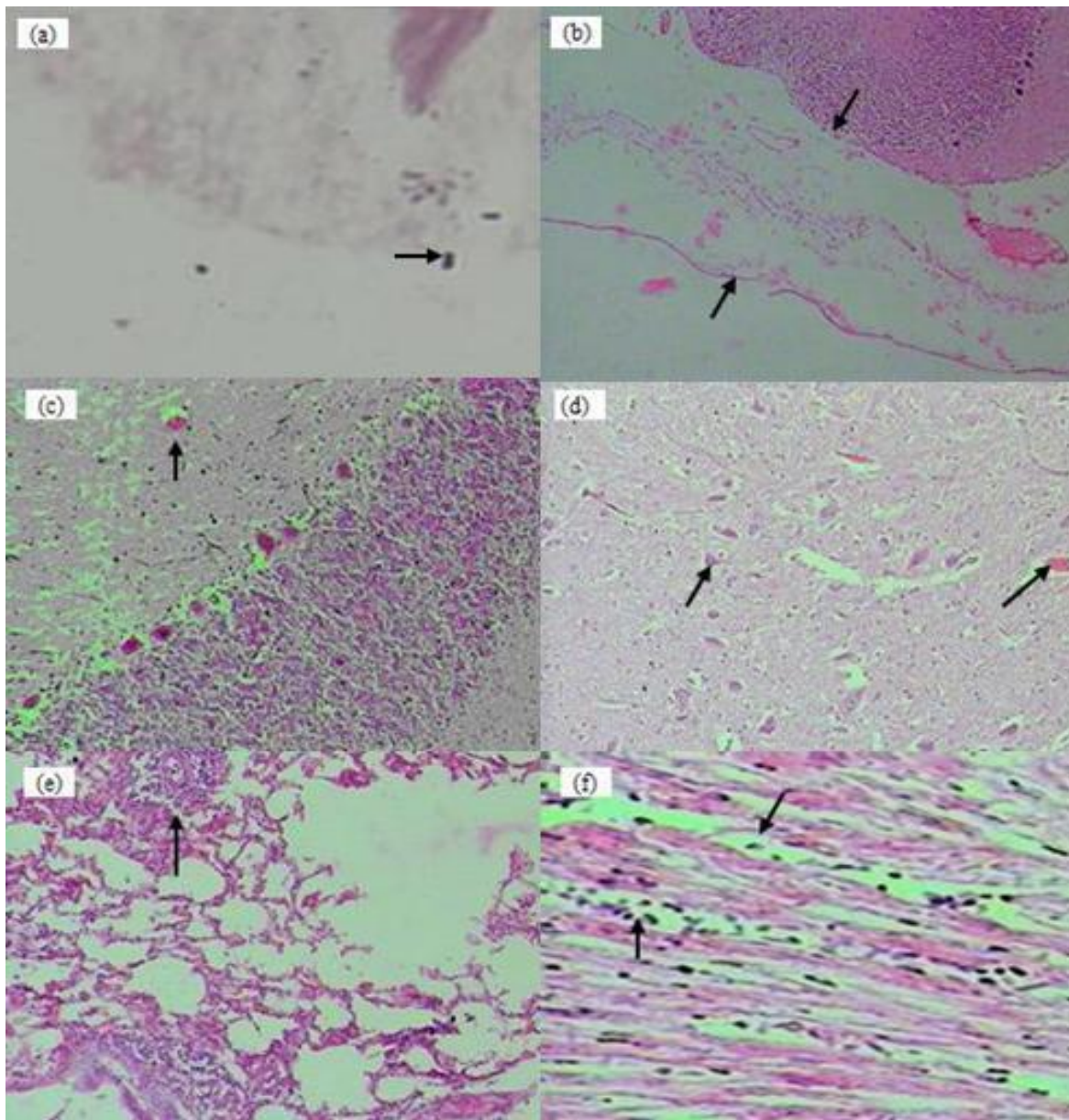
(Figure 1)].

### Detection of virulence factor genes

Genes encoding *esp*, *cylA*, *asa1*, *ace*, *efa*, *ef0591* and *ef3314* were detected in 5 *E. faecalis* isolates; genes encoding *esp*, *geE*, *asa1*, *ace*, *efa* and *ef3314* were detected in 1 *E. faecalis* isolate; genes encoding *cylA*, *asa1*, *ace*, *efa*, *ef0591* and *ef3314* were detected in 1 *E. faecalis* and genes encoding *esp*, *cylA*, *asa1*, *ace*, *ef0591* and *ef3314* were detected in 1 *E. faecalis* isolate. Only 1 isolate carried the *esp* gene only and 2 strains did not possess any of the virulence genes examined (Table 2) and 8/11 virulence factor genes were detected in 11 *E. faecalis* isolates (Figure 2).

### Virulence gene homology analysis

Eight virulence factor gene fragments corresponding to *ace*, *efa*, *cylA*, *geE*, *asa1*, *esp*, *ef3314* and *ef0591* from the isolates were sequenced, revealing 96.64 to 99.90% homology to corresponding GenBank sequences (Table 3).



**Figure 1.** Wright's staining of semears of tissues infected by *E. faecalis*. Tissues from the encephalon of a dead lamb infected by pathogenic strain: **(a)** (100X). *E. faecalis* were found scattered in short chains of two to three bacteria with or without capsule. Arrow indicates three *E. faecalis*. H&E staining of lamb cerebellum sections from infected lambs with the arrow indicating nerve cell edema and satellite phenomenon; **(b)** (40X) and microthrombosis in a cerebellum capillary; **(c)** (40X). Histopathologic changes in the cerebrum from infected lambs following H&E staining. Arrow denotes nerve cell necrosis and gliocyte nodular in the cerebrum **(d)** (40X). Histopathologic changes in lung tissue harvested from infected lambs and stained with H&E. The arrow indicates lymphocyte infiltration around blood capillaries and bronchioles and congestion of alveolar wall capillaries **(e)** (10X). Histopathologic changes in heart muscle harvested from infected lambs and stained with H&E. The arrow indicates a widened myocardial fiber gap and red blood cells **(f)** (10X).

## DISCUSSION

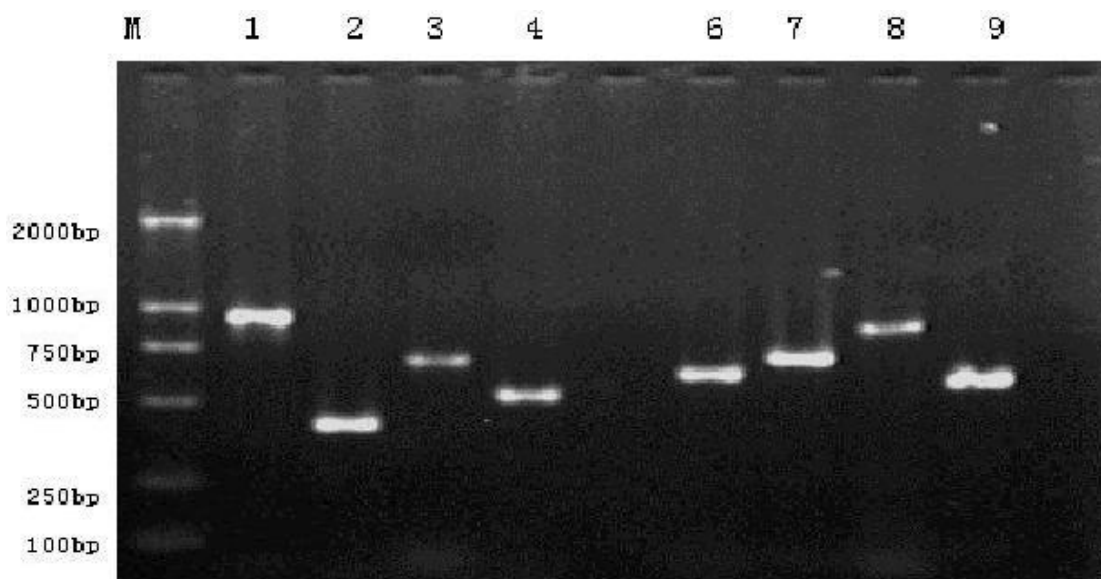
*Enterococcus* species colonize the intestines of both animals and humans and are considered to be part of the natural intestinal flora. However, infections caused by

*Enterococcus* species are becoming more serious due to increased antibiotic resistance and a growing immune-suppressed population (Ma et al., 2005a; Fisher and Phillips, 2009). *Enterococcus* species infections in veterinary clinic have also been reported (Han and Zhang, 2003;

**Table 2.** Virulence factor genes amplified.

Virulence gene	<i>E. faecalis</i> isolates number										
	1	2	3	4	5	6	7	8	9	10	11
<i>esp</i>	+	+	+	+	-	+	+	+	-	+	-
<i>gelE</i>	+	-	-	-	-	-	-	-	-	-	-
<i>cylA</i>	-	+	+	+	+	+	+	-	-	+	-
<i>asa1</i>	+	+	+	+	+	+	+	-	-	+	-
<i>asa373</i>	-	-	-	-	-	-	-	-	-	-	-
<i>ace</i>	+	+	+	+	+	+	+	-	-	+	-
<i>efa</i>	+	+	+	+	+	+	+	-	-	-	-
<i>ef0591</i>	-	+	+	+	+	+	+	-	-	+	-
<i>ef3314</i>	+	+	+	+	+	+	+	-	-	+	-

+, Detection of virulence gene; -, virulence factor could not be detected.



**Figure 2.** Virulence factor gene amplification. Eight virulence factor genes were detected from 11 *Enterococcus faecalis* isolates obtained from sheep presenting with encephalitis. Lanes M-9 corresponds to Marker DL2000, *esp*, *gelE*, *cylA*, *asa1*, *ace*, *efaA*, *ef0591* and *ef3314*, respectively.

**Table 3.** The lowest homology analysis of virulence factor gene fragments (%).

	Sequences from GenBank							
	AF 159247 <i>ace</i>	EFU 03756 <i>efa</i>	AD1 CYLYL <i>cylA</i>	M 37185 <i>gelE</i>	SFP ASA1 <i>asa1</i>	AF 03779 <i>esp</i>	NC 005668 <i>ef3314</i>	NC 00468 <i>ef0591</i>
Sequences of pathogenic <i>E. faecalis</i>	99.30	99.56	99.30	97.85	96.64	99.90	99.29	98.11

Stalker et al., 2003). Infections of 20 to 40 day-old lambs presenting with a short course of neurological symptoms (regardless of lamb breed) have occurred across different sheep farms in the northern Xinjiang province with seasonal frequency in recent years. These infections have

led to encephalitis and death, resulting in severe economic losses. In this study, 11 *E. faecalis* isolates were recovered from sick lambs. It demonstrated that *E. faecalis* was the causative agent of the encephalitis epidemic spreading across sheep farms in the Xinjiang pro-

vince through a series of tests.

Numerous studies have demonstrated an increase in nosocomial *Enterococcus* infections related to the heavy use of broad-spectrum antibiotics that have selected antibiotic-resistant *Enterococcus* strains, in combination with the increased frequency of invasive treatments, poor health and the severe illness that many hospitalized patients present with, making control of *Enterococcus* species infections more difficult (Arias et al., 2010; Han and Zhang, 2003; Hidron et al., 2008). For example, vancomycin-resistant *Enterococcus* isolates around the world were described shortly after the first vancomycin-resistant *Enterococcus* was reported in Britain (Perencevich et al., 2004; Willems et al., 2005). The mechanism of drug-resistance acquisition in *Enterococcus* species is complex and resistant strains possess natural and acquired drug resistance to a variety of antibiotics. *Enterococcus* resistance to ampicillin and penicillin G results from the production of a low-affinity penicillin-binding protein and resistance to lactamase and aminoglycoside resistance is conferred by plasmid-encoded enzymes which weaken the role of lactam and aminoglycoside based combination therapies (Thouverez and Talon, 2004; Yazgi et al., 2002).

Our results show that the *E. faecalis* isolates described were highly resistant to penicillin, ampicillin, streptomycin, gentamicin and erythromycin but extremely sensitive to vancomycin, probably because this antibiotic has not been highly used in this region to date. Since *Enterococcus* species are used in human food fermentation, the risk of humans coming in contact with highly drug-resistant *Enterococcus* species will pose a threat to human food safety (Charles et al., 2003; Kayser, 2003; Khan et al., 2005; Hammerum et al., 2010). Therefore, the use of antibiotics for both humans and animals should be carefully monitored and regulated.

Pathogenic *E. faecalis* can produce a variety of virulence factors encoded by various virulence genes, and these virulence factors comprise a complex and diverse arsenal and include *ace*, *efaA*, *cylA*, *gelE*, *asa1* and *asa373*, *esp*, *ef0591* and *ef3314* (Dupont et al., 2008; Harada et al., 2005; Vankerckhoven et al., 2004). These virulence factor genes play an important role in mediating disease severity and presentation. Additional virulence determinants have also been associated with a pathogenicity (Leavis et al., 2004), encoding the production of toxins that facilitate host cell invasion, in addition to genes encoding adhesins that facilitate extra-intestinal infections (Coburn et al., 2004). Studies have shown that some virulence genes were important component of the 150 kb pathogenicity island (Leavis et al., 2004), play critical role in the disease process respectively (Charles et al., 2001; Heikens et al., 2007; McBride et al., 2009). In addition, it was shown that clinical isolates possessed combinations of virulence factor genes (Bittencourt de Marques and Suzart, 2004). Ma et al. (2005a, b) demon-

strated that there was an increased likelihood that *gelE* and *efaA* would be present in respective isolates and that *gelE*, *efaA* and *ace/esp/cylA* could also be present in different clinical specimens, suggesting that disease-causing genes may be present in clusters and that *gelE*, *efaA* and *cylA* likely played primary pathogenicity roles in clinics (Ma et al., 2005b).

The strains described in this report in veterinary clinic had additional virulence factor gene combinations. *gelE* and *esp* did not present together in the same strain, while *esp*, *cylA*, *asa1*, *ace*, *efa*, *ef0591* and *ef3314* were present in 5/11 *E. faecalis* lamb isolates. How these virulence factors interact and mediated disease needs to be further addressed. Two strains did not possess any of the virulence genes examined, but still led to infection, the reason need to be studied further.

## ACKNOWLEDGMENT

The first author acknowledges the financial support received from China National Natural Science Fund (NSFC, Grant No: 31160507), Department of Human Resources and Social Security in China (Grant No: 2010LX007) and National Students Innovation and Entrepreneurship Training Program (Grant No: 201210759021).

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