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Full Length Research Paper

Identification and genotypic analysis of *Streptococcus* spp. isolated from Giant Pandas in China by PCR-based methods

Li Wan[1#], Shi-jie Chen[2#] Cheng-dong Wang[3#], De-Sheng Li[3#], Zhi-he Zheng[4], Cheng-dong Wang[4], Qi-gui Yan[1,5]*, Wan-zhu Guo[1], Xiao-yu Wang[1] and Ling Guo[1]

[1]College of Veterinary Medicine, Sichuan Agricultural University, Ya'an 625014, P. R. China.
[2]Entry-exit Inspection and Quarantine of Sichuan province, Cheng Du 610041, P. R. China China.
[3]Conservation and Research Center for Giant Panda, Ya'an 625000, P. R. China.
[4]Chengdu Research Base of Giant Panda Breeding, Chengdu 610081, P. R. China.
[5]Key laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an 625014, P. R. China.

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PCR-based methods were frequently used for identification and analysis of microorganisms. In this study 8 *Streptococcus* strains isolated from Giant Pandas in different parts of China were identified by amplification of 16S ribosomal RNA sequence by PCR. Genomic diversities of 8 identified *Streptococcus* strains were analyzed by repetitive extragenic palindromic PCR (REP-PCR) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR). REP-PCR and ERIC-PCR fingerprinting showed different patterns of each *Streptococcus*. The cluster analysis revealed there is a low level of similarity among these isolated *Streptococci* which were separated into different subtypes and the same serotype *Streptococci* were divided into sub-genotypes. REP-PCR and ERIC-PCR fingerprinting results were correlated with serotype. The results also demonstrated the two PCR methods have good discriminative ability and have potential as rapid methods for typing *Streptococcus* as well as high genomic diversity of *Streptococci* of pandas.

Keywords: *Streptococcus*, REP-PCR, ERIC-PCR, serotype, Giant Panda.

INTRODUCTION

The Giant panda, *Ailuropoda melanoleuca*, is one of the most rare species in the world and are unique to China, they are the First-grade state protected animal in China, who are praised as "Living Fossil" and "National Treasure" of our country. *Streptococcus* species are widely distributed in nature, such as water, dust, alimentary tract, respiratory and genitourinary tracts, milk, etc. They are major pathogens for animals and human beings. The species of *Streptococcus* spp. are diversified including non-pathogenic normal bacteria in human or animal, and those which cause human or animal diseases. The cases of Giant Panda infected by β-hemolytic *Streptococcus* have been reported (Zheng et al., 1999).

Identification of streptococcal species is currently based on observation of the cultural and morphological characteristics, determination of the biochemical pattern or antigenic characteristics. There are different typing methods for *Streptococcus* spp. The traditional classification is based on hemolytic or antigenic characteristics. However, these methods have their own limits. With the development of molecular biology, methods based on PCR technique had been recognized as having the great discriminative ability to differentiate isolated strains by

*Corresponding author. E-mail: yanqigui@126.com.

#Authors contributed equally.

producing DNA fingerprints that are specific for individual strains. REP-PCR and ERIC-PCR methods were widely used for analyzing *Streptococcus* spp. (Alam et al., 1999; Coffey et al., 1995; Harrington et al., 2007). Analysis of 16SrRNA squence of *Streptococcus* spp. by restriction fragment length polymorphism PCR (RFLP-PCR) can be a reliable method to distinguish different species of *Streptococcus* (McDonald et al., 2005). A new gene glucose kinase (gki) –PCR-denaturing gradient gel electrophoresis (DGGE) technique used to discriminate different *Streptococcus mitis* group strains had been reported van Vliet et al. (2009). These methods were proved to be fast, sensitive and reliable for differentiation of microorganisms.

The aim of this study was to isolate *Streptococcus* from Giant pandas in China, identify them by amplifying 16S ribosomal RNA sequences, and trying to analyze the genetic diversity among *Streptococcus* spp. isolated in Giant pandas by REP-PCR and ERIC-PCR techniques.

MATERIALS AND METHODS

Bacterial isolation

Streptococcus strains were isolated from samples collected from different locations of different Giant pandas in Giant Panda Conservation and Research Center in Ya'an Bi Feng Xia Base of China Suspected *streptococcus* strains were purified and initially identified by biochemical tests and kept at -20°C in 30% glycerol sera medium to be further identified.

DNA preparation

Genomic DNA of *Streptococcus* spp. was extracted by the CTAB/NaCl mini-prep protocol (Barney et al., 2001). All *streptococcus* isolates were grown in 5mL of Luria-Bertani (LB) broth (Oxoid) containing 10% sera with 160 rpm shaking for 18 h at 37°C. Then, 1.5 ml LB broth culture was centrifuged at 10,000 g for 2 min. The cell pellet was re-suspended in 567 µl TE and mixed with 30 µl 10% SDS and 3 µl 20 mg/ml protase K; then the mixture was kept for 1 h at 37°C; 100 µl CTAB and 100 µl 5 mol/L NaCl were added in the mixture, 65°C for 10 min, DNA templates were extracted by phenol/chloroform method, and DNA were stored at -20°C for amplification of 16S ribosomal DNA sequence.

Identification by 16SrRNA PCR amplification

The primers used for amplification of 16S rDNA sequence were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3' (Greisen et al., 1994). The per PCR reaction (50 µl) contained 10×buffer 5 µl, dNTPs(2.5 mmol/L) 5 µl, TaqDNA polymerase (TaKaRa) (5 U/µl) 1µl, $MgCl_2$ (25 mmol/L) 3 µl, primers(10 µmol/L) each 2 µl, template DNA 3 µl and add ddH_2O to 50 µl volumes. Amplification was performed in a Thermal Cycler (Bio-BRI) with temperature ramping as follows: denatured for 10 min at 95°C, followed by 30 cycles of 30 s at 95°C, 3 min at 53°C, and 1.5 min at 72°C; then extension at 72°C for 8 min.

The purified PCR product was cloned into pMD18 Simple vector; the recombinant plasmid was transformed into competent cell DH5α, the positive plasmid screened were sent to Invitrogen in Shanghai to sequence.

Serotyping

Determinations of the serotypes of Streptococcus strains identified by16S rRNA was performed according to the procedures of specification of Strep Latex Slide Agglutination Test Kit (purchase from MICROGEN). This Kit was used for only separating A, B, C, D, F and G serotype of *Streptococcus* spp.

REP-PCR

The primers REP1R (5′-III ICG ICG ICA TCI GGC-3′) and REP2I (5′-ICG ICT TAT CIG GCC TAC-3′) (Versalovic et al., 1991) were synthesized by Invitrogen. The PCR reaction (25 µl) contained 10×buffer 2.5 µl, dNTPs (2.5 mmol/L) 2.5 µl, TaqDNA polymerase (5 U/µl) 0.5 µl, $MgCl_2$ (25 mmol/L) 1.5 µl, primers (10 µmol/L) each 1 µl, template DNA 1 µl and add ddH_2O to 25 µl total volumes. The amplification was carried out by incubation of the mixture for 5 min at 95°C for pre-denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 65°C for 8 min. A final extension was performed at 65°C for 16 min.

The reaction products were stored at 4°C until they were electrophoresed on a 1.5% (w/v) agarose gel containing 1×TAE and 0.5 µg ml^{-1} ethidium bromide along with the DNA marker IV (TIANGEN) in 2 to 3 h.

ERIC-PCR

DNA of *Streptococci* identified by 16SrRNA was used as templates of ERIC-PCR. The primers 5'-ATG TAA GCT CCT GGG GAT TCA C-3' (ERIC1); 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (ERIC2) (Versalovic et al., 1991) were synthesized by Invitrogen. The PCR reaction (25 µl) contained 10×buffer 2.5 µl, dNTPs (2.5 mmol/L) 2.5 µl, TaqDNA polymerase (5 U/µl) 0.5 µl, $MgCl_2$ (25 mmol/L) 1.5 µl, primers (10 µmol/L) each 1 µl, template DNA 1µl and add ddH_2O to 25 µl total volumes. Amplification was performed in a Thermal Cycler (Bio-BRI) with temperature ramping as follows: denatured for 7 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 48°C, and 5 min at 72°C; and the final extension at 72°C for 7 min.

The reaction products were stored at 4°C until they were electrophoresed on a 2%(w/v) agarose gel containing 1×TAE and 0.5 µg mL^{-1} ethidium bromide along with the DNA markers IV (TIANGEN) in 2 to 3 h.

ERIC-PCR and REP-PCR fingerprints analyses

ERIC-PCR and REP-PCR fingerprints of amplified DNA fragments obtained from the agarose gel electrophoreses were recorded. REP-PCR and ERIC-PCR fingerprints were converted to a two-dimensional binary matrix and analyzed by using the biostatistical analysis program NTSYS-pc (Version 2.10). From bottom to top of the gel, if a band was present, it was assigned a value of 1, if it was absent at same horizontal place, it was assigned a value of 0. According to previous rules, the DNA sequence data was recorded in Ntedit and saved with format of nts. Dendrograms were generated based on Dice's similarity coefficient (SD) and the un-weighted pair group method using arithmetic averages (UPGMA).

RESULTS

Identification by 16SrRNA

16S rDNA production about 1500 bp was amplified from all suspected *Streptococcus* (Figure 1). The results of

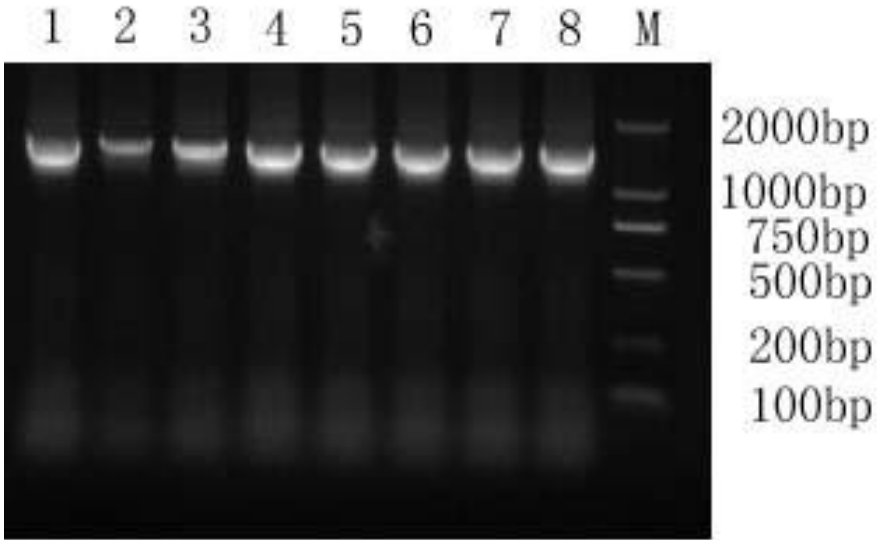

Figure 1. The profiles of 16Sr DNA amplication production of all suspected *Streptococcus* spp. 1. *S. pluranimalium* (isolated from anua of panda 3); 2, *S. dysgalactiae* (isolated from sole of panda 2); 3, Unknown (isolated from uterus of panda 1); 4, Unknown(isolated from vaginal of panda 1); 5, *S. dysgalactiae* (isolated from teeth of panda 2); 6. *S. suis* (isolated from throat of panda 5); 7. *S. alactolyticus* (isolated from nasal of panda 4); 8. *S. alactolyticus* (isolated from anus of panda 4).

Table 1. The 8 *Streptococcus* strains identified by 16SrRNA in this study.

Giant pandas	Symptom	Position	Species	Serotype	No.
Panda1	Endometritis	Uterus	*Streptococcus* spp.	B	1
Panda1	Endometritis	Vaginal	*Streptococcus* spp.	B	1
Panda2	Diapyetic sole	Sole	*Streptococcus dysgalactiae*	C	1
Panda2	Paradentitis	Teeth	*Streptococcus dysgalactiae*	C	1
Panda3	Alvei profluvium	Anus	*Streptococcus pluranimalium*	D	1
Panda4	/	Nasal cavity	*Streptococcus alactolyticus*	D	1
Panda4	/	Anus	*Streptococcus alactolyticus*	D	1
Panda5	Laryngopharyngitis	Throat	*Streptococcus suis*	/	1
Total					8

sequence analysis showed that 8 strains were identified as *Streptococcus* strains including 2 *Streptococcus dysgalactiae* isolated from panda 2, 1 *Streptococcus pluranimalium* isolated from panda 3, 2 *Streptococcus alactolyticus* isolated from panda4, 1 *Streptococcus suis* isolated from panda 5, and 2 other species of *streptococcus spp.* isolated from panda 1 .These strains were listed in Table 1.

The two *Streptococci* isolated from panda 1 are sharing 97, 97, 96, 96, 96 and 96% homology with *Streptococcus canis*, *Streptococcus pyogenes*, *Streptococcus uberis*, *Streptococcus iniae*, *S. dysgalactiae and S. dysgalactiae subsp. equisimilis* respectively. Generally, 16S rDNA sequence of bacteria sharing homology about 93% to 95% each other belong to the same genus (Fry et al., 1991) so the 2 isolations from panda1 are belong to *Streptococcus* spp.

Serotyping

The serotype results were listed in Table 1. *S. dysgalactiae* was serotype C, *S. alactolyticus* and *S. pluranimalium* were serotype D, 2 other species of *Streptococcus* spp. were serotype B, the serotype of *S. suis* was not determined because of the limitation of the Kit. 8 *Streptococcus* strains were divided into 4 groups according to the serotype of each strain.

REP-PCR

REP-PCR fingerprinting patterns of *Streptococcus* consisted of 4 to15 amplification bands ranging in size from 100 to 5000 bp (Figure 2). The reproducibility of this technique was demonstrated by repeated study. Each

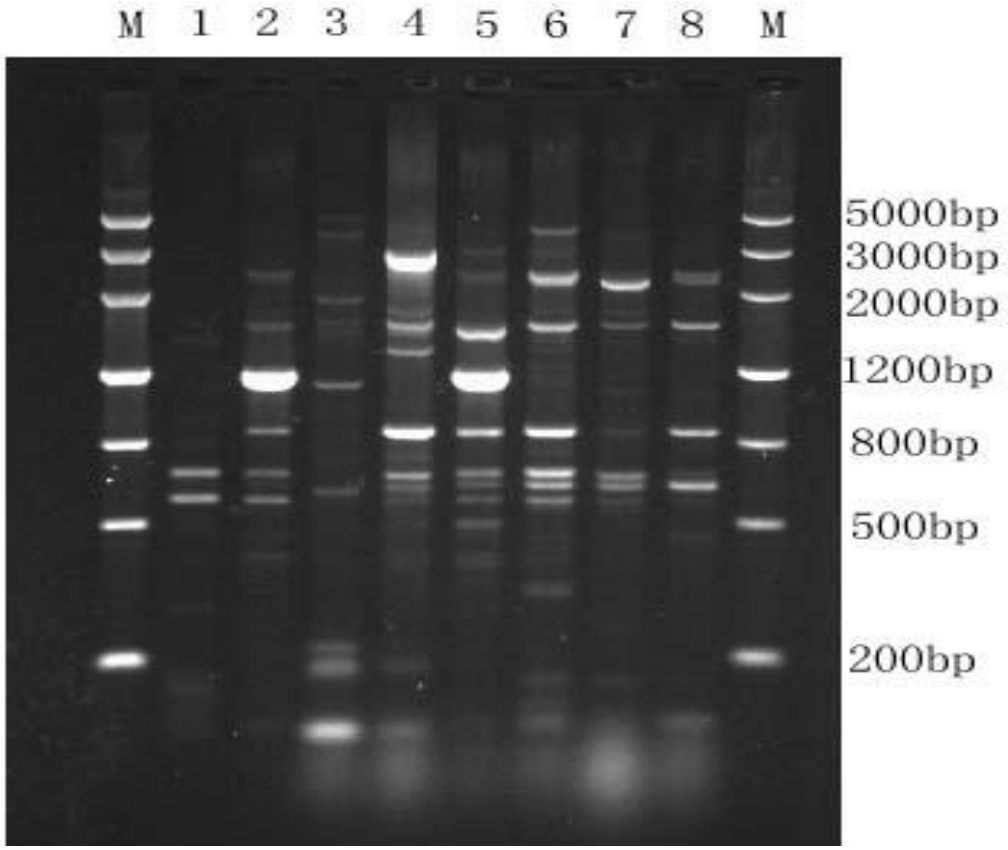

Figure 2. REP-PCR fingerprinting patterns abtained for 8 *Streptococcus* strains isolated from Giant panda. Lanes: M, Marker IV; 1.*S. pluranimalium* (isolated from anua of panda 3); 2. *S. dysgalactiae* (isolated from sole of panda 2); 3. Unknown (isolated from uterus of panda 1); 4. Unknown (isolated from vaginal of panda 1); 5. *S. dysgalactiae* (isolated from teeth of panda 2); 6. *S. suis* (isolated from throat of panda 5); 7. *S. alactolyticus* (isolated from nasal of panda 4); 8, *S. alactolyticus* (isolated from anus of panda 4).

strain gave almost a different REP-PCR fingerprinting although a common band about 700 bp was observed in 7 strains. Dendrogram was constructed by analysing the similarity among the different profiles with the cluster analysis software (Figure 3). According to dendrogram analysis, 2 *S. dysgalactiae* with 0.88 similarity formed cluster as one type, *S. suis* and one of *S. alactolyticus* shared 0.84 similarity were clustered as a type with another *S. alactolyticus* at 0.81 similarity. The remaining strains were distinct with similarity of lower than 0.80.

ERIC-PCR

With primer ERIC1and ERIC2, ERIC-PCR finger-printing profiles were obtained (Figure 4). Analysis of these strains with ERIC-PCR yielded six to eleven bands ranging in size from 50bp to 2500bp. ERIC-PCR fingerprinting profiles of individual strain was different. The profiles were repeated three times, however, some of minor amplification bands were inconsistent making the analysis more difficult. Dendrogram was constructed by the same method (Figure 5). Similar to REP-PCR, only few strains formed clusters as most strains have unique REP-PCR fingerprinting profile. The first cluster sharing 0.9 similarities consisted of 2 *S. dysgalactiae* isolated from feet and teeth of Giant panda 2, respectively. The second cluster with 0.85 similarities was composed of 2 *S. alactolyticus* isolated from anus and nasal of Giant panda4. The remaining strains were distinct with similarity of lower than 0.80.

DISCUSSION

Giant pandas as the First-grade state protection animal in China are important for biological diversity. In order to protect Giant pandas, we have conducted experiments to isolate and identifying microorganisms in Giant panda. *Streptococcus* spp. widely existing in nature may cause suppurative inflammation. In our study eight strains of *Streptococcus* spp. were isolated from different locations of Giant pandas some of which belonged to the same species such as *S. dysgalactiae* or *S. alactolyticus*.

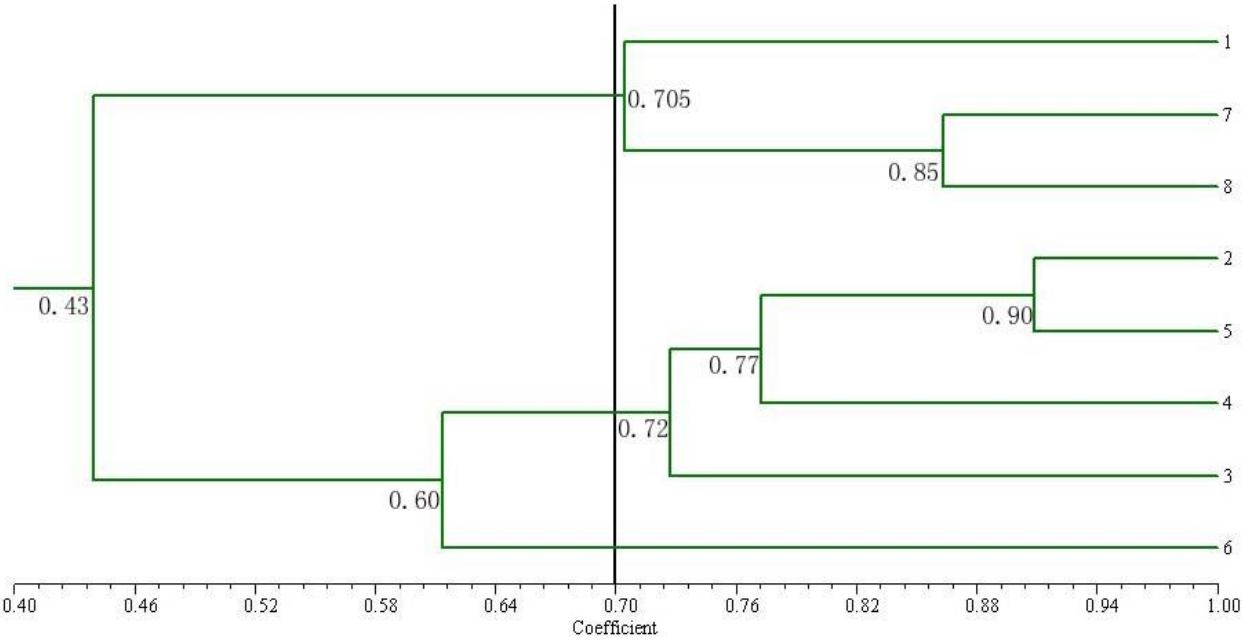

Figure 3. Dendrogram derived from REP-PCR profiles obtained with primers REP1R and REP2I. 1. *S. pluranimalium* (isolated from anua of panda 3); 2. *S. dysgalactiae* (isolated from sole of panda 2); 3. Unknown (isolated from uterus of panda 1); 4. Unknown (isolated from vaginal of panda 1); 5. *S. dysgalactiae* (isolated from teeth of panda 2); 6. *S. suis* (isolated from throat of panda 5); 7. *S. alactolyticus* (isolated from nasal of panda 4); 8. *S. alactolyticus* (isolated from anus of panda 4).

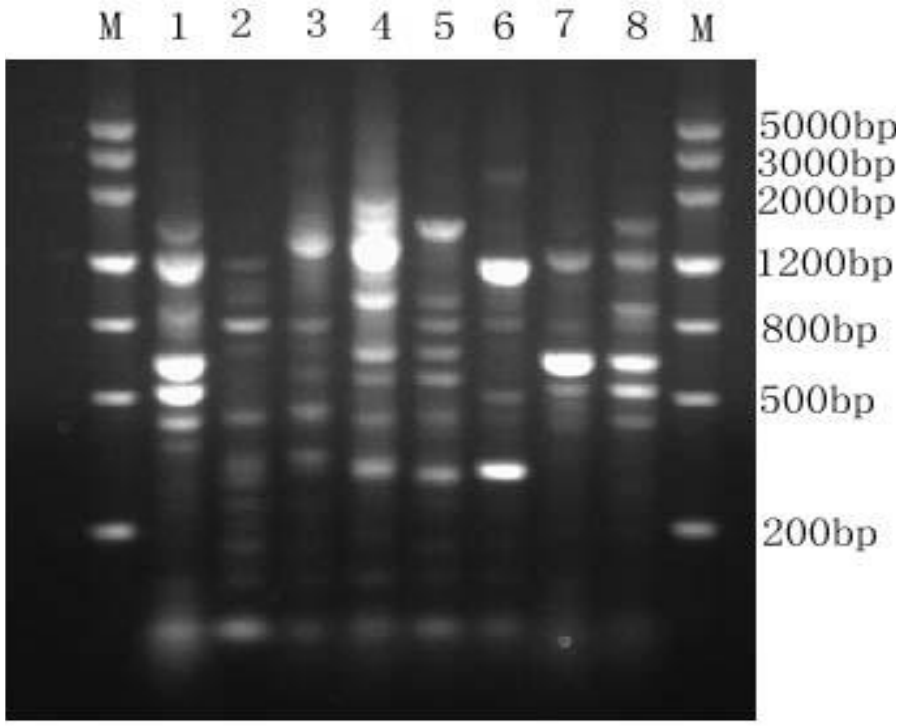

Figure 4. ERIC-PCR fingerprinting patterns obtained for 8 *Streptococcus* strains isolated from Giant pandas. Lanes: M, Marker IV; 1. *S. pluranimalium* (isolated from anua of panda 3); 2. *S. dysgalactiae* (isolated from sole of panda 2); 3. Unknown (isolated from uterus of panda 1); 4. Unknown (isolated from vaginal of panda 1); 5. *S. dysgalactiae* (isolated from teeth of panda 2); 6. *S. suis* (isolated from throat of panda 5); 7. *S. alactolyticus* (isolated from nasal of panda 4); 8. *S. alactolyticus* (isolated from anus of panda 4).

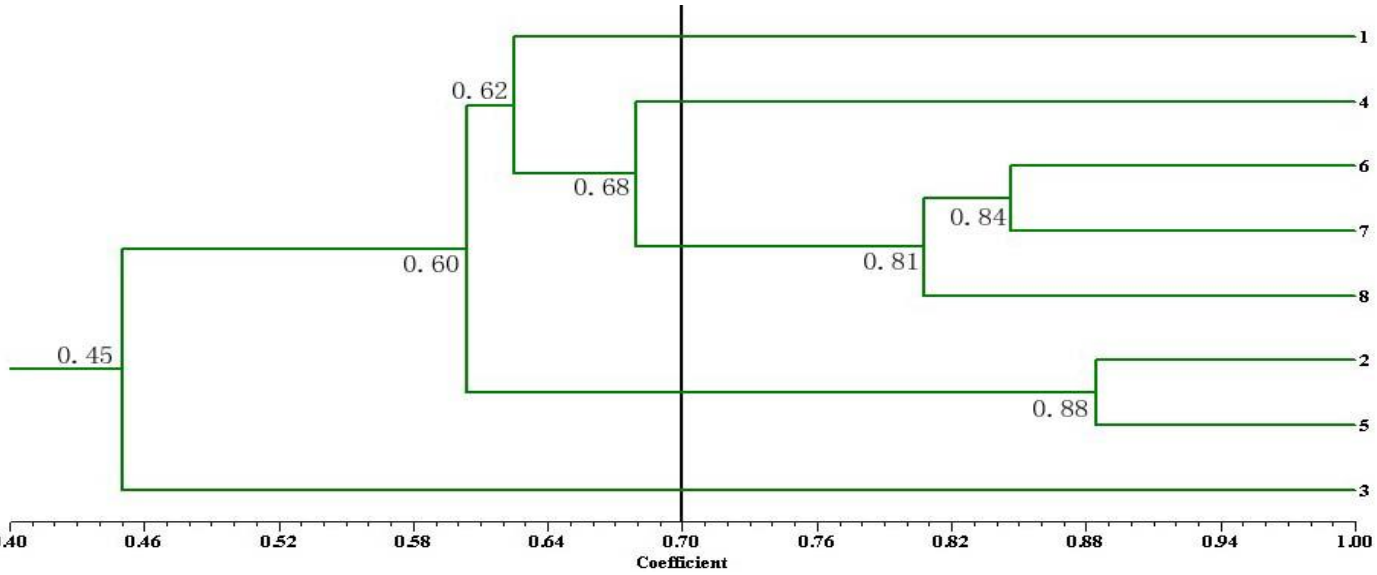

Figure 5. Dendrogram derived from ERIC-PCR profiles obtained with primer ERIC1 and ERIC2. 1. *S. pluranimalium* (isolated from anua of panda 3); 2. *S. dysgalactiae* (isolated from sole of panda 2); 3. Unknown (isolated from uterus of panda 1); 4. Unknown(isolated from vaginal of panda 1); 5. *S. dysgalactiae* (isolated from teeth of panda 2); 6. *S. suis* (isolated from throat of panda 5); 7. *S. alactolyticus* (isolated from nasal of panda 4); 8. *S. alactolyticus* (isolated from anus of panda 4).

However, their ERIC-PCR and REP-PCR finger-printing profiles were inconsistent. *Streptococci* isolated from different Giant pandas were different. Edwards *et al* had reported the entire gene of 16S rDNA about 1.5kb of *Streptococcus* which had been used for identification of *Streptococcus* because of its conservation and specificity for *Streptococcus* species. Identification of *Streptococci* by analysis of 16S rRNA had been reported (Hassan et al., 2001).

According to the results, 8 *Streptococcus* ware isolated from 5 Giant pandas. 2 *Streptococcus* were isolated from uterus excreta and vaginal discharge of panda 1 troubled with endometritis by 16SrRNA PCR method, these two *Streptococcus* are sharing 97, 97, 96, 96, 96 and 96% homology with *S. canis*, *S. pyogenes*, *S. uberis*, *S. iniae*, *S. dysgalactiae* and *S. dysgalactiae subsp. equisimilis* respectively. Those *Streptococcus* sharing high homology are all pathogenic to animal and human (Facklam et al., 2005; Galperine et al., 2007; Hughes et al., 2009; Lamagni et al., 2008; Ward et al., 2009). Panda 2 was troubled with paradentitis and its sole was diapyesis, 2 *S. dysgalactiae* were obtained from teeth and sole, respectively.

S. dysgalactiae is an important pathogenic bacterium which is reason of acute or chronic mastitisthe for cattle, goats and sheep (Chenier et al., 2008) even for fish (Nomoto et al., 2006). *S. pluranimalium* was isolated from panda 3 when it was Alvei profluvium. Seimiya et al. (2007) has reported *S. pluranimalium* was isolated from the brain and cerebrospinal fluid of a neonatal calf (Seimiya et al., 2007), so the isolated *S. pluranimalium* may cause Giant panda Alvei profluvium. 2 *S. alactolyticus* was isolated from nasal cavity and anus of panda4 who is a wild Giant panda. It is not confirmed that *S. alactolyticus* is pathopoiesis to Giant pandas. *S. suis* is another important pathogenic bacteria that can cause both animal and human arthrophlogosis and meningitis, it was outbreak that human was infected in Sichuan, China in 2006 (journal.shouxi.net), follow by human infected *S. suis* was reported in Vietnam (2006), Thi Hoang Mai et al. (2008), the United States (2008), Willenburg et al. (2006), etc.

Only Zhang et al has reported that panda infected β-hemolytic streptococci, however, *Streptococcus* isolated in this study has not been reported so for. Accordingly, this study has considerable contribution to *Streptococcicosis* of Giant panda.

PCR-based methods for genotypic analysis of *Streptococcus* spp. are widely applied. Valdes et al had used different molecular typing methods including REP-PCR and ERIC-PCR to evaluate the genetic diversity of *S. phocae* (Valdes et al., 2009). In this study, REP-PCR and ERIC-PCR were used for genotypic analysis of 8 *Streptococcus* REP-PCR fingerprinting profiles of each *Streptococcus* were inconsistent. It demonstrated genetic diversity of *Streptococcus* of Giant pandas. According to REP-PCR dendrogram analysis results, 2 *S. dysgalactiae* belonged to serotype C were divided into 2 sub-genotypes at 0.88 similarity, 2 *S. alactolyticus* and 1 *S. pluranimalium* belonged to serotype D were also separated into 3 sub-genotypes, the species of *S. pluranimalium* is not the same as *S. alactolyticus*, so they were separated from each other with lower similarity. Unfortunately, *S. alactolyticus* and *S. suis* sharing

relatively high similarity with 0.84 forming one cluster. Although the 2 unknown *Streptococcus spp.* belonged to identical serotype D, they did not form into the same cluster due to genetic variability.

Compared with REP-PCR, ERIC-PCR has a higher discriminative ability. 2 *S. alactolyticus* and 2 *S. dysgalactiae* were divided into 2 sub-genotypes with similarity 0.90 and 0.85, respectively. The remaining strains were distinct with similarity of lower than 0.80.

REP-PCR can be used for molecular epidemiologic analysis of *Streptococcus* (Al-Ghamdi et al., 2000; Matsumoto et al., 2001). REP-PCR and ERIC-PCR fingerprinting profiles suggested that there are genetic differences among to the identical *Streptococcus* species isolated from the same Giant pandas. It was demonstrated that REP-PCR and ERIC-PCR analysis can rapidly type 8 *Streptococcus*, and the results indicated that the genetic diversity of *Streptococcus spp.* in Giant pandas is high.

As a result, The REP-PCR and ERIC-PCR fingerprinting analysis results were correlated with serotype results. REP-PCR and ERIC-PCR are rapid methods for typing as well as investigating of the molecule epidemiology of *Streptococcus* spp.

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