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Sequence analysis of the *groEL* gene and its potential application in identification of pathogenic bacteria

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This study aims to develop a rapid method for identifying pathogenic bacteria based on the sequence difference of pathogenic bacteria *groEL* gene. A pair of universal degenerate primers was designed to amplify the *groEL* genes of 34 major species of 16 genus's of pathogenic bacteria. The conservation, variation and the interspecies phylogenetic relations of the *groEL* gene sequences were informatically analysed. Our results showed that the *groEL* gene fragments of the selected pathogenic bacteria could be amplified using the degenerate primer. The results of the sequence comparative study indicated that the *groEL* gene is characterized by conservation and variation, while the variation regions are distributed separately among the conserved regions, just like the mosaic. This conservation and variation coexistence of pathogenic bacteria *groEL* genes could provide a solid basis for the rapid identification of pathogenic bacteria and the development of a diagnostic microarray.

Key words: *groEL,* homology, phylogenesis.

INTRODUCTION

Although most infectious diseases were controlled to a low level with the development of medical science, the new and re-emerging infectious diseases continue to appear. And infectious diseases remain the major cause of death worldwide and the leading cause of illness (Franklin et al., 2004). In order to control infectious diseases more effectively, laboratory methods for the rapid and accurate identification of emerging and reemerging pathogens must be presented. The routine approaches for pathogen identification are cultivation, biochemistry reaction and serologic or immunological test. Those approaches are trivial, time-consuming and labor-intensive, especially for those slow-growing and hard-to-culture pathogens. Recently, typing methods began to evolve from phenotype-based methods toward genotype-based methods, including restriction endo-nuclease analysis (REA) of the total bacterial genome, pulsed-field gel electrophoresis (PFGE), arbitrarily primer PCR (AP-PCR) and PCR ribotyping (George et al., 2008). Ribosomal 16S rRNA, 23S rRNA, *slpA*, *selC,* and the *pheU* tRNA locus are usually selected as the target genes for successful ribotyping subgroups of bacteria (Gelsen et al., 1994; Gurtler et al., 1996; Anthony et al. 2000; Hayden et al., 2001; Corinne et al., 2008; Ghebremedhin et al., 2008). The *groEL* gene, which encodes a 60 kDa subunit (known as HSP60, 60 kDa chaperonin and heat shock protein), also has the potential to serve as a general phy-logenetic marker because of its ubiquity and conservation in nature. The heat shock protein is of great importance for maintaining cellular normal physiological function and is also the major antigen protein of many bacterial pathogens. It has been well documented that the *groEL* gene is one of the most conservative systems in nature (Kwok et al., 1999; Rebecca et al., 2002; Karuna et al., 2003; Jung-Hee et al., 2003; Yu-Hsiu et al., 2003; Eric et al., 2004; Sensu et al., 2004; Giuseppe et al., 2008). Despite the conserved nature of the *groEL* gene, the level of interspecies variation of *groEL* sequence is greater. Therefore, the *groEL* gene may be a good target gene for species classification. In the present study, *groEL* genes of 34 major species of 16 genus bacterial pathogens were analyzed for homology, variation and gene distribution.

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This study may provide better resolution for the rapid identification of pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains

34 species of 16 genuses of bacterial pathogens of medical interest were selected, and their *groEL* gene sequences were extracted from GenBank. The bacterial strains used in this study are listed in Table 1.

The conservation analysis of *groEL* gene

Given conservative sequence length parameter and variation value, *groEL* gene sequences of the bacteria strains were edited and compared to find out the conservative region of the gene and analyze conservative distribution according to bio-software ClustalX and Bio-edit.

Homology analysis of *groEL* gene

The *groEL* gene sequences of bacteria strains were analyzed for homology using bio-software DNAStar. The phylogenetic tree was drawn based on the homology analysis of *groEL* gene. The phylogenetic relationship among the bacterial classification and the importance of rapid diagnosis were analyzed at the same time.

The variation analysis of *groEL* gene

The variation analysis of *groEL* gene was conducted bas-ed on the homology analysis of *groEL* gene. The map of conservative sequence and variant sequence distribution was drawn accordingly. Furthermore, the variation rule of *groEL* gene sequence was analyzed.

PCR amplification of *groEL* gene

Using bio-software primer premier 5.0 (PREMIER Biosoft International, USA), a proper pair of universal primers were designed based on the homology analysis of the conserved sequence of *groEL* gene. Because of the lower homology within the region, a degenerate upstream primer was designed. The sequences of primers were P1:-5'AGTTACCCT XGG YCCZ AAAG-3', X is C or T, Y is T or C, Z is A or G; P2: -5'CAGCAAC-CACGCCTTCTTC-3', with an amplified fragment of approximately 600 bp in length. The template used for amplification was prepared as follows: bacteria grown in nutrient broth for 24 h were plated on selective agar plate and single colony was picked up after cultivation for 24 – 36 h. Bacteria suspension was made with 100 µl of re-distilled water, heated in 95℃ for 10 min, centrifugated at 12000 rpm for 1 min and the supernatant was used as PCR template. The PCR amplification was performed in 50 µl reaction volume of mixture containing 5 µl of 10 × PCR buffer, 4 µl of 20 mM dNTP, 3 µl of 2 mM, $MgCl_2$, 1 µl of 25 pmol forward and reverse primers, 5 µl of temple DNA, and 1 µl of 5U *Taq* DNA polymerase (Takara Bio). The condition for PCR thermal cycling was as follows: the PCR mixture was held at 95℃ for 4 min prior to the 35 cycles of PCR amplification in a thermal cycler (Eppendorf, Hamburg, Germany), the first 5 cycles are consisted of denaturation at 94℃ for 40 s, annealing at 46℃ for 50 s and extension at 72℃ for 1.5 min and followed the other 30 cycles: denaturation at 94℃ for 30 s, annealing at 53℃ for 45 s and extension at 72℃ for 1.5 min.

RFLP analysis of *groEL* gene

The *groEL* genes were amplified, purified and then digested with restriction enzymes Hae III (Takara Bio). The digested products were electrophoresed with a 1% TBE agarose gel in 0.5x TBE and visualized under UV light.

Hybridization analysis of *groEL* gene in oligonucleotide membrane array

The nucleic acid hybridization oligonucleotide probes were selected from the target region of different species, respectively (Table 2). All probes were synthesized in Takara Bio. Positive charge nylon membrane (Takara Bio) was used as array base. A 3 × 3 mm grid was formed with blunt pencil. The nylon membrane was immersed in distilled water for 10 min and then dried in Whatsman paper. Each probe was suspended to make a 25 pmol/µl solution. The solution was heated at 95℃ for 3 min and 1 µl of the solution was spotted on a corresponding position of the nylon membrane. Membranes with the probes were immersed in a Petri dish containing 0.5 ml DIG Easy Hyb solution (Roche, Indianapolis, USA) pre-warmed at hybridization temperature and pre-hybridization was perfomed at 50℃ for 30 min with gentle shaking. 5 µl PCR products were heated at 95℃ for 5 min, immediately cooled on ice and then added to newly pre-warmed hybridization solution. The membranes were hybridized in the solution at 50℃ for 2 h with gentle shaking. After hybridization, the membranes were washed 4 times with 0.25 × SSC-0.1% SDS for 2 min at 37℃. Before blocking, the membranes were washed in washing buffer (Roche) for 1 min, then immersed in 10 ml blocking buffer (Roche) for 30 min, and put to react with anti-digoxigenin antibody for another 30 min with gentle shaking. Color development was made with NBT-BCIP for 30–60 min in the dark without shaking. The reaction was stopped with tap water. The resulting images were visible and photographed.

RESULTS

The conservative sequences of *groEL* gene

The *groEL* gene sequences of pathogenic bacteria were analyzed by the bio-softwares ClustalX and Bioedit for the presence of the conservative regions in the genomes. It was found that the distribution of the conserved regions showed a characteristic pattern, that is, the mutant regions inserted into the conserved regions, just like mosaic (Figure 1). The conserved regions were mainly distributed in C region (nt900 - nt1500) and D region (nt1500 - nt1900), in which most conserved regions with high conservation were distributed in D region.

Variation of *groEL* gene

By software analysis, the variation map of *groEL* gene was obtained (Figure 1). As demonstrated in this map, it is evident that the *groEL* gene appears to be conserved. However, there are still considerable variations exist, especially in the A region (nt1- nt600). And these variations are found to be inserted among the conserved

Table 1. Bacteria strain resources used in the study.

Bacteria strain	Genbank accession No.	Source
E. coli	M11294	Guangzhou CDC
S. enterica	AY044102	Guangzhou CDC
S. typhi	U01039	Guangzhou CDC
S. typhimurium	AY044105	Guangzhou CDC
S. flexneri	AY044103	FuTian CDC
S. sonnei	AY044104	FuTian CDC
S. boydii	AY044101	FuTian CDC
E. aerogenes	AF306521	NanShan CDC
E. amnigenus	AB008140	NanShan CDC
E. asburiae	AB008137	NanShan CDC
K. pneumoniae	U81143	FuTian CDC
Listeria monocytogenes	AY922346	FuTian CDC
Vibrio parahaemolyticus	AF230952	Guangzhou CDC
Vibrio cholerae	AF230940	Guangzhou CDC
Yersinia enterocolitica	X59367	ShenZhen CDC
Yersinia pestis	AB064592	ShenZhen CDC
Campylobacter jejuni	AY628401	ShenZhen CDC
Helicobacter pylori	AJ558222	ShenZhen CDC
Aeromonas hydrophila	AY922365	GuangDong CDC
Aeromonas jandaei	AY922357	GuangDong CDC
Mycobacterium marinum	AF271346	GuangDong CDC
Brucella melitensis	L09273	GuangDong CDC
S. aureus	AF053568	GuangDong People Hospital
S. epidermidis	AF029245	GuangDong People Hospital
S. haemolyticus	U92809	GuangDong People Hospital
S. saprophyticus	AF029246	GuangDong People Hospital
S. warneri	AF053569	GuangDong People Hospital
S. anginosus	AY344497	GuangDong People Hospital
S. bovis	AY346155	FuTian CDC
S. mutans	AY344496	FuTian CDC
S. pneumoniae	AY344504	GuangDong People Hospital
S. sanguinis	AY344503	GuangDong People Hospital
N. gonorrhoeae	U64996	GuangDong People Hospital
N. perflava	AY837569	GuangDong People Hospital

regions, just like a mosaic with serrated appearance.

Homology of *groEL* gene

According to the comparative analysis of homology in different species of pathogenic bacteria, the phylogenetic tree was drawn using DNAstar (Figure 2). The phylogenetic relationships derived from comparisons of the *groEL* sequences are presented in Figure 2. The phylogenetic analysis revealed that the sequences of the *groEL* genes from bacteria species were divided into 5 clusters. From this phylogenetic tree, it is apparent that the highest homology exists in the same genus of bacteria with closest interrelationship, such as *Shigella*, *Salmonella* and *Staphylococcus* of the same genus. The homology of bacteria belonging to the same family is higher than those of bacteria of different families, such as, the interrelationship of *Salmonella* and *Shigella* in the same family is closer than that of *Salmonella* with *Mycobacterium* in the different family. However, intercross in interrelationship occurs among different genus of bacteria, such as *Staphylococcus* and *Streptococcus*. *Coccus* and *Bacillus* belong to different branch of bacteria. The phylogenetic tree also showed that the relationship between bacteria *groEL* genes and eukaryotics *groEL* genes is close. For example, *Aspergillus clavatus*, *Aspergillus fumigatus, Drosophila melanogaster* and *Mycobacterium marinum* are highly related. But *Cryptococcus neoformans* and *Plasmodium vivax* are relative far to

Table 2. Probes used in the study.

Probe name detection range	Probe sequence (5' to 3')	Genbank accession number
Universal probes	*CTAAAGCGATTGCTCAGGTTG*	X62914
Salmonella spp	*TGAACCCGATGGACCTGAAACG*	AY044102
Salmonella enterica	*TGAACCCGATGGACCTGAAACG*	AY044102
Salmonella typhi	*AAGGCGTGGTTGCTGGTGGTGG*	U01039
Salmonella typhimurium	*CTCCGCTAACTCCGACGAAACC*	AY044105
Shigella spp	*ACCATCTCCGCTAACTCCGACG*	AY044103
Shigella flexneri	*TCTCCGCTAACTCCGACGAAA*	AY044103
Shigella sonnei	*ACCATCTCCGCTAACTCCGACG*	AY044104
Shigella boydii	*ACCATCTCCGCTAACTCCGACG*	AY044101
Streptococcus spp.	*CAAGTAGGTGCGATTTCTGC*	AF053568
S. aureus	*ATCGTGCTAAACCGTATGCGTG*	AF053568
S. haemolyticus	*AAACCGTATGCGTGGAAC*	U92809
S. epidermidis	*TAAACCGTATGCGTGGAAC*	AF029245
Streptococcus spp.	*GAGGATGCTCTAAATGCCACA*	AY344504
S. pneumoniae	*TCCAATCGGTATTCGTCGTG*	AY344504
Positive control	-	
Negative control	-	

Postive control is digoxigenin labeling plasmid *pBR328/BamHI*. Negative control is plasmid *pBR328/BamHI*.

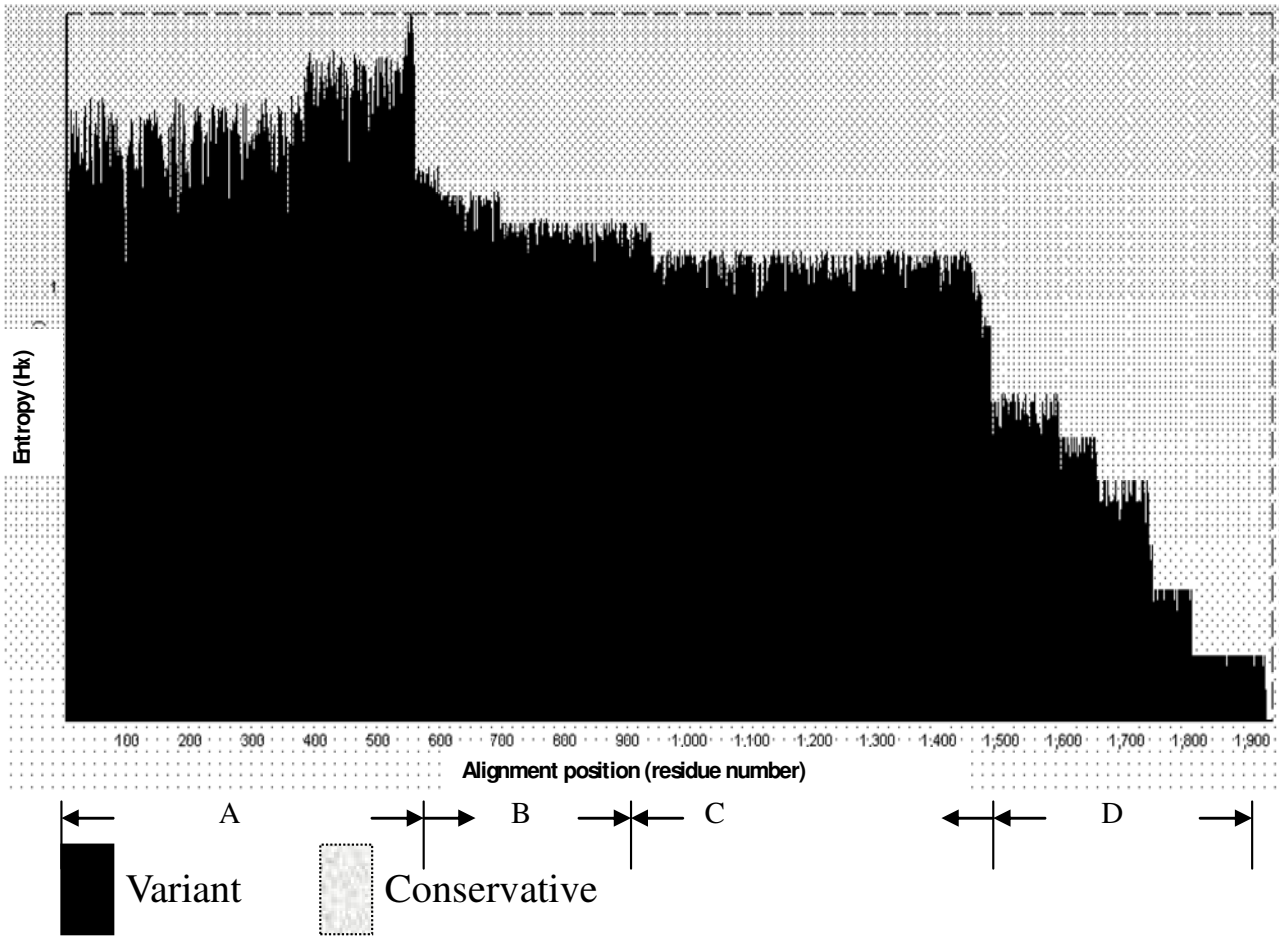

Figure 1. Analysis of *groEL* gene divergence. There are 4 variant regions in *groEL* gene. A region is the most mutable one; D region is the least mutable one.

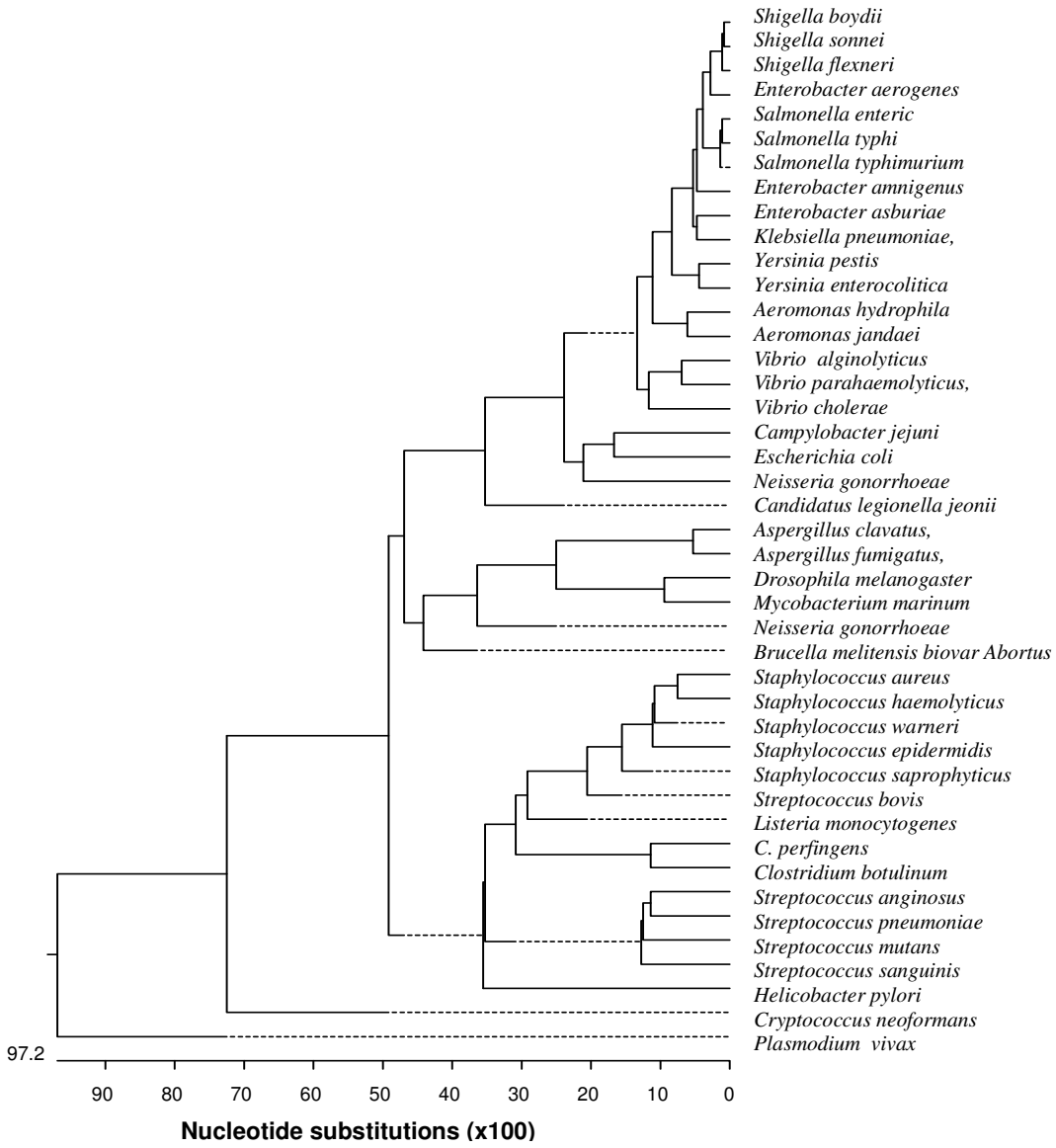

Figure 2. Phylogenetic tree of bacteria and eukaryotics *groEL* gene.

bacteria.

PCR amplification of *groEL* gene sequence

The *groEL* gene in intestinal pathogens, such as *E. coli*, *Salmonella enterica*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Vibrio parahaemolyticus*, *Campylobacter jejuni* was successfully amplified by PCR and a DNA fragment of 600bp could be obtained from all the pathogenic bacteria (Figure 3), indicating that the universal degenerate primer can amplify the *groEL* gene effectively.

PCR-RFLP analysis of *groEL* gene products

The PCR-amplified products of *groEL* gene of *S. enterica*, *S. typhi*, *S. typhimurium*, *S. flexneril, Shigella sonnei* and *Shigella boydii* were identified by RFLP analysis. It is apparent that the RFLP patterns were rather clear with definite DNA bands in size except *S. typhi* and *S. typhimurium* because of their similar patterns (Figure 4). So this method can be used for partially identification of pathogenic bacteria.

Hybridization analysis of *groEL* gene in oligonucleotide membrane array

The probes were designed, synthesized and dotted on the oligonucleotide membrane (Figure 5). The PCR products were then hybridized with probes on the membrane followed by color development under appropriate condition. Some bacterial species were tested with the

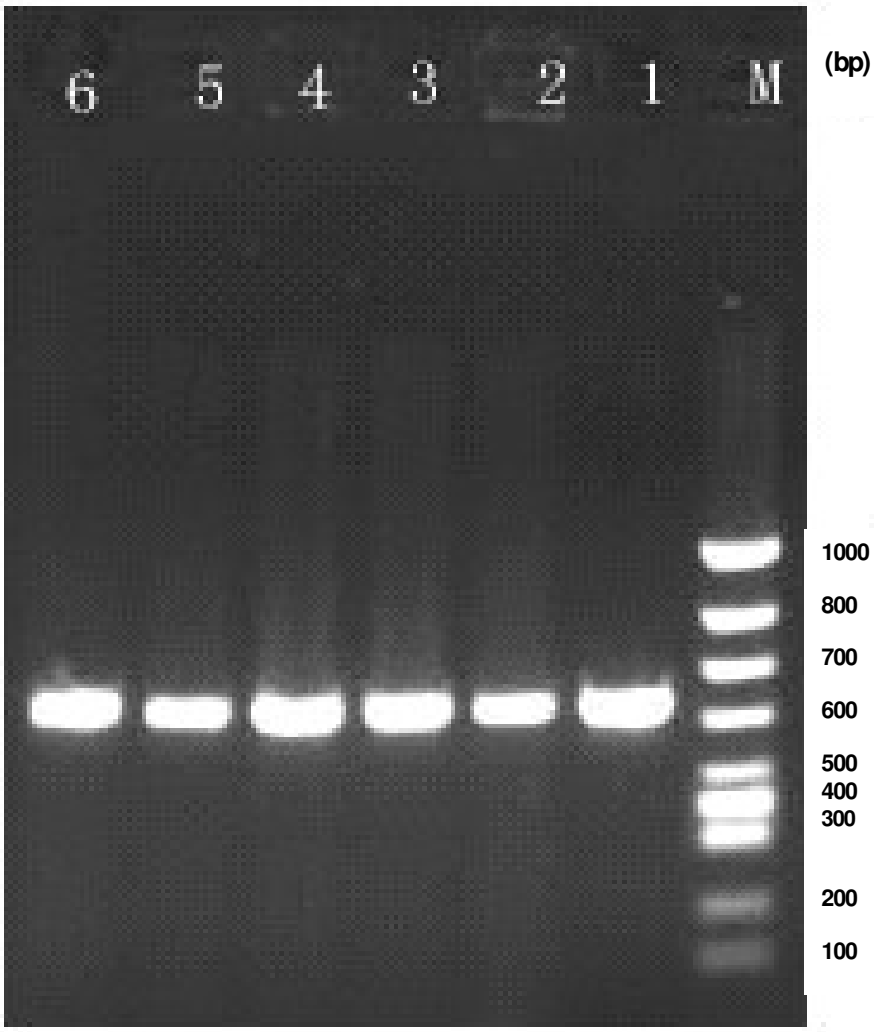

Figure 3. Amplification of *groEL* gene fragment of some pathogenic bacteria. Lanes 1–6 are PCR products. 1: *E. coli*; 2: *S. enterica*; 3: *S. flexne*; 4: *K. pneumonia*; 5: *V. parahaemolyticus*; 6: *C. jejuni*; Lane M: DNA molecular marker.

oligonucleotide array method. The results showed that high sensitivity and specificity of hybridization results were obtained with these species of bacteria, including *S. flexneri, S. sonnei, S. pneumoniae, S. aureus, Staphylococcus haemolyticus, Staphylococcus epidermidis, S. enterica*, *S. typhimurium, S. typhi.* As to *S. boydii*, we found poor signals because of the low amount of oligon-ucleotide dotted on the membrane (Figure 6).

DISCUSSION

The traditional methods involved in the discovery of the infective agents are cultivation, isolation, biochemical identification and the serological tests to the suspected pathogens. However, these methods are more or less trivial and time-consuming with a rather low detection rate. This is disadvantageous especially during the outbreak of an epidemic infection when the pathogens should be rapidly identified to meet the requirement for effective control of infection or disease. Molecular biological technology provides a better resolution in the rapid identification of pathogens.

At present, various DNA-based techniques have been developed to identify pathogenic bacteria, such as PCR-RFLP, PCR-SSCP, APPCR, PF-GE, multiple PCR and PCR aiming at speci-fic gene. In addition, the high through-out biochip is now being available for rapid identification of microbes (Hyun-Hee et al., 2002; Bangxing et al., 2004; Carl et al., 2004; Gerard et al., 2004; Kensuke et al., 2004; Rousseaux et al., 2004; Nonglak et al., 2005; Ana et al., 2006; Il-Jin et al., 2007; Ling-Xiang et al., 2007; Zhengshan et al., 2008). The

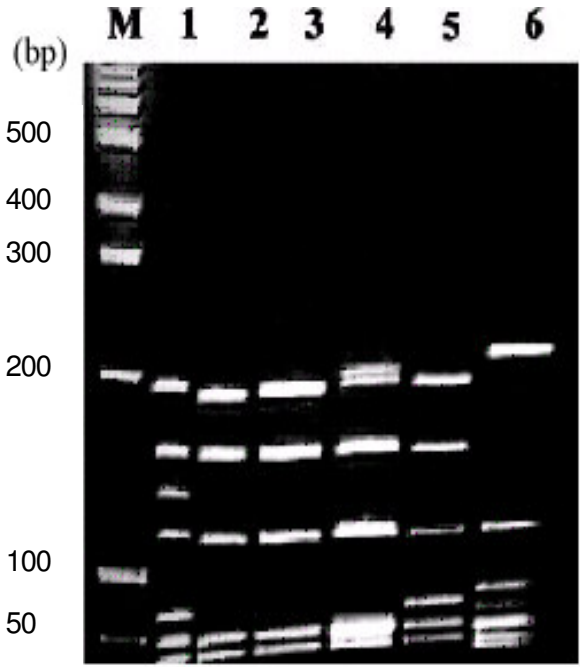

Figure 4. RFLP analysis of *groEL* gene fragment of some pathogenic bacteria. Lanes 1 – 6 are RFLP analysis patterns. 1: *S. enteric;* 2: *S. typhi*; 3: *S. typhimurium*; 4: *S. flexneri*; 5: *S. sonnei*; 6: *S. boydii*; Lane M: DNA molecular marker

p	b	j	f	q
	c	k	g	
a	d	l	h	
	e	m	i	
p		o	n	q

Figure 5. The probe position of the oligonuc-leotide array. The letter in the grids is probe name as indicated in Table 2.

conserved genes of pathogenic micro-organisms are usually used as the target gene in the identification of pathogens, such as 16S rRNA gene, 23S rRNA gene, 16-23S rRNA space region. In these con-served genes, 16-23S rRNA space region is a short gene fragment with large extent of variations; 23S rRNA also has great variation in bacterial species, but its sequence is incomplete; 16S rRNA is most conservative among species and its sequences is the most complete which can be used for the classification of bacteria (Gelsen et al., 1994; Gurtler et al., 1996; Anthony et al., 2000; Hayden et al., 2001; Corinne et al., 2008; Ghebremedhin et al., 2008). Heat shock protein is a highly conserved protein, whose encoding gene *groEL* constitutes to be the most conserved component in evolution. The *groEL* gene has been used as the target gene in the typing and identification of *Salmonella*, *C. jejuni* and *Staphylococcus* on account of its complete database (Goh et al., 1996; Satheesh et al., 2002; Karenlampi et al., 2004). Thus, in this study, we select the groEL gene as the target gene to investigate its significance in the identification of pathogenic bacteria.

It was found that the *groEL* gene appeared to be conserved and mutable, and therefore can be used as a general phylogenetic marker to construct the phylogenetic tree. In this way, the phylogenetic tree of various pathogenic bacteria can be drawn based on *groEL* gene using the bio-software and the phylogenetic tree constructed was found to be consistent with those based on 16S rRNA gene and 23S rRNA gene. Even though, the *groEL* gene analysis has several characteristics different from those of analysis of other genes. Compared with the 16S rRNA gene and 23S rRNA gene, the *groEL* sequences have higher divergence for species. The *groEL* gene is more heterogeneous than 16S rRNA gene and 23S rRNA gene and it is useful in intraspecies differentiation. So the *groEL* sequence analysis is useful not only in interspecies differentiation but also in intra-species differentiation. The species were characterized by phylogenetic analysis based on *groEL* gene sequences revealed that species within a group are highly related. For example, *S. flexneri, S. sonnei* and *S. boydii* are highly related. Similarly, *S. enterica*, *S. typhimurium* and *S. typhi* are highly related. From this phylogenetic tree, it is apparent that the homology of bacteria belonging to the same family is higher than those of bacteria of different families, such as, the interrelationship of *Salmonella* and *Shigella* in the same family is closer than that of *Salmonella* with *Mycobacterium* in the different family. From the result of variance analysis on *groEL* gene, it is evident that its variation is rather high, especially among interspecies. There are 4 variant regions in *groEL* gene, in which the A region appears to be the most mutable one, while the D region is the least mutable one. According to the distribution of *groEL* gene, we have designed the PCR universal primers and by the use of a pair of primers thus synthesized, all the corresponding gene fragments in pathogenic bacteria could be amplified by PCR under the proper amplification conditions. Meanwhile, the detection probe for genus and species of bacteria can be also designed based on the variant region.

Subsequently, we can use PCR-RFLP or PCR-SSCP in the classification of pathogenic bacteria as well, and can also take advantage of special probes for spot hybridization detection. Above all, we can still design genus, species or type probes based on the variant region as well as construct the high throughput, high effective and parallel disposal microarray for bacterial identification from genus to species and type in the same membrane array. Our study showed that satisfactory hybridization results with good sensitivity and specificity were obtained with these tested bacteria. *S. boydii* gave weak signal

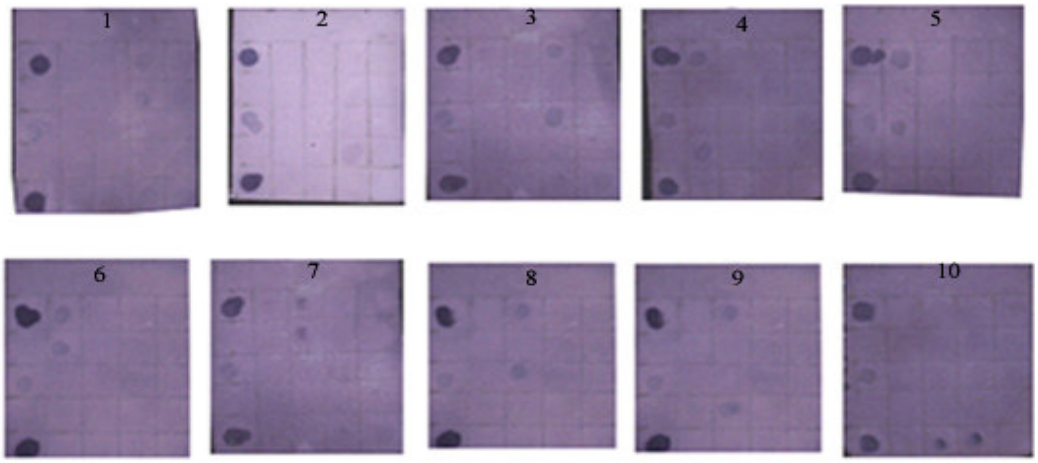

Figure 6. Membrane array hybridization assay results of some pathogenic bacteria. Lanes 1 - 10 are hybridization results of different bacterial species. 1: *S. flexneri*; 2: *S. boydii*; 3: *S. sonnei*, 4: *S. typhimurium*; 5: *S. typhi*; 6: *S. enterica*; 7: *S. aureus*; 8: *S. haemolyticus*; 9: *S. epidermidis*; 10: *Streptococcus pneumonia.*

because of the low amount of oligonucleotide dotted on the membrane. Proper design of probes is the key factor for successful oligonucleotide array hybridization. Hybridization conditions and bacteria gene mutations often affect the sensitivity and specificity of the detection. In addition to probes, different labeling methods also influence the result of detection.

In conclusion, the *groEL* gene is useful for the identification and characterization of pathogens.

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