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Full Length Research Paper

Characterization of pathogenic or non-pathogenic *Enterococcus faecalis* isolated from lambs from Xinjiang, a remove North-west province of China

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The majority of the 11 pathogenic *Enterococcus faecalis* from lambs developed encephalitis and 45 non-pathogenic *E. faecalis* from intestinal and respiratory microbiota of healthy lambs were belonged to Streptococcus serotype D. Haemolytic study revealed that 8 of 11 pathogenic stains had stable haemolyticus; 8/30 strains of intestinal normal microbita and 3 of 15 strains from respiratory system showed unstable haemolyticus. Of 11 pathogenics *E. faecalis*, 8 of 9 virulence factor genes were detected in all the strains; 5 of 11 expressed Esp, CylA, Asa1, Ace, efa, EF0591 and EF3314 simultaneously and 1 of 11 expressed GelE; Two of 11 did not express any of the 9 virulence factor genes. Among 30 strains isolated from the intestinal microbita, only one had 2 (GelE, EF3314) and one had 3 (GelE, EF3314 and Asa1) of the 9 virulence factor genes. The homology of these 3 common virulence gene fragments (GelE, EF3314 and Asa1) was more than 95% between *E. faecalis* from GenBank and intestinal microbita and 96% when comparing the *E. faecalis* isolated from intestinal microbita and from those of pathogenic strains. Antibiotic sensitivity study indicated that all of the 11 pathogenic strains were resistance to a variety of antibiotics in various degrees. In comparison, Only 2 strains from normal flora were resistance to individual antibiotics. *In vivo* challenge study showed that all of the 11 the pathogenic strains could lead to the death of mice, whereas none of the isolates from normal flora could cause the death of the experimental animals.

Key words: Biochemical characteristic, *Enterococcus faecalis*, lamb,virulence factor gene.

INTRODUCTION

Enterococcus faecalis are an important part of normal flora in humans and animals. It is the second regular bacteria, following to *E. coli* as ecological agents (Yuan and Fu, 2003; Drahovska et al., 2004). Recent studies have confirmed that the pathogenicity of *E. faecalis* is an important nosocomial infectious pathogen after *Staphylococcus* in the aerobic Gram-positive *cocci* (Schaberg et al., 1991). *E. faecalis* today is ranked second to third in frequency among bacteria isolated from hospitalized patients (Kayse, 2003). Treatment for *E. faecalis* is difficult due to the possession of a large number of virulence factors and drug resistant. So the study on *E. faecalis* has become one hot point in medical research (Giridhara Upadhyaya et al., 2009; Katie Fisher, 2009). There are also reports of infection in livestock and poultry in the veterinary practice, but the relationship between pathogenic and normal flora *E. faecalis* and its pathogenic mechanisms are not yet very clear.

We have 11 isolated *E. faecalis* from Lamb encephalitis recently, but the relationship between pathogenic strain and normal flora isolates from healthy lamb is unclear.

So our purpose is to make a comparative study on different sources of *E. faecalis* from lamb, such as culture characteristics, biochemical features, hemolytic charac-terristics, drug susceptibility, animal corresponding pathogenicity and the types of virulence factor genes and

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the fragments nucleotide sequence, which can provide information for pathogenic mechanism of animal *E. faecalis* infection.

MATERIALS AND METHODS

Reagents

TaqDNA polymerase and DNA gradient ladders were obtained from Shanghai Sangon Bio-Engineering Company. *Streptococcus* Grouping Kit was obtained from the France BioMerieux Inc. Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engeering Material Co., Ltd. China. Antibiotics were purchased from Hangzhou Tianhe Microorganism Regent Co., Ltd China.

Isolation of *E. faecalis*

Eleven pathogenic *E. faecalis* were isolated from lamb brains which had developed encephalitis and were identified. Thirty and fifteen *E. faecalis* of normal flora were isolated from intestinal microbiota and respiratory microbiota of healthy lambs, respectively.

Growth characteristics of isolates

The purified *E. faecalis* were inoculated on ordinary nutrient agar plate, *Streptococcus* selective blood agar medium, LB blood agar medium, and incubated at 37°C under 10% CO_2 for 24 h.

Culture characteristics of the isolates

A single bacteria colony was picked up from blood agar plate after 24 h and was inoculated in LB broth with 5% fetal bovine serum with or without 10% CO_2 in order to test the requirement for oxygen. To test the tolerance to temperature, the isolates were inoculated in LB broth with 5% fetal bovine serum at either 40°C or 10°C for 24 ~ 48 h. To test the tolerance to high-salt or high-alkali, the isolates were inoculated in LB broth containing 5% fetal bovine serum with either 6.5% NaCl or pH 9.6 at 37°C for 24 ~ 48 h.

Biochemical characterization of the isolates

Isolates from blood agar after 24 h were identified by VITEK-AMS32 Bacterial Biochemical Identification System. Hemolysis to sheep red blood cells were determined by plate assay (PA) (Li and Li, 1999). Serotype of the isolates was identified by use of *Streptococcus* Grouping Kit.

Antimicrobial susceptibility test (disk diffusion method)

15 to 20 mm zone of inhibition is high-sensitive, 10 to 14 mm is the medium-sensitive, 10 mm below is low- sensitive, 0 mm is non-sensitive according the standards adopted from United States Committee for Standardization of Clinical Trials. Antibacterial drugs include gentamicin, tetracycline, norfloxacin, streptomycin 2000, penicillin vancomycin, nitrofurantoin, chloramphenicol, rifampicin and erythromycin.

Mice and infection

Group of 3 Kunming out-bred mice, 6 to 8 weeks old, female, were obtained from Xinjiang Medical University, China. For infection study, all of the 11 pathogenic strains, 10 randomly picked strains of intestinal isolates and 10 stains of respiratory isolates were injected and was infected with intra-peritoneally (i.p.) with 3×10^8 bacteria per mouse in Martin broth. Control group was injected with LB broth alone.

Detection of 9 types of virulence factor genes and sequence analysis

Virulence factor genes include haemolysin activator (Cyl A), gelatinase (GelE), enterococcal surface proteins (Esp), endocarditis antigen (EfaA), collagen-binding protein(Ace), aggregation substances(Asa373 and Asa1), another 2 protein EF0591 and EF3314. The PCR primers synthesized according to reference (Roberta et al, 2004) were showed in Table 1. and normal flora strains reference Molecular Cloning (Sambrook and Russell, 2002) with a slight modification. The homologous of DNA fragments were analyzed with ClustalX sequence analysis software.

RESULTS

Growth and cultivation characteristics of isolates

Most of the *E. faecalis* isolated from the three different sources were long-chain and Gram-positive coccus in 2 kind of liquid cultures (Figure 1). In aging cultures they sometimes became Gram-negative. Colonies were round, smooth, moist, medium-sized with neat edge in blood agar LB plate after anaerobic culture. They also produced the colonies which are colorless, transparent, round, surface wet, slightly smooth, neat edge, like tip in *Streptococcus* select agar medium. Those isolates almost can not grow or grow poorly in the ordinary medium and make *Streptococcus* enrichment medium become slightly turbid and a small amount of flocculent precipitate at the bottom of tube. All strains can grow at 45°C and 10°C and also grow in 6.5% NaCl or pH 9.6 LB broth with serum. These features are consistent with characteristics of *E. faecalis.*

Biochemical characteristics of isolates

Biochemical reactions of 45 strains from normal flora were the same as that of 11 pathogenic strains when assayed with VITEK-AMS 32 systems. Butyl-diphenyl-pyrazole dione (PYR) test is positive; Cyclic Adenosine monophosphate test, Optochin test and bacitracin inhibition test are negative; These isolates can ferment glucose and produce L-lactic acid mainly; Catalase test is negative, but some strains can produce false catalase; Benzidine test is negative. Eight of 11 pathogens produce β-hemolysis in sheep blood agar, which is relatively stable after continuous passage. Eight of 30 strains from intestinal microbiota and 3 of 15 strains from respiratory microbiota also produce hemolysis, but these characteristics disappear after continuous passage. The results of serotypes showed that 8 of 11 were type D, 1 of

Table 1. Information of PCR primers for detection of different virulence factor genes.

Name of virulence vector genes	Sequence of primer	Length of Segment (bp)	GenBank accession No.	Location
Esp	TTG CTA ATG CTA GTC CAC GAC C GCC TCA ACA CTT GCA TTG CCG A	932	AF034779	1217-1249
GelE	ACC CCG TAT CAT TGG TTT CAG CAT TGC TTT TCC ATC	405	M37185	762-1163
CylA	GAC TCG GGG ATT GAT AGG C GCT GCT AAA GCT GCC CTT AC	688	AD1CLYL	6656-7344
Asa1	CCA GCC AAC TAT GGC GGA ATC CCT GTC GCA AGA TCG ACT GTA	529	SFPASA1	3122-3651
Asa373	GGA CGC ACG TAC ACA AAG CTA C CTG GGT GTG ATT CCG CTG TTA	619	AJ132039	3094-3713
Ace	GGA ATG ACC GAG AAC GAT GGC GCT TGA TGT TGG CCT GCT TCC G	616	AF159247	160-776
EfaA	GCC AAT TGG GAC AGA CCC TC CGC CTT CTG TTC CTT CTT TGG C	688	EFU03756	312-1000
EF0591	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C	844	NC_004668	99-1003
EF3314	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C	566	NC_004668	35-601

Note: Primer references (Roberta et al., 2004).

Figure 1. Gram staining of the pathogenic *E. faecalis s* cultivated in broth（100×）The *E. faecalis* were isolated from brains of infected lamb. They were cultured in broth for 18 h. The bacteria were stained long-chain and Gram-positive coccus.

11 is type G, 2 of 11 is not identified in pathogens; Twenty-four of 30 are type D, 6 of 30 are not identified from intestinal microbiota; Fifteen of 15 are type D from respiratory microbita.

Antibiotic susceptibility test

Eleven pathogenic isolates were highly sensitive to nitro-furantoin and moderately sensitive to chloramphenicol,

Table 2. Antibiotics susceptibility test of the *E. faecalis* isolated from the three sources.

Antibiotic category		Nitrofurantoin	Chloramphenicol	Rifampicin	Vancomycin	Norfloxacin	Penicillin	Tetracycline	Streptomycin	Gentamicin	Erythromycin
					Pathogenic strains						
	1-11	S	S	S	S	R	R	R	R	R	R
					Intestinal strains						
E. faecalis strains from lambs	1-29	S	S	S	S	S	S	S	S	S	S
	30	S	S	S	S	S	S	R	R	S	R
					Respiratory strains						
	1-14	S	S	S	S	S	S	S	S	S	S
	15	S	S	S	S	S	R	S	S	S	S

Table 3. *In vivo* study of the pathogenicity of the stains isolated from different sources. Each of the *E. faecalis* strains isolated from 3 sources was infected into three mice (see Material and Methods). By 24 h post-infection, the numbers of the dead mice were recorded. No more death of the mice was observed after 24 h.

	Numbers of mice died / numbers of mice infected										
Infected with pathogenic strains	2/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/3	2/3	1/3
Infected with intestinal strains	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
Infected with respiratory strains	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	

rifampicin and vancomycin. These isolates were resistant to norfloxacin, penicillin, tetracycline, streptomycin, gentamicin and erythromycin to various degrees. The rest of strains from normal flora were sensitive to the other antibiotic except that 1 of 30 strains from intestinal microbiota was resistant to tetracycline, erythromycin and streptomycin, 1 of 15 strains from respiratory microbita is resistant to penicillin (Table 2).

Experimental infection of mice

All mice infected with pathogenic strains appeared apathetic and back hair handstand 16 h after infection. Of 33 mice infected with the 11 pathogenic strains (3 mice per strain), 25 of 33 the mice died within 24 h post-infection. The mice infected with normal flora strains appeared temporary apathetic and loss of appetite, but they gradually recovered after 12 h. The control group looked normal during the experiment (Table 3 and Figure 2).

Results of virulence factor genes

Nine virulence factor genes were listed in Table 1. Our gene analysis showed that 8 of 9 virulence factors were tested in the 11 pathogenic *E. faecalis*. Among the 11 pathogenic *E. faecalis*, five took Esp, CylA, Asa1, Ace, efa, EF0591 and EF3314 virulence factors genes, one strain had GelE only. Two strains did not take any of the 9 virulence factor genes (Figure 3 and Table 4). Of the 30 *E. faecalis* isolated from intestinal microbiota, three out of the nine virulence factor genes were detected in all of them. One of the normal strains had virulence factor genes GelE and EF3314 and one took virulence factor genes GelE, EF3314 and Asa1. Of the 15 isolated from respiratory microbita, none of the virulence factor genes were detected among them (Figure 4 and Table 4). The Homology of amplified fragments of three common virulence factor genes is 99.53, 96.2 and 99.12%, respectively between normal flora and pathogenic *E. faecalis*. Similarly, the homology from normal flora *E. faecalis* is 98.03, 95 and 99.3% compared to the information of GenBank from medical the cultural and biochemical characteristics of *E. faecalis* from normal flora were basically the same as that of pathogens except that the individual biochemical characteristics of individual strains were instable. These results were basically consistent with biochemical characteristics of *E. faecalis*. Haemolyticus is an important virulence indicator of certain bacteria. Eight of 11 pathogenic stains showed signifi-

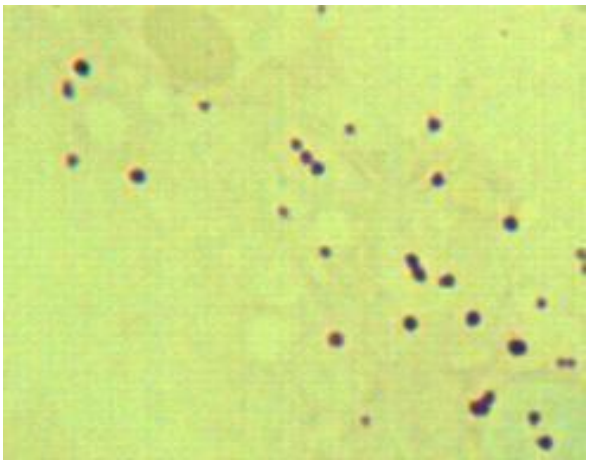

Figure 2. Wright's staining of the *E. faecalis* in encephalon of dead mice infected with pathogenic strains（100×). The brain smear of the infected mice was subject to Wright's staining. The *E. faecalis* were scattered or a very short strain including 2 to3 bacteria in brain smear of mice infected with pathogenic strains.

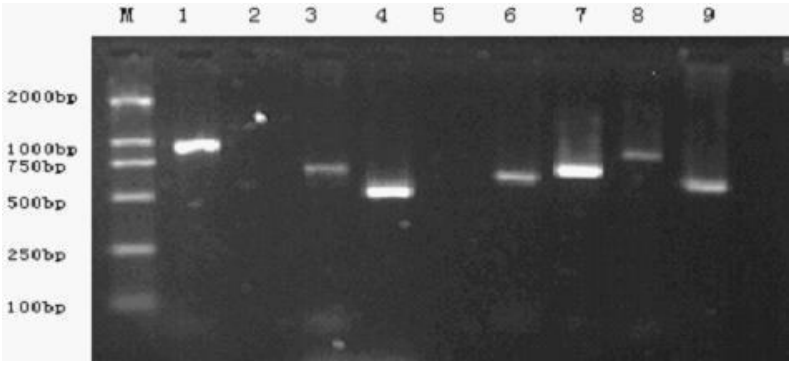

Figure 3. Virulence factor genes detected in stains No. 4 of pathogenic strains by PCR**.** M: Marker DL2000；1: Esp; 2: GelE; 3: CylA; 4: Asa1; 5: Asa373; 6: Ace; 7: Efa; 8: EF0591; 9: EF3314. Nine virulence facter genes from the No. 4 pathogenic strains were amplified with PCR and the products were analysed by gel electrophoresis in 0.8% (w/v) agarose gel.

Table 4. Detection of virulence factor genes by PCR. Nine virulence factor genes were tested in *E. faecalis* from 3 sources of lambs. Pathogenic strains have more combination of virulence factors gene than that of normal flora.

Virulence factors gene	Pathogenic strains												Intestinal strains			Respiratory strains
	1	2	3	4	5	6	7		8	9	10	11	1	2	3-30	1-15
Esp	+	+	+	+	-	+	+	+	-	+		-	-	-	-	-
GelE	+	-	-	-	-	-	-	-	-	-		-	+	+	-	-
CylA	-	+	+	+	+	+	+	-	-	+		-	-	-	-	-
Asa1	+	+	+	+	+	+	+	-	-	+		-	-	+	-	-
Asa373	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-
Ace	+	+	+	+	+	+	+	-	-	+		-	-	-	-	-
efa	+	+	+	+	+	+	+	-	-	-		-	-	-	-	-
EF0591	-	+	+	+	+	+	+	-	-	+		-	-	-	-	-
EF3314	+	+	+	+	+	+	+	-	-	+		-	+	+	-	-

Note: + mean there is the virulence factor gene; - mean there is no the virulence factor gene.

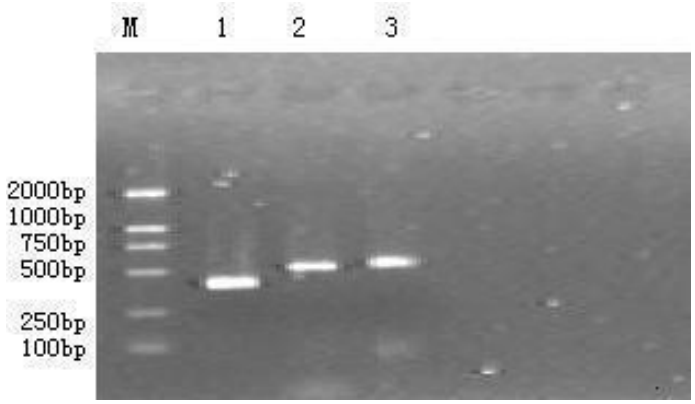

Figure 4. Virulence factor genes GelE, Asa1, and EF3314 were detected in stain No. 2 of the non-pathogenic stains isolated from the intestine by PCR. M: DL2000; 1: GelE; 2: Asa1; 3: EF3314. Nine virulence facter genes from the No. 2 non-pathogenic stains were amplified with PCR and the products were analysed by gel electrophoresis in 0.8% (w/v)agarose gel.

Table 5. The homology (%) of the 3 common virulence factor gene fragments from normal flora are compared among pathogenic *E. faecalis* and Genbank.PCR products of 3 common virulence factor gene fragments (GelE, Asa1 and EF3314)cloned into vector, and sent them to Sangon Bio-Engineering Company for sequencing, then the homologous of DNA fragments were analyzed with ClustalX sequence analysis software.

	***E. faecalis* from normal Flora**		
	GelE	**Asa1**	**EF3314**
E. faecalis from GenBank	98.03	95.00	99.30
Pathogenic *E. faecalis*	99.53	96.20	99.12

cant hemolysis, and this feature did not disappear upon passages. While only 8 of 30 strains of normal flora showed hemolytic clinical isolates (GelE（M37185, Asa1（X17214）, and EF3314（NC004668) (Table 5).

DISCUSSION

The results of biochemical characteristics showed that properties and it's hemolytic disappeared after the limited passages. Experimental infection of mice also verified this. All these evidences indicated that hemolytic of *E. faecalis* still was an indispensable factor in lambs encephalitis caused by *E. faecalis*.

The mechanism of drug resistant in *E. faecalis* is more complex, which include natural resistance, acquired drug resistance and multi-drug resistance (Zhang et al., 2001; Li and Zhang, 2004). Susceptibility test results indicated that the pathogenic strains were resistant to most antibiotics in various degrees,and individual strains from normal flora is also resistance to certain antibiotics, the reason for these phenomena in *E. faecalis* may be due to the complete manifestation of natural resistance and acquired drug resistance under the pressure of a large number of antibiotics used in clinic. Pathogenic strains were resistant to streptomycin, gentamicin and erythromycin. Interestingly, there were also individual strains of normal flora *E. faecalis* which were resistant to tetracycline, erythromycin and streptomycin, suggesting that drug resistance of *E. faecalis* was very universal. Researchers had confirmed that some drug resistant plasmid could be transferred each other among different sources *E. faecalis* or between *E. faecalis* and other bacteria (Launay et al., 2006; Simjee et al., 2006; Jacobsen et al., 2007). Multi-drug resistance genes could also be horizontally transferred between humans and poultry (Lim et al., 2006). Antibiotics are used widespread in animal husbandry and agriculture, which may increase the transmitted opportunity of *E. faecalis* drug resistance, including the *E. faecalis* in normal flora of humans and animals, these situation may give some *E. faecalis* from normal flora more opportunity to become pathogenic strains. Therefore, one should be very cautious when come to use any antibiotics in human medicine and veterinary practice.

Pathogenicity island usually was found in *E. faecalis*, there were several virulence factor genes in those pathogenicity island, different virulence factor genes had different roles in the course of infectious disease (Heikens et al., 2007, 2008; Dupont et al., 2008). Some

researchers found that *E. faecalis* from different sources had different combination of virulence factor genes in medical clinic (Mannua et al., 2003; Roberta et al., 2004). The pathogenicity of *E. faecalis* from clinical isolates was more virulent than from that animal manure and dairy products. Our study indicated that virulence factor genes could be detected both in *E. faecalis* causing lambs encephalitis and in normal flora *E. faecalis*. But pathogenic isolates had more combination of virulence factor genes than the isolates of normal flora. This was basically consistent with the results which Roberta reported (Roberta et al., 2004). Our study showed that EF3314 and GelE appear in normal flora *E. faecalis* simultaneously, Maria also reported that GelE can be easily detected in *E. faecalis* from dairy (Lopes et al., 2006). All these suggested that GelE has a high frequency in the non-pathogenic *E. faecalis*. Sequencing results showed that sequence homology of these 3 virulence factor gene fragments in normal flora *E. faecalis* (GelE, Asa1 and EF3314) was over 95 and 96% compared with corresponding sequence from GenBank and from *E. faecalis* causing lamb encephalitis. These results showed that the 3 virulence factor gene fragments had high homology among 3 *E. faecalis* from different sources of lamb. It suggests to us once again that there is a close relationship among *E. faecalis* from veterinary clinic, medical clinic and animal normal flora. We still do not know clearly the detailed mechanisms how *E. faecalis* from normal flora becomes pathogenic strains.

It is well known that *E. faecalis* is commonly used as microbial agent that has played a positive role on prevention of intestinal infectious diseases and regulation of microbiota homeostasis balance in recent years (Garcia et al., 2004). But we should also realize that *E. faecalis* as an ecological agent may artificially increase the opportunity to delivery the drug resistance and transfer virulence factor gene in the process of using these ecological agent considering its particularity.

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