Original Article

Establishment and Comparison of Pulsed-field Gel Electrophoresis, Multiple-locus Variable Number Tandem Repeat Analysis and Automated Ribotyping Methods for Subtyping of *Citrobacter* Strains*

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Abstract

Objective To establish and compare the pulsed-field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat analysis (MLVA) and automated ribotyping for subtyping of *Citrobacter* strains.

Methods PFGE protocol was optimized in terms of plug preparation procedure, restriction enzymes and configuration of electrophoretic parameters. MLVA method was evaluated by finding variable number tandem repeats in two genomes of *Citrobacter* strains. The ribotyping was performed by using the automated RiboPrinter system.

Results We optimized the plug preparation procedure, focused on the cell suspension concentration (turbidity of 2.5 to 3.5), SDS addition (no SDS needed) and lysis time (1 h), and selected the appropriate restriction enzyme (*Xba*I) and the electrophoretic parameters (1.0 s-20.0 s for 19 h) of PFGE. There was nearly no discriminatory power of MLVA between *Citrobacter* strains. For 51 *Citrobacter* strains, automated ribotyping gave a *D*-value of 0.9945, while PFGE gave a *D*-value of 0.9969. Both PFGE and automated ribotyping clustered strains from the same sources (with the same species from the same place at the same time identified as the same source) and divided strains from different sources (from different years, places and hosts) into different subtypes.

Conclusion PFGE protocol established in this paper and automated ribotyping are suitable for application in *Citrobacter* subtyping.

Key words: *Citrobacter*; Pulsed-field gel electrophoresis; Multiple-locus variable number tandem repeat analysis; Ribotyping; Molecular subtyping

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INTRODUCTION

Citrobacter is a genus of Gram-negative facultative pathogens which can be found almost everywhere in soil, water, food as well as the intestinal tracts of humans and animals. *Citrobacter* is an opportunistic pathogen which has been recognized as an important cause of nosocomial infection, especially in neonates and immunocompromised patients[1-2]. The *Citrobacter* genus contains 11 species, several of which could cause human diseases. The clinical manifestations of

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Citrobacter infection are diverse, including urinary tract infection, superficial wound infection, respiratory tract infection, intra-abdominal infection, bacteraemia, endocarditis, infant meningitis and sepsis[2-5]. Two important sources of *Citrobacter* infection are healthcare-associated and community-acquired[6]. *Citrobacter* is now one of the most important causes of nosocomial infections in China[6-9].

Genetic clustering of microorganisms is necessary for identification of sources of contamination and determination of routes of transmission; this could in turn enable us to more accurately detect and limit the spread of *Citrobacter* infections. Molecular typing techniques have been widely used in molecular epidemiological studies of bacterial infectious diseases to investigate the source and relationship between strains. Among them, the pulsed-field gel electrophoresis (PFGE), the multiple-locus variable number tandem repeat analysis (MLVA) and the rRNA gene fingerprinting (ribotyping) show good utility in investigation of outbreaks and sporadic cases[10-11].

PFGE is a high-performance molecular typing method and is used most frequently for subtyping of bacteria. The PFGE method involves the use of restriction enzymes which cleave the genome of bacteria to generate a limited number of high-molecular-weight restriction fragments. These fragments are then separated by gel electrophoresis with programmed variables in both direction and duration of the electric field (the pulsed field). The use of standardized PFGE protocols allows for rapid comparison of DNA fingerprints from pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae* and *Vibrio parahaemolyticus* between different laboratories to enhance disease surveillance[12-14]. A number of *Citrobacter* PFGE protocols have been described in the literature[15-18]; however, most laboratories that use PFGE to subtype *Citrobacter* cannot compare their results because the protocols differ from each other in critical parameters, such as the restriction enzymes and electrophoresis conditions used to generate the DNA fingerprints. To enhance our ability to monitor this pathogen, there is a need for an optimized *Citrobacter* PFGE protocol which can readily be implemented in different laboratories for information interpretation. MLVA and ribotyping are the other two mostly used molecular typing techniques for bacteria. In this study, we optimized the PFGE protocol and established MLVA and ribotyping methods for subtyping of *Citrobacter* strains, and further assessed and compared the ability of typing of PFGE, MLVA and ribotyping.

MATERIALS AND METHODS

Bacterial Strains

68 strains of *Citrobacter* isolated from China were used in this study, including 52 *Citrobacter freundii*, 9 *Citrobacter youngae* and 7 *Citrobacter braakii* . The isolates were obtained from different sources (years, places and hosts) in China and were stored at -80 °C in brain heart broth with 20% sterile glycerol. The bacteria were streaked onto Luria-Bertani (LB) agar plates, and typical colonies were picked and identified by biochemical tests with API®-20E test kits (bioMérieux, Lyons, France) and 16S RNA sequence analysis. The source and date of isolation of all the strains are described in Supplementary Table S1. To establish the PFGE method for *Citrobacter* subtyping, 14 *C. freundii*, 9 *C. youngae* and 7 *C. braakii* of various origins (Panel 1) were used. Eighteen strains (Panel 2) were selected to test variable number tandem repeat (VNTR) loci and establish the MLVA method for *Citrobacter* subtyping. Sixteen strains (Panel 3) were selected to establish and assess the automated ribotyping method. To compare the three methods, 51 strains (43 *C. freundii*, 4 *C. youngae* and 4 *C. braakii*) from different years, places and hosts (with no epidemiological correlation observed, Panel 4) were selected from the test strains first to compare the discriminatory power of different methods, and then 16 strains associated with 5 independent sources (with the same years and places) (Panel 5) were included to evaluate the epidemiologic concordance of different subtyping methods.

PFGE

The PFGE protocol used in this study was based on the PulseNet 1-day standardized PFGE protocol for *Escherichia coli* O157: H7[14]. The cell suspension was adjusted to a particular optical density between 2.0 and 4.5 units with a bioMérieux DENSIMAT. Slices of *Citrobacter* plugs were digested in a volume of 200 μL with corresponding amount per slice enzymes (New England Biolabs, Ipswich, USA) for 4 h at 37 °C. The electrophoresis was run on CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA, USA). Images were captured on the Gel Doc 2000 system (Bio-Rad) and converted to TIFF files for the

computer analysis. *Salmonella* serotype Braenderup H9812 was used as a DNA size marker in the PFGE analysis, as recommended by PulseNet[19].

The preliminary enzymes were selected with DNASTAR 5.01 software (DNASTAR, Inc., WI, USA) based on the complete nucleotide sequence of *Citrobacter* strains (NC009792 and NC013716) published in the GenBank and two Chinese *Citrobacter* strains (CF72 and CF74, unpublished data). The primary enzymes were then selected. A pilot test with three strains (CF0702, CY038, 091844) was conducted for further evaluation and the candidate enzymes for *Citrobacter* PFGE were selected based on the distribution of the bands. The candidate enzymes were further evaluated for use in PFGE of *Citrobacter*.

Thirty isolates were analyzed with each candidate enzyme digested with three EPs: EP-a, EP-b and EP-c. EP-a was recommended by the CHEF Mapper equipment manufacturer. EP-b was fine-tuned based on EP-a to provide the best possible resolution. EP-c is the parameter used in PulseNet 1-day standardized PFGE protocol for *Escherichia coli* O157:H7 *and Shigella sonnei*[14]. Simpson diversity index (*D*-value)[20] and similarity coefficients[21] were used to compare the discriminatory power under each parameter. *D*-value was given by the equation $D=1-\{\sum [n_j (n_j-1)]\}/ [N (N-1)]$, where n_j is the number of strains belonging to the *j*th type and *N* is the number of strains in the population. The similarity coefficients of every two PFGE patterns were compared. Two-tailed probability was calculated by using the Friedman test by SPSS 11.5 for multi-group comparisons. The Friedman test is a non-parametric test for analyzing randomized complete block designs and testing the null hypothesis that the treatments have identical effects. If significance was found among groups, the Friedman test was performed for two-group comparisons with the adjusted significance level of 0.007. An EP with higher discriminatory power can distinguish patterns better, thus yielding a smaller similarity coefficient. Accordingly, the EP with higher *D*-value and minimal similarity coefficients was considered optimal for distinguishing strains and was used as the standard to evaluate the discriminatory power of the enzymes selected.

Automated Ribotyping

Automated ribotyping was performed as previously reported[22] by using the automated RiboPrinter system and following the manufacturer's recommendations (Qualicon, Wilmington, DE, USA). All consumables and equipment used in further analyses were supplied by the manufacturer. Strains were grown on Luria-Bertani broth. Bacterial samples were obtained with a colony pick and resuspended in lysis buffer. After heat treatment at 80 °C for 10 min, lytic enzymes were added and samples were loaded in a carrier into the instrument. After the strain information was entered into the instrument, the further analysis was carried out automatically. The restriction enzyme *Eco*RI (Qualicon) was recommended with the RiboPrinter system for typing *Citrobacter*. Gel cassettes contained 13 wells, 5 of which contained molecular size standard DNA. After automatic electrophoresis, electroblotting, hybridization with a sulfonated *Escherichia coli* rrnB rRNA operon probe and detection of the signals in the system, patterns were converted to TIFF files for the computer analysis.

MLVA

The genomes of *Citrobacter* strains CF72 and CF74 were screened for repetitive DNA with the Tandem Repeats Finder, Version 3.21[23], which are available for free at tandem.bu.edu/tools.html. The program generates an output file giving the repeat location, the repeat segment size, the nucleotide composition, and the copy number of the array. Seventeen VNTR loci were selected on the basis of a repeat length between 6 and 46 bp and 100% matched. PCR primers were designed from the sequences flanking the repeats of the 17 loci. Primers were designed by using the Primer Premier 5 program (Premier Biosoft International, CA, USA). Amplification was conducted in a 30 μL reaction mixture. After an initial denaturation at 94 °C for 5 min, the reaction was performed for 30 cycles at 94 °C for 1 min, at different annealing temperatures depending on the primers for 1 min, and at 72 °C for 1 min and 30 s, followed by an extension at 72 °C for 5 min. PCR reactions were prepared in a 20 μL volume with 10× PCR buffer, 0.5 U Taq polymerase, 200 μmm/L each of the four dNTPs, 10 μmm/L each primer set, 1 ng template DNA and filtered sterile water. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, and then 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 45 s, followed by a final polymerase extension at 72 °C for 5 min. Strains CF72 and CF74 were used as positive controls. The PCR products were detected by electrophoresis in 0.5×Tris-borate-EDTA (TBE) buffer and visualized by UV light. The positive PCR products

were analyzed by capillary separation along with an internal size standard (GeneScan® ROX-500 size standard, PE Applied Biosystems) on a PE Applied Biosystems ABI Prism® 3730 instrument. Copy numbers were calculated by size and assigned according to the number of repeats for each locus. The PCR products of samples, randomly selected from each number of repeats in each locus, were sequenced and the sequences were compared.

Data Analysis

The PFGE patterns, ribotyping patterns and MLVA data were analyzed with the BioNumerics Version 5.10 software (Applied Maths, Kortrijk, Belgium). For PFGE and ribotyping, the similarity between two patterns was expressed as a Dice coefficient. The Dice coefficient (S_D)[24] is calculated as $S_D = [2(n_{xy})] ? (n_x + n_y)$, where n_{xy} is the number of bands common to isolates *X* and *Y*, n_x is the total number of bands for isolate *X*, and n_y is the total number of bands for isolate *Y*. The clustering and construction of dendrograms were performed by the unweighted pair group method and using arithmetic averages (UPGMA).

Discriminatory Index

The discriminatory power of subtyping methods was compared with Simpson's index of diversity[20]. It was given by the equation $D=1-\sum[n_j (n_j - 1)]/[N(N - 1)]$, where n_j is the number of strains belonging to the *j*th pattern and *N* is the number of strains in the population. The *D*-value from this equation can be applied to directly compare typing methods.

RESULTS

Optimization of PFGE Method

A theoretical enzyme selection with the DNASTAR 5.01 software was based on the two completed nucleotide sequences and included all satisfactory enzymes. The number of bands required for the analysis should not be too large. Many of the enzymes produced too many bands (more than 50 bands); therefore, *Fse*I, *Sbf*I, *Psp*XI, *Spe*I, *Bln*I, *Xba*I, and *Not*I were chosen as the candidate enzymes for the pilot study. We obtained three images using the seven enzymes and found that the cuts by *Xba*I were clear enough to meet our needs (Figure 1).

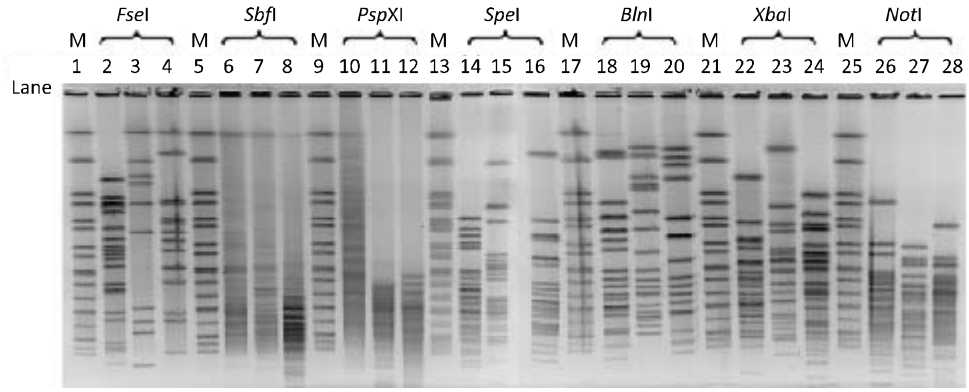

Figure 1. Pulsed-field gel electrophoresis (PFGE) images of three *Citrobacter* strains (CF0702, CY038, 091844) restricted with *Fse*I (lanes 2-4), *Sbf*I (lanes 6-8), *Psp*XI (lanes 10-12), *Spe*I (lanes 14-16), *Bln*I (*Avr*II) (lanes 18-20), *Xba*I (lanes 22-24), and *Not*I (lanes 26-28). The size standard (Salmonella enterica serotype Braenderup strain H9812, *Xba*I digestion) was loaded in Lanes 1, 5, 9, 13, 17, 21, and 25. The electrophoretic parameter was 6.8 s-34.8 s for 19 h.

Adjusting the cell suspensions to an optical turbidity of 2.5 to 3.5 worked best in our experiments. Suspensions adjusted to a higher cell concentration yielded DNA concentrations that were too high, resulting in high background of the PFGE patterns, partial digestion, and poor resolution (Figure 2). Plugs made with SKG agarose containing 0.5% and 1% SDS showed no difference from those made with the agarose without SDS (with data not shown), so SDS was not used in further experiments. Three lysis incubation times (1, 2, and 4 h) were used to determine the shortest incubation time required for optimal lysis of cells in agarose plugs. The results showed that optimal lysis occured within the first hour of incubation at 54 °C and there was no difference in the quality of the plugs when the lysis step was allowed to proceed for up to 4 h (with data not shown).

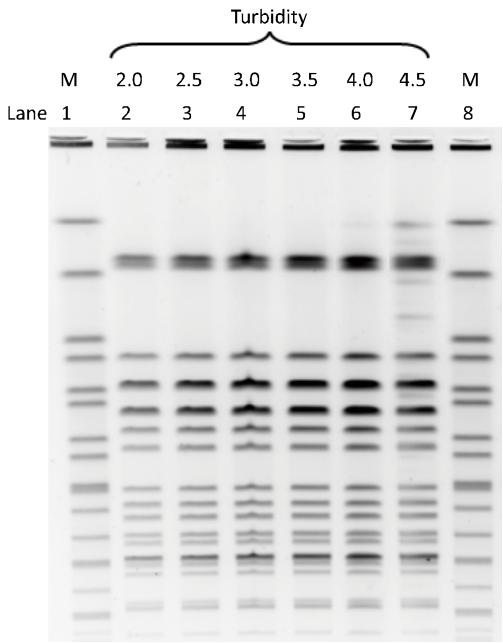

Figure 2. PFGE gels showing a comparison of plugs made with different cell suspension concentrations (Lanes 2-7, strain CF72, *Xba*I digestion). The size standard (*Salmonella enterica* serotype Braenderup strain H9812, *Xba*I digestion) was loaded in Lanes 1 and 8. The electrophoretic parameter was 6.8 s-34.8 s for 19 h.

For every enzyme, an EP could be recommended by the CHEF Mapper equipment based on the size of restriction fragments. The EP recommended for *Xba*I digesting of *Citrobacter* was 6.8 s-38.4 s for 19 h (EP-a). However, with this EP, the bands were not well distributed, and it was not sufficient to distinguish fragments under 300kbp. We fine-tuned the EP (EP-b: 1.0 s-20.0 s for 19 h) to provide the best possible resolution. These two EPs were selected to compare with EP-c (2.2 s-54.2 s for 19 h). Slight differences in the patterns produced by the *Xba*I digestions by using each of the three EPs were simultaneously evaluated. For example, the distribution of the strain 091798 restriction fragments under each EP was different with each digestion (Figure 3). As shown by the figure, EP-b exhibited the highest discriminatory power for small fragments <300 kbp. EP-a and EP-c exhibited stronger discriminatory power for fragments >300 kbp. The two largest fragments (783 kbp and 585 kbp) were distinguished with EP-a and EP-c but not with EP-b. At the same time, the small fragments (near 77 kbp) were distinguished with EP-b but not with EP-a and EP-c. The three EPs divided the 30 *Citrobacter* strains into 22 patterns and gave the same discrimination index (*D*-value) of 0.9747 (Figure 4). Five similar patterns containing more than one strain were analyzed with different EPs. To compare the similarity coefficients, multi-group comparisons were made by using SPSS 11.5. Nonparametric tests were performed because one-sample Kolmogorov-Smirnov tests demonstrated that the data were not normally distributed. The Friedman test showed that for the 30 test isolates (N=465, Chi-Square=441.792, df=2), there were significant differences among three groups (Asymp. Sig. 0.000). Two-group comparisons were performed by using the Friedman test with the adjusted alpha value of 0.007. Similarity coefficients generated by EP-b were significantly smaller than those obtained with EP-a and EP-c. So EP-b (1.0 s-20.0 s for 19 h) was declared the optimal EP.

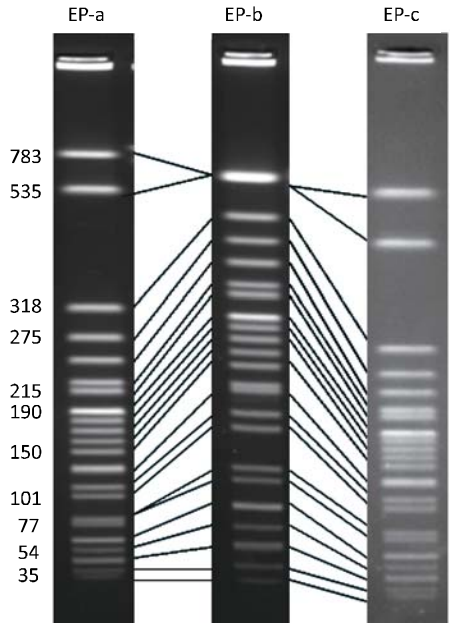

Figure 3. PFGE gels under three EPs of strain 091798 (*Xba*I digestion; EP-a: 6.8 s-38.4 s, 19 h; EP-b: 1.0 s-20.0 s, 19 h; EP-c: 2.2 s-54.2 s, 19 h).

PFGE Analysis

The chromosomal DNA of all 62 *Citrobacter* strains (including 52 *C. freundii*, 6 *C. youngae* and 4 *C. braakii* from the same or different sources) tested with PFGE in this study were successfully cleaved with XbaI and gave clear patterns, and 17-24 bands between 20 kb and 700 kb were obtained for each

strain. All fragments were polymorphic among the 62 strains.

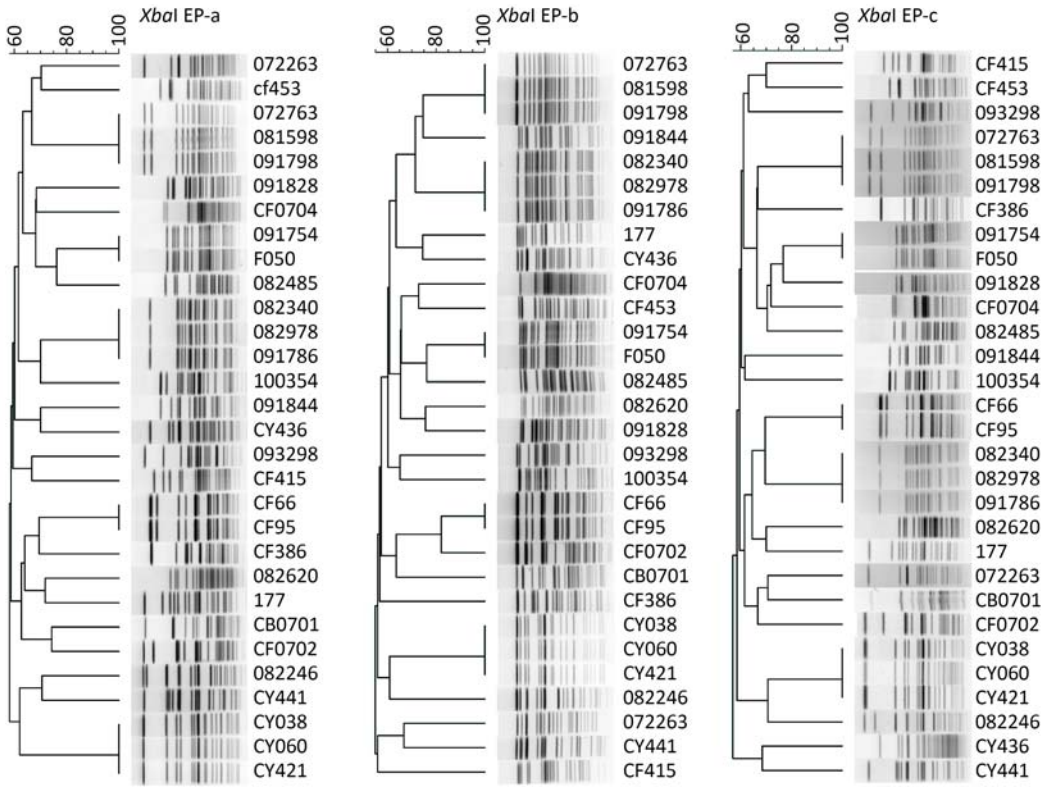

Figure 4. Clustering results of patterns obtained by using three EPs with XbaI digestion. The charts are shown for 30 *Citrobacter* strains.

The 51 *Citrobacter* strains (including 43 *C. freundii*, 4 *C. youngae* and 4 *C. braakii*) from different sources could be distinguished by *Xba*I restriction digestions at the strain level, producing 48 different PFGE patterns with similarity values between 43.8%-100% (Figure 5). The *D*-value of PFGE subtyping of 51 *Citrobacter* strains was 0.9969. Of the 16 *Citrobacter* strains associated with 5 independent sources analyzed with PFGE, most of the strains from each source had the same PFGE type, except strain CF78, which had one band different from the other 5 strains from same source (Figure 6).

MLVA

There were 17 VNTR loci found in genomes of strains CF72 and CF74 according to the standard for locus selection (Supplementary Table S2), and 9 loci were found in both of the two genomes. We designed primers to amplify 17 VNTR loci in 18 *Citrobacter* strains (Panel 2). There were 7 loci that could not be amplified in all other strains except CF72 or CF74; 2 loci showed non-specific amplification; 2 loci could be amplified in parts of strains and had no polymorphism; 6 loci could be amplified in all strains and were combined and used as MLVA method in the further analysis.

All 62 *Citrobacter* strains from different or the same sources were analyzed by using 6 VNTR loci. The 6 VNTR loci could be amplified in all strains; however, only 2 had polymorphism. For 51 strains from different sources, loci CF72VNTR03 and CF74VNTR01 divided 51 *Citrobacter* strains into 2 types with 50 strains and 1 strain in the two types, respectively. By combination and using two loci, 51 strains were divided into 3 types with a *D*-value of 0.077. Of the 16 strains associated with 5 independent sources analyzed with MLVA, all strains had the same MLVA type, except 6 strains from one source which could be distinguished from others. So the MLVA method using the 6 loci selected in this study does not have enough discriminatory ability to distinguish *Citrobacter* strains from both different and the same sources.

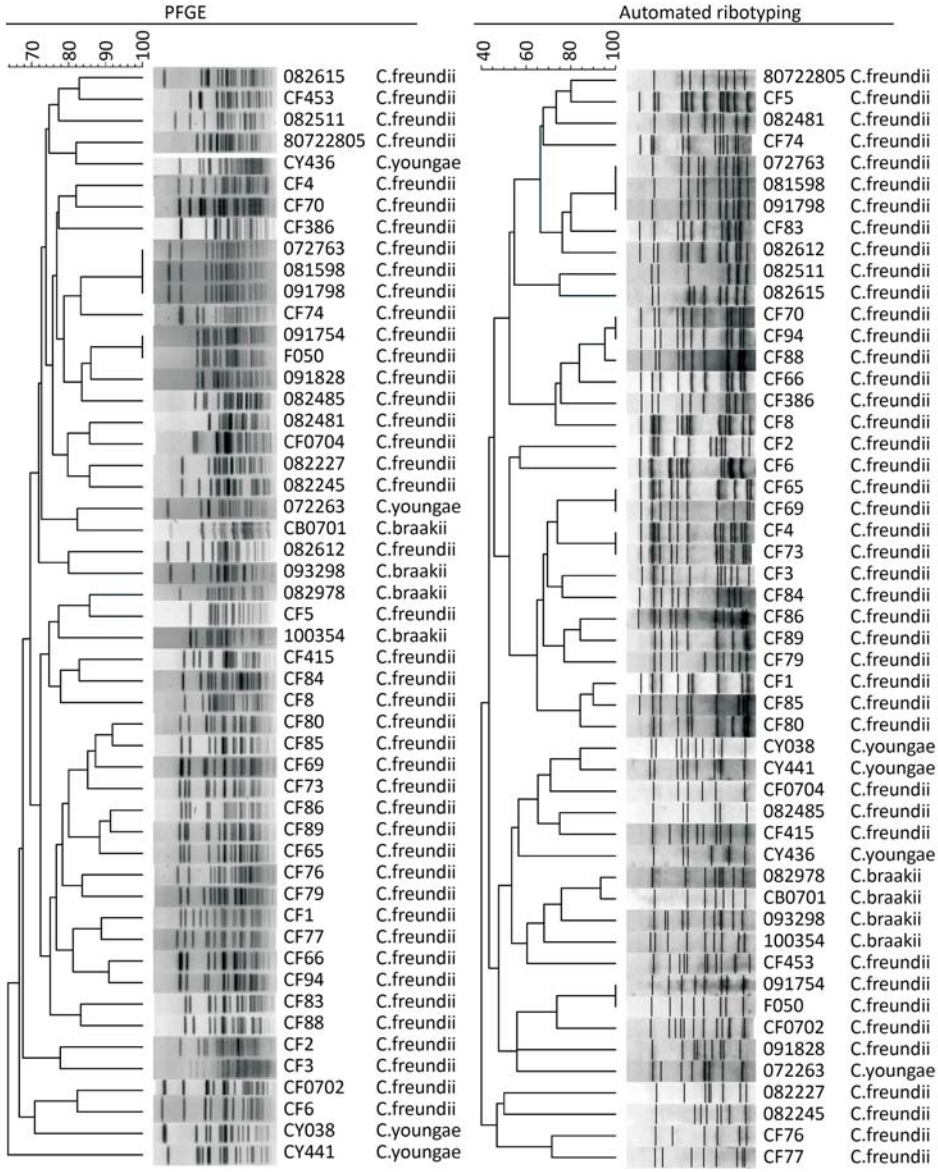

Figure 5. Clustering results of 51 epidemiologically unrelated *Citrobacter* strains by using PFGE and automated ribotyping.

Automated Ribotyping Analysis

All 62 *Citrobacter* strains analyzed by using automated ribotyping with *Eco*RI digest gave clear patterns with no untypable strains present. The patterns contained 6-13 fragments between 1 kb and 48 kb with similarity values of 43.8%-100%. The 51 *Citrobacter* strains from different sources could be distinguished at the strain level, producing 45 different ribotypes with *D*-value of 0.9945 (Figure 1) (Table 2). For 16 *Citrobacter* strains associated with 5 independent sources, automated ribotyping gave strains from one source one unique ribotype while strains from different sources had different ribotypes.

Comparison of PFGE and Automated Ribotyping in Citrobacter Subtyping

PFGE and automated ribotyping had the ability to type all *Citrobacter* strains and showed typability of 100%. For 16 *Citrobacter* strains associated with 5 independent sources, both PFGE and automated ribotyping classified strains from the same sources together and divided strains from different sources into different subtypes.

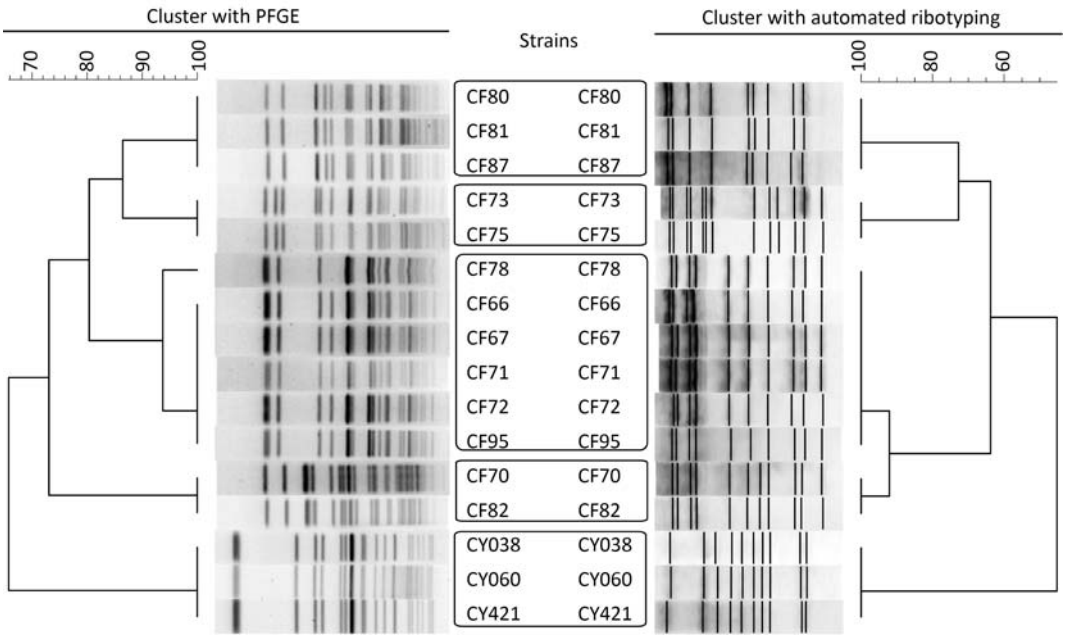

Figure 6. Clustering results of patterns obtained with PFGE and automated ribotyping of 16 *Citrobacter* strains from five sources. The strains in each frame were from the same source.

For 51 *Citrobacter* strains from different sources, both PFGE and automated ribotyping gave high discriminatory power with the *D*-value greater than 0.99. No strains with different species (*C. freundii*, *C. youngae* and *C. braakii*) were clustered together. PFGE distinguished the 51 strains into 48 patterns, whereas 45 patterns were obtained by automated ribotyping. Forty-six patterns, accounting for 95.8% (46/48) of PFGE subtyping, only contained one strain in each. These patterns contained 90.2% (46/51) of test strains. Forty patterns of automated ribotyping, accounting for 88.9% (40/45), contained one strain in each. These patterns contained only 78.4% (40/51) of test strains. 2 and 5 groups of strains have identified patterns in PFGE and automated ribotyping analyses, respectively. Both of the 2 groups of strains that clustered in the same PFGE patterns also had the same ribotypes. Three other groups of strains with the same ribotypes were divided into different PFGE patterns, which showed lower discriminatory power of automated ribotyping compared to PFGE.

DISCUSSION

Genetic subtyping with powerful discrimination has made it feasible to identify bacterial strains from different ecosystems. However, these techniques have not been widely used for subtyping of *Citrobacter* strains. Among the subtyping methods, PFGE is widely used in subtyping of bacteria. The power of this typing technique for enteric pathogens, such as *E. coli* O157:H7, *Vibrio cholerae*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Clostridium perfringens*, has also been demonstrated in previous studies[13,25-28]. PulseNet, an early warning system for outbreaks of foodborne diseases, was set up based on the PFGE technique and databases of patterns[29]. Standardization of protocols is a prerequisite for the implementation of any molecular method as a practical tool for epidemiological surveillance. Previous studies have shown that several factors could influence PFGE results, including plug preparation, enzymatic digestion and electrophoresis[28,30]. In this study, we optimized the cell concentration, SDS concentration in agarose and incubation time in plug preparation procedure, and the cell suspensions with an optical turbidity of 2.5 to 3.5 worked best for PFGE of *Citrobacter*. The “ghost” “phantom” bands were observed when the turbidity was adjusted to 4.0 or higher. The SDS helped to lyse cells; on the other hand, SDS may cause shearing of the DNA and high background of the PFGE patterns. Some researchers believe that the addition of SDS to the plug agarose is detrimental and unnecessary[28]. In this study, there was no difference in the patterns of agarose with or without SDS. In terms of the incubation time,

our result showed that one hour was sufficient for *Citrobacter* lysis in PFGE subtyping.

Several criteria have been proposed for evaluating the performance of typing systems including typability, reproducibility, and most importantly, discriminatory power. Discrimination indices and the similarity coefficients are the indices usually used to compare discriminatory power. The key factors influencing discriminatory power in PFGE subtyping are restriction enzyme and electrophoretic parameters used. Different restriction enzymes produce different banding patterns and often different band numbers, which would influence the typing effect of PFGE[31]. With the aid of software analyses, *Xba*I were chosen. In this study, all three EPs used gave the same *D*-value for *Xba*I digestion. However, the similarity coefficients generated by different EPs were significantly different. EP-b (1 s-20 s for 19 h) gave the highest discriminatory power and was selected as the optimal EP for PFGE of *Citrobacter*. With this EP, *Xba*I-restricted PFGE could distinguish 51 epidemiological unrelated *Citrobacter* strains at the strain level and give a *D*-value greater than 0.99. So PFGE protocol optimized in this study is suitable for subtyping of *Citrobacter* strains with the generally accepted probability of 1% of type I errors[32].

Traditional ribotyping techniques are commonly time-consuming and labor-intensive. The automated ribotyping, using RiboPrinter™ system, has overcome many shortcomings of the traditional methods and is used in identification and subtyping of bacterial pathogens[33]. The restriction enzyme used in automated ribotyping is the key factor for discriminatory power[22]. *EcoR*I is recommended for subtyping of *Citrobacter* by the manufacturer of RiboPrinter™. 51 *Citrobacter* strains were used to evaluate the discriminatory power of automated ribotyping. In comparison with PFGE, the automated ribotyping in this study showed lower discriminatory power, which was also reported in subtyping of other bacteria, such as *V. cholerae*[22] and *Clostridium botulinum*[34].

MLVA showed equivalent discriminatory power to PFGE in subtyping of pathogens, such as *E. coli* O157:H7, *V. cholerae*, *Streptococcus suis* and *Salmonella*[35-38]. However, the discriminating power of MLVA was significantly lower than that of PFGE and automated ribotyping for *Citrobacter* subtyping in this study. Only 2 loci showed polymorphism among the 17 VNTR loci in the *Citrobacter* strains analyzed. Among possible reasons for the lower discriminating power of the MLVA analysis in subtyping of *Citrobacter* strains, one may be that we have not found the high-resolution VNTR loci. So more genomes should been sequenced to find novel VNTR loci suitable for MLVA. Another reason could be the intrinsic limitations of MLVA, since MLVA exclusively focuses on tandem repeats within genomes.

The PFGE and automated ribotyping methods used in this study are feasible for three serotypes of *Citrobacter* strains (*C. freundii*, *C. youngae* and *C. braakii*) based on the appropriate bands distribution, good typability and discriminatory power. So PFGE and automated ribotyping could be used for subtyping of *Citrobacter* strains.

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