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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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 septicemia 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, 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 reseasons that enterococcus infections is becoming more serious is it’s 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 datads 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% CO₂ 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 erythromycin 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 MgCl₂, 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 30 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 15 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% CO₂ or the presence of O₂, 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 |
|--------|----------------------------------------------------|-----------------------|--------------|
| esp    | TTG CTA ATG CTA GTC CAC GAC C GCC TCA ACA CTT GCA TTG CCG A | AF034779              | 932          |
| geE    | ACC CCG TAT CAT TGG TTT CAG CAT TGC TTT TCC ATC    | M37185                | 405          |
| cylA   | GAC TCG GGG ATT GAT AGG C GCT GCT AAA GCT GCC CTT AC | AD1CLYL               | 688          |
| asa1   | CCA GCC AAC TAT GGC GGA ATC CCT GTC GCA AGA TCG ACT GTA | SFPASA1               | 529          |
| asa373 | GGA CGC ACG TAC ACA AAG CTA C CTG GGT GTG ATT CCG CTG TTA | AJ132039              | 619          |
| ace    | GGA ATG ACC GAG AAC GAT GGC GCT TGA TGT TGG CCT CCT C   | AF159247              | 616          |
| efaA   | GCC AAT TGG GAC AGA CCC TC CGC CTT CTG TGT TGG CCT TGG C | EFU03756              | 688          |
| ef0591 | AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C | NC_004668            | 844          |
| ef3314 | AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C | 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, gelE, 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, gelE, asa1, esp, ef3314 and ef0591 from the isolates were sequenced, revealing 96.64 to 99.90% homology to corresponding GenBank sequences (Table 3).
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 | E. faecalis isolates number |
|---------------|---------------------------|
|               | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| esp           | + | + | + | + | - | + | + | - | + | - | - |
| gelE          | + | - | - | - | - | - | - | - | - | - |
| cylA          | - | + | + | + | + | + | - | - | + |
| asa1          | + | + | + | + | + | + | + | + | - | - |
| asa373        | - | - | - | - | - | - | - | - | - |
| ace           | + | + | + | + | + | - | - | - | + |
| efa           | + | + | + | + | + | - | - | - | - |
| ef0591        | - | + | + | + | + | + | - | - | + |
| ef3314        | + | + | + | + | + | - | - | - | + |

+, 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  | EFU | AD1 | M   | SFP | AF  | NC  | NC  |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
|                        | 159247 | 03756 | CYLYL | 37185 | ASA1 | 03779 | 005668 | 00468 |
| ace                    | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| efa                    | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| cylA                   | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| gelE                   | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| asa1                   | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| asa373                 | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| esp                    | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| ef0591                 | 99.30 | 99.56 | 99.30 | 97.85 | 96.64 | 99.90 | 99.29  | 98.11 |
| ef3314                 | 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 neurologic 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-
ulence 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) demonstrated 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.

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