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Full Length Research Paper

Genetic diversity of *Escherichia coli* isolated from commercial swine farms revealed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and repetitive extragenic palindrome PCR (REP-PCR)

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The objective of this study was to use enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and repetitive extragenic palindrome PCR (REP-PCR) for the analysis of genetic diversity among *Escherichia coli* strains isolated from commercial swine farms in Sichuan province of China. Thirty four strains of *E. coli* were selected by selective medium and conventional biochemical test from fresh stool samples of swines in five farms in Sichuan province. The isolates were identified by 160 kinds of *E. coli* O serums. The results show that 30 strains were determined among 34 *E. coli* isolates, 12 kinds of O serogroups were obtained on the basis of the agglutination test. The predominant types are O_{23}, O_{113} and O_{120}, representing 35.4%. Furthermore, the genotypes and phylogenetic relationship of all isolates were analysed by Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and repetitive extragenic palindrome PCR (REP-PCR), 34 *E. coli* isolates were clustered to 19 ERIC-PCR genotypes and 13 REP-PCR genotypes. The isolates from the same farm or sharing the same serotyping showed different genotype. And the isolates which could not be serotyped were genotyped by ERIC-PCR and REP-PCR. The analysis of genetic type and original source revealed that isolates from different farms had different genetic types. The subtypes of *E. coli* are also different within a single farm. Genetic variability with *E. coli* strains isolated from swine farms in China has been demonstrated. The presence of ERIC-PCR and REP-PCR sequences in the genome of *E. coli* was confirmed. ERIC-PCR and REP-PCR techniques are more rapid methods for molecular typing of *E. coli* strain. They are also useful methods for diversity survey of *E. coli* and the two methods analyzes genetic diversity of *E. coli* isolated in Sichuan of China.

Key words: *Escherichia coli*, serotype, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), repetitive extragenic palindrome PCR (REP-PCR).

INTRODUCTION

Escherichia coli is one of the strain widely distributed in nature and intestinal tract of animal. Genetic diversity exists widely among strains, so that using traditional bacterial taxonomy proves problematic when differentiating between strains that share a close genetic relationship. The traditional method for subspecies typing among the *E. coli* has been serotyping. The serotype is based on the antigenic properties of the O-antigen (surface polysaccharide) and H antigen(s) (flagellar). Typing the O-antigen itself denotes the serogroup, and

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Table 1. The results of *E. coli* isolation.

Swine house	Serial number	Original	King of sample	Number
A	A1	Farrow	Stool	1
A	A2	Stag	Stool	1
A	A3, A4, A5, A6	Weaner	Stool	4
B	B1, B3, B4	Pregnancy sow	Stool	3
B	B2, B5	Farrow	Stool	2
B	B6	Stag	Stool	1
C	C1, C2	Pregnancy sow	Stool	2
C	C3, C6, C7	Weaner	Stool	3
C	C4, C5	Farrow	Stool	2
D	D1, D6	Pregnancy sow	Stool	2
D	D2, D4, D7	Weaner	Stool	3
D	D3, D5	Stag	Stool	2
E	E1, E2, E6, E7, E8	Pregnancy sow	Stool	5
E	E3, E4, E5	Weaner	Stool	3
Total				34

the serotype is obtained by characterizing the flagella, which is often biphasic (Popoff and Le Minor, 2001). Although, all the methods are reliable, they present some disadvantages either in the time needed to perform the test or in the cost of sera and reagents. Moreover, readings are not always easy to interpret. With the development of molecular biology, some classification methods based PCR technique had been recognized as having great ability to differentiate separate strains by producing DNA fingerprints that are specific for individual strains. These methods were proved to be fast, sensitive and reliable for differentiation of microorganism. Several of them had been used widely for studying genetic relationship of different bacteria such as the random amplified polymorphic DNA PCR (RAPD-PCR) (Welsh and McClelland, 1990), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) (Wei et al., 2004; Wenz et al., 2006; Chen et al., 2010) and repetitive extragenic palindromic PCR (REP-PCR) (Trombert et al., 2007; Bonacorsi et al., 2009), other analysis methods such as pulsed-field gel electrophoresis (PFGE) (McLellan et al., 2003; Casarez et al., 2007), amplified fragment length polymorphism (AFLP) (Engstrom et al., 2003) and ribotyping also had been applied widely. REP consensus sequence consist of 38 bp inverted repeat sequence, ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome (Versalovic et al., 1991). REP-PCR and ERIC-PCR were used for studying bacterial source tracking in the field and analysis of genetic diversity. Identification of the spread of airborne *Escherichia coli* using ERIC-PCR and REP-PCR (Dombek et al., 2000; Duan et al., 2009; Yuan et al., 2010) has been reported using ERIC-PCR fingerprinting to assess *E. coli* strains diversity isolated from natural water (Casarez et al., 2007).

Sichuan is one of the biggest pig raising provinces in China. The infectious diseases caused by *E. coli* are very serious. Analysis of *E. coli* strains genetic diversity is important for epidemiology. The objective of this study was to use REP-PCR and ERIC-PCR for the analysis of genetic diversity among *E. coli* strains isolated from commercial swine farms in Sichuan province of China.

MATERIALS AND METHODS

Five commercial swine farms in Sichuan were chosen, fresh stool samples were aseptically collected from sick pig that have being alo laxata from the five farms, respectively, a total of 40 stool samples were collected, they are listed in Table 1. Samples were diluted and spread onto the surface of MacConkey agar plates which were incubated at 37℃ for 18 to 20 h. Colonies with typical pink on MacConkey agar plates were picked and streaked onto eosin methylene blue (EMB) agar plates. After overnight incubation at 37℃, one or two colonies with a metallic sheen on EMB agar were selected and cultured in Luria-Bertani broth. All cultures were stored at -70℃ in Luria-Berintani broth with 30% glycerol. Then, the strains species identifications were made using conventional biochemical tests (not shown in the paper). A total of 34 *E. coli* strains were chosen due to their varying sources.

Serotyping procedures

All the 160 standard *E. coli* O factor serums (O_{17}, O_{23}, O_{24} O_{198}, etc) were kindly provided by China Institute of Veterinary Drug Control, and the serotyping procedures used including the production of heat-stable antigen extracts by heating at 121℃ for 2 h have been described in detail elsewhere (Biao et al., 2009).

DNA preparation

Genome DNA of all *E. coli* was isolated by the CTAB/NaCl mini-prep protocol (Barney et al., 2001) used for all PCR-based methods. All *E. coli* isolates strains were grown in 5 ml of Luria-

Table 2. The serological result of *E. coli* O antigen by slide agglutination.

Serotype	Strain	Numbe (%)
O_{198}	A1, A2, C1	3 (8.8%)
O_{150}	A3, B2, E4	3 (8.8%)
O_{46}	A5, D6, E7	3 (8.8%)
O_{113}	A6, C3, D3, E8	4 (11.8%)
O_{26}	B4	1 (2.9%)
O_{120}	B5, D2, E1, E2	4 (11.8%)
O_{23}	B6, C2, D4, E3	4 (11.8%)
O_{17}	C4, C5	2 (5.9%)
O_{68}	C6, C7	2 (5.9%)
O_{24}	D5	1 (2.9%)
O_{78}	D7	1 (2.9%)
O_{129}	E5, E6	2 (5.9%)

Bertani (LB) broth (Oxoid) with moderate shaking for 18 h at 37℃. 1.5 ml LB broth was removed and centrifuged at 10,000 g for 2 min. The cell pellet was resuspended in 567 µl TE and mixed with 30 10% SDS and 3 µl 20 mg/ml protein K; and the mixture reacted for 1 h at 37℃; followed by 100 µl ABTC and 100 µl 5mol/L NaCl were added in the mixture, 65℃ for 10 min, and DNA templates were extracted by phenol/chloroform method, and DNA preparation were stored at -20℃ until used by PCR amplifications. Three microlitres of DNA preparation was used as the source of DNA template in the PCR reaction.

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 el al., 1991) were synthesized by Invitrogen. The PCR reaction (25 µl) contained 10× buffer 2.5 µl, dNTPs (2.5mmol/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 µ| and addition ddH_2O to 25 µl. Amplification was performed in a Thermal Cycler (Bio-BRI) with different temperatures: denatured for 7 min at 94℃, followed by 40 cycles of 1 min at 94℃, 1 min at 48℃ and 5 min at 72℃; and then extension at 72℃ for 7 min.

The reaction products were stored at 4℃ until they were electrophoresed on a 2% (w/v) agarose gel containing 1×TAE and 0.5 µg ml^{-1} ethidium bromide together with the DL2000 DNA markers (TaKaRa) for 2 to 3 h.

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 (2.5mmol/L) 2.5 µl, *Taq*DNA 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 addition of ddH_2O (25 µl). The amplification was carried out by incubation of the mixture for 5 min at 95℃ for pre-denaturation, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 45℃ for 1 min and extension at 65℃ for 8 min. A final extension was performed at 65℃ for 16 min.

The reaction products were stored at 4℃ until they were electrophoresed on a 1.5% (w/v) agarose gel containing 1×TAE and 0.5 µgml^{-1} ethidium bromide together with the DNA marker III (TaKaRa) for 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. The observed bands in the gels were evaluated based on the presence (coded 1) or absence (coded 0) of polymorphic fragments for the ERIC and REP-PCR primers. Cluster analysis was performed with NTSYS-pc (Version 2.10), a numerical taxonomy and multivariate analysis software package (Rohlf, 2000), based on Dice's similarity coefficient (S_D) with a 1% position tolerance and the unweighted pair group method using arithmetic averages (UPGMA). In addition, each isolate was considered as an operational taxonomic unit (OTU). In order to reduce the number of OTUs in the dendrogram, to facilitate interpretation, isolates of ≥70% similarity were treated as a single isolate.

RESULTS

Serotyping

As noted earlier, 30 strains are determined among 34 *E. coli* isolates, 12 kinds of O serogroups were obtained on the basis of slide agglutination. The results are listed in Table 2. The predominant types are O_{23}, O_{113} and O_{120}, representing 35.4%, but 3 strains are nontypeable by the test. The nontypeable isolates may either exist due to the fact that some isolates do not likely contain a sufficient level of serovar-specific antigens or that there exist novel serovars that have not been identified yet. The remaining serovars were detected at low frequencies, ranging from 2.9 to 8.8% (Table 3).

As indicated in Table 3, 5 serovars were identified among 6 strains isolates from A farm, 4 serovars present in B farm, 7 serovars present in C farm, 6 serovars present in D farm, 8 serovars present in E farm, and O-type 23 was reported to be the most prevalent serotype in B, C, D and E, followed by O_{113} (A, C, D and E).

Comparing the type strains, many field isolates with mixed serotypes showed that there are many kinds of serogroups in the same pig farm and they have predominant types in most pig farms. Some predominant

Table 3. The distribution of O serogroups of *E. coli* in different pig farms.

Pig farm	Number of *E. coli* isolate	Numberof identified isolate	Serotype (Number of isolate)
A	6	5	O_{198}(2) O_{150}(1) O_{46}(1) O_{113}(1)
B	6	4	O_{150}(1) O_{26}(1) O_{120}(1) O_{23}(1)
C	7	7	O_{198} (1) O_{23}(1) O_{113}(1) O_{17}(2) O_{68}(2)
D	7	6	O_{120}(1) O_{113}(1) O_{23}(1) O_{24}(1) O_{46}(1) O_{78}(1)
E	8	8	O_{120}(2) O_{23}(1) O_{150}(1) O_{129}(2) O_{46}(1) O_{113}(1)

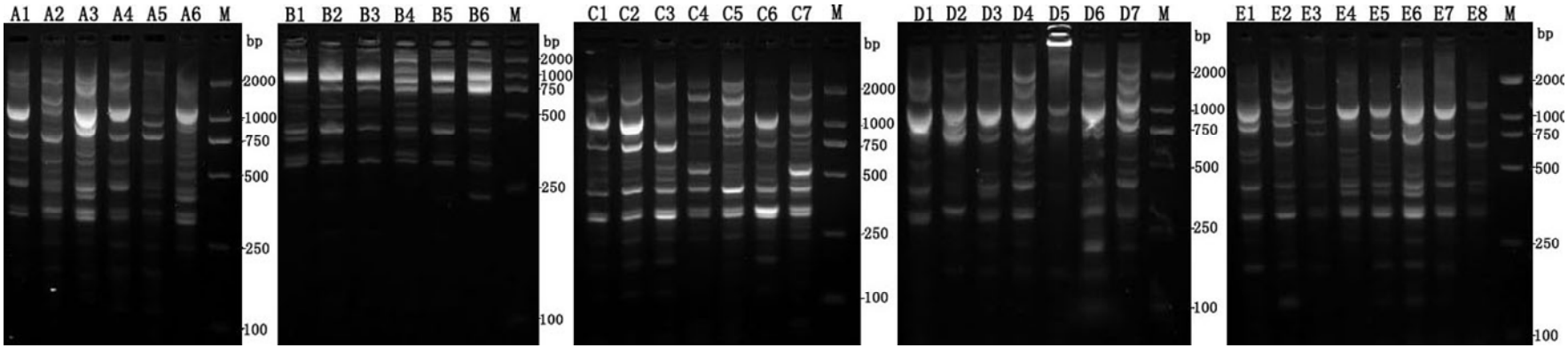

Figure 1. ERIC-PCR fingerprints obtained for *E. coli* isolate from A to E farms with primers ERIC1 and ERIC2. Lane: M, DNA marker DL2000; lane: A1 to A6, *E. coli* strains isolated from A pig house of Sichuan; lane: B1 to B6, *E. coli* strains isolated from B pig house of Sichuan; lane: C1 to C7, *E. coli* strains isolated from C pig house of Sichuan; lane: D1 to D7, *E. coli* strains isolated from D pig house of Sichuan; lane: E1 to E8, *E. coli* strains isolated from E pig house of Sichuan.

serogroups in different districts are identical, others are different. Serogroups of different pig farms in the same district are generally different.

ERIC-PCR

The ERIC-PCR fingerprints analysis showed that there were 6 to 19 bands with molecular weight ranging from 100 bp to more than 3 kb generated by ERIC primers (Figure 1), and a common intensive band of about 270 bp in most *E. coli* (Figure 1). The data matrices based on the DNA fragments and the dendrogram using the NTSYS-pc software were constructed, all *E. coli* isolates obtained in this study were grouped into different clusters or branches based on the ERIC-PCR pattern similarity.

According to the UPGMA dendrogram (Figure 2), all generated DNA patterns are relatively complex. Each subjects exhibited a unique banding pattern with similarity ranging from 0.17 to 0.93. These 34 isolates were grouped into 19 different groups with the similarity coefficients 0.78, designated as I-XIX. The cluster X includes 5 strains isolated from D farm with similarity coefficients 0.83. This second level of clustering suggested that a remarkable polymorphism exists among all strains in these groups. The two samples collected at different farms showed much higher similarity, 0.89 between C6 and E5 and0.81 between A1 and B5.

REP-PCR

REP-PCR fingerprinting profiles and cluster

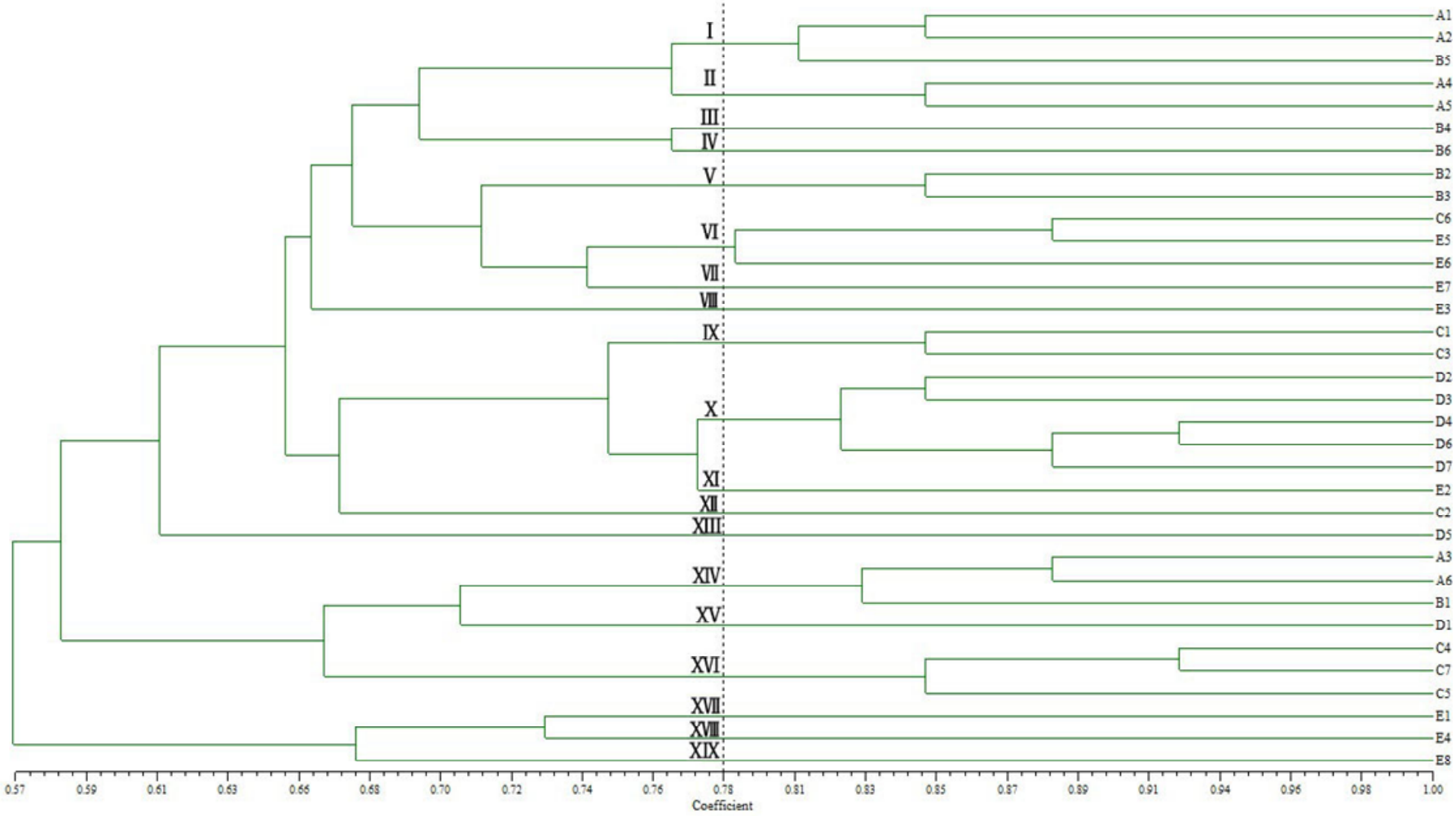

Figure 2. Dendrogram established by the biostatistical analysis program NTSYS-pc using the dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profile of *E. coli* strains obtained with primers ERIC1 and ERIC2.

analysis of 34 *E. coli* strains are listed in Figure 3. With primers REP1R and REP2I, REP-PCR fingerprinting profiles were obtained. Analysis of the strains with REP-PCR yielded one to fourteen bands depending on the strains. The size of these bands ranged from 200 bp to more than 4500 bp. REP-PCR fingerprinting profiles of *E. coli* strains were different from each other. According to UPGMA dendrogram, 34 *E. coli* strains were separated into thirteen types with 0.81 similarity named 1 to 13 (Figure 4); however, *E. coli* strains of every pig house were not in the same type.

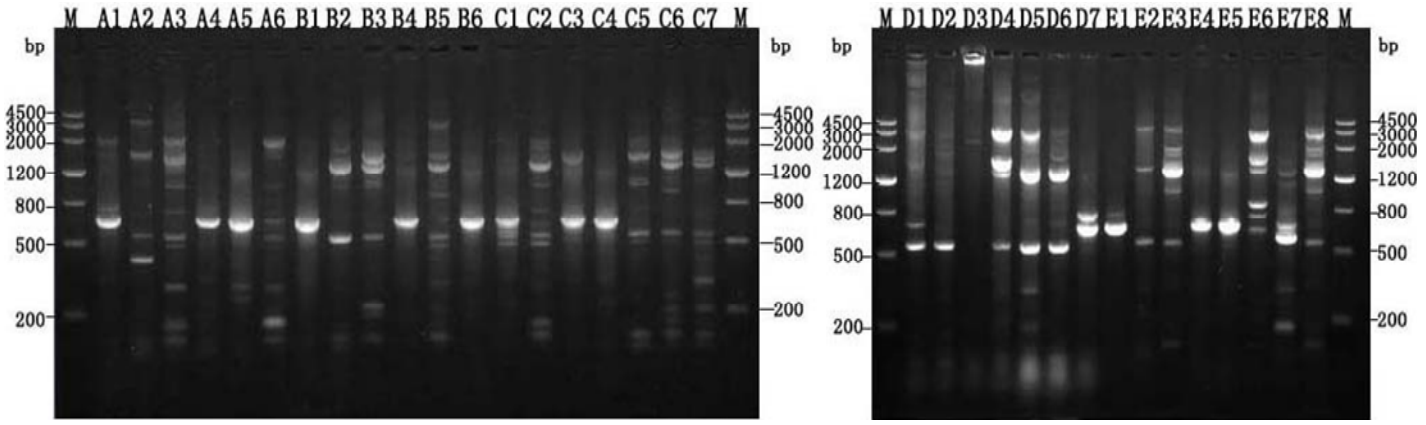

Figure 3. REP-PCR fingerprints for 34 *E. coli* strains A to E farms with primers REP1R and REP2I. Lane: M, DNA marker III; A1 to A6, *E. coli* strains isolated from A pig house of Sichuan; B1 to B6, *E. coli* strains isolated from B pig house of Sichuan; C1 to C7, *E. coli* strains isolated from C pig house of Sichuan; D1 to D7, *E. coli* strains isolated from D pig house of Sichuan; E1 to E8, *E. coli* strains isolated from E pig house of Sichuan.

Thirteen *E. coli* strains (A1, A4, A5, B1, B4, B6, C1, C3, C4, D7, E1, E4 and E5) sharing common band of about 700 bp were clustered together and belonged to type 1; among them A4, D7, E5 and B1, E4, E5 shared 1.0 similarity, however, they were not from simple pig house. Type 2 only had one strain E7. Four *E. coli* isolates D2, D4, D5 and D6 isolated from D pig house were clustered to type 3. Other *E. coli* isolates sharing high similarity were separated into same type such as B2 and B3, E3 and E8, C5 and C6, A6 and C2.The remaining *E. coli* strains were unique.

DISCUSSION

REP-PCR and ERIC-PCR techniques that prove to be fast, sensitive and reliable for the differentiation of microorganism were applied for analysis of genetic diversity and epidemiology. REP-PCR and ERIC-PCR technique were established from highly conserved repetitive DNA sequence distributed among the gene of microorganism. Combined with PCR and gel electrophoresis, highly complex and specificity genome fingerprintings profile produced could discriminate spices and strains. REP-PCR and ERIC-PCR technique are widely used method for DNA typing analysis and has been shown to successfully differentiate strains of *Bradyrhizobium japonicum* (Judd et al., 1993), *Lactobacillus johnsoni* (Ventura and Zink, 2002) and *Clostridium perfringens* (XiaoLi et al., 2009). Many genotyping studies were done on clinical isolates of *E. coli*, as well as environmental and veterinary isolates identified via biochemical and/or culture based approach (Carvalho de Moura et al., 2001; Gordon and Cowling, 2003; Higgins et al., 2007).

In this study, two molecular methods, REP-PCR and ERIC-PCR, were applied and their power to discriminate 34 *E. coli* strains was compared with serogrouping method. Difference in PCR fingerprinting profile existed among identical swine farms. It was indicated that the diversity of *E. coli* strains of swine farms in Sichuan province were high.

Genetic diversity of *E. coli* isolated from different five swine farms demonstrated in dendrogram based on ERIC-PCR fingerprinting profiles, namely, *E. coli* strains from different sources showed certain difference at the level of gene and were separated into different sub-genetypes. *E. coli* isolated from the same swine farms were clustered into several groups, and had low cross with other farms. According to analysis of the dendrogram of ERIC-PCR, it can be seen that farm A was mainly concentrated in I, II and XIV, farm B was mainly concentrated in III, IV and V, farm C was mainly concentrated in IX, XII and XVI, farm D was mainly concentrated in X, XV and XIII, farm D was mainly concentrated in VI, VII, VIII, X, XI, XVI, XVII and XIX.

When compared with ERIC-PCR, REP-PCR has lower discrimination power. Although, genetic differences of *E. coli* of farms were demonstrated on dendrogram by REP-PCR, the sub-genetypes of identical farm has higher cross with others. Especially, genetypes of *E. coli* from farm A were crossed with other farms, and it was difficult to analyze the major subgroup of *E. coli* of some farms. It was determined that the major groups of farm D were 3 and 6, farm E were 2, 5 and 8.

Each *E. coli* strain has only one O serovars, so it is usually chosen for serotype identification. The results show that *E. coli* O serovars diversity was presented in five swine house. The method of analysis of *E. coli* diversity by O serovars diversity is a practical method indeed. But the quantity of *E. coli* O serovars is much and it is difficult to collect all identified O serovars. Accordingly, O serovars of 4 strains were not identified because only 160 kinds of O serovars were provided.

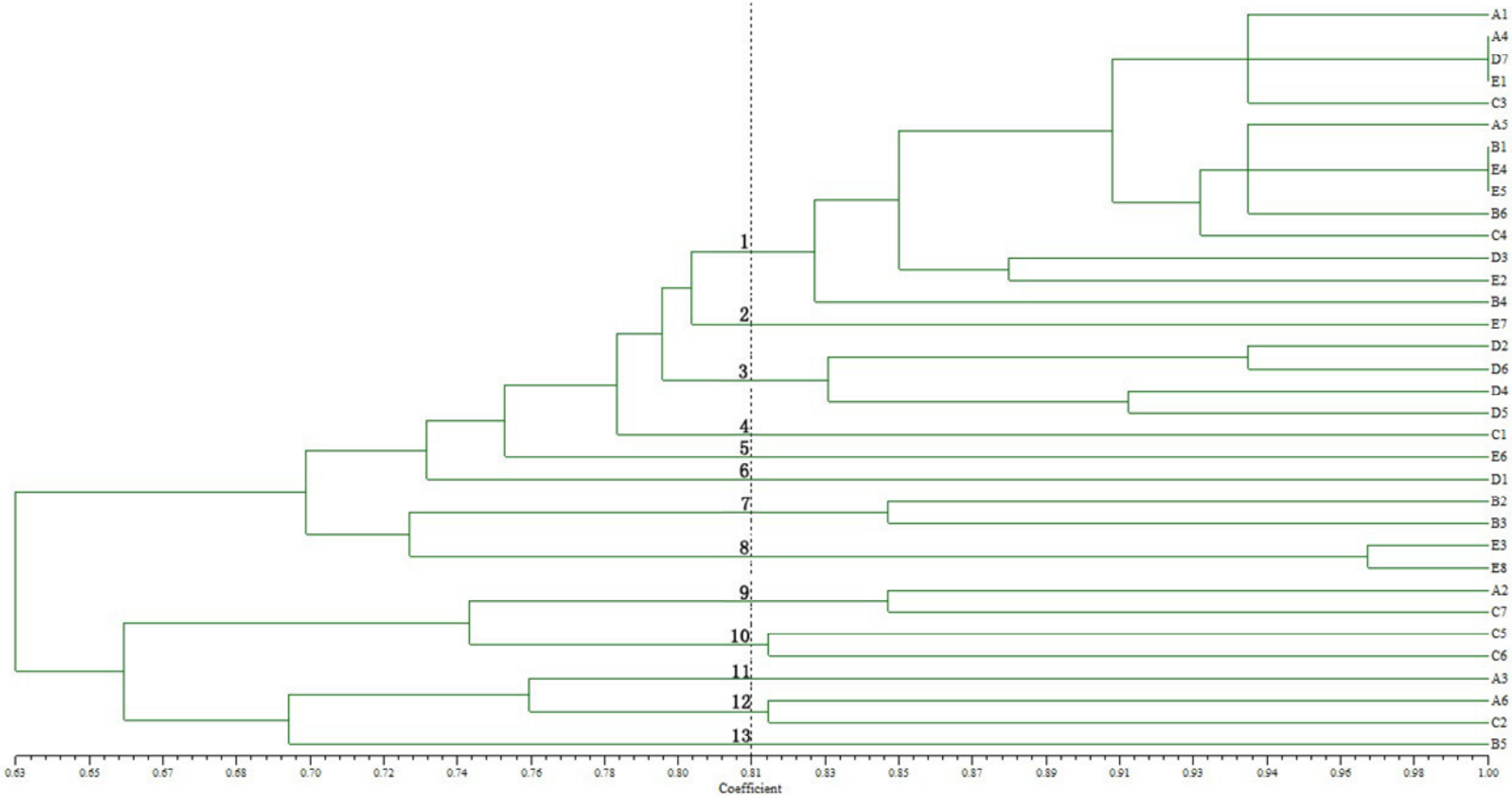

Figure 4. Dendrogram established by the biostatistical analysis program NTSYS-pc using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profile of *E. coli* strains obtained with primers REP1R and REP2I.

Predicting *Salmonella enteric* serotypes by repetitive sequence-based PCR has been reported recently successfully and it was discovered that REP-PCR and PADF analysis have good correlation with O antigen serotyping. When compared with both sides, O antigen serotypes of *Salmonella* strains studied by Albufera were relatively single (Albufera et al., 2009; Wise et al., 2009).

However, O antigen serotypes of *E. coli* in this study were quite complex, which made it difficult for ERIC-PCR and REP-PCR to identify the O antigen serotypes of *E. coli*. For example, C6 and E5 are closer that E6 and E5 on the dendrogram, but E5 and E6 belonged to the same serogroup

and C6 to another. It was consistent with previous study.

Hence from this study, the results of PCR fingerprinting profiles with the primers REP and ERIC revealed that genetic variability of *E. coli* strains isolated from the five swine farms was obvious. It was indicated that the diversity of *E. coli* strains of swine farms in Sichuan province were high.

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