

## The clonal relationship among the *Citrobacter freundii* isolated from the main hospital in Kermanshah, west of Iran

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### ABSTRACT

**Background and Objectives:** *Citrobacter freundii* is an opportunistic pathogen causing nosocomial infections and resistant to various antibiotics. We aimed to determine the clonal relationship of *C. freundii* isolates using Pulsed-Field Gel Electrophoresis (PFGE).

**Materials and Methods:** Fifty clinical isolates of *C. freundii* were collected from the main hospital in Kermanshah. After antibiotic susceptibility testing and screening for extended-spectrum beta lactamase (ESBL), all isolates were genotyped by PFGE. The DNA fragment patterns were analysed using Gelcompar II version 6.6 software. The Dice coefficient was used to calculate similarities for cluster analysis.

**Results:** The PFGE results of 12 (24%) and 38 (76%) ESBL positive and negative isolates, respectively, produced 39 clusters ( $X_{1-39}$ ) with different genotype patterns. The  $X_1$  and  $X_2$  clusters were the major clusters, each contained 3 isolates from different hospital wards. However, the majority of isolates showed a high genotypic diversity.

**Conclusion:** Results revealed the genotypic diversity of *C. freundii* isolates indicating the various sources for the bacterial isolates. However, the presence of isolates with similar genotypes indicates the common origin for these strains and may reflect the strain dissemination within the hospital wards, in particular in infectious ward and intensive care unit.

**Keywords:** Clonal dissemination, *Citrobacter freundii*, PFGE

### INTRODUCTION

The genus *Citrobacter* is a member of the *Enterobacteriaceae*

*bacteriaceae* family and ubiquitous in soil, sewage and water. This group of bacteria can also colonize human and animal gastrointestinal tract (1). Several species of *Citrobacter* have been identified and a few of them, including *Citrobacter freundii*, have been recognized as opportunistic pathogens (2). Although *C. freundii* is often considered as a commensal bacterium of the human intestinal microbiota, it can cause opportunistic infections in hospitalized patients (3). It has been associated with nosocomial infections of urinary, respiratory, biliary, gastritis,

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meningitis, brain abscesses and neonatal sepsis (2-4). *Citrobacter* spp. are bacteria with low virulence and can persist as a population for a long time and thus accumulate resistance determinants, which may make the treatment of their infections more difficult (4). *Citrobacter* infections can be fatal, with 33-48 % overall death rates, and 30% for neonates (4). Resistance to antibiotics may develop by several mechanisms in *Citrobacter* species, but the most important one is the production of  $\beta$ -lactamases that hydrolyze the  $\beta$ -lactam ring of antibiotics (5). Plasmid-mediated extended-spectrum  $\beta$ -lactamases (ESBLs) were first reported in the mid 1980s (6). There are now over 200 distinct ESBLs that have been characterized and classified on the basis of amino acid gene sequences (7).

The dissemination of nosocomial infection caused by *C. freundii* has been attributed to the various ways of bacterial spreading including medical staff hands and other objects shared in hospitals (8). Molecular typing of microorganisms is a tool to identify the sources of contamination and to determine the routes of transmission for pathogenic bacteria that could enable us to more accurately detect and limit the spread of hospital-acquired infections. The pulsed-field gel electrophoresis (PFGE) has been used with a good discriminatory power for investigation of outbreaks and sporadic cases of *Citrobacter* spp. (9, 10). Given the increased rate of nosocomial infection, we aimed to determine the clonal relationship of *C. freundii* isolates from the main hospital in Kermanshah city.

## MATERIALS AND METHODS

**Bacterial isolates.** Fifty isolates of *C. freundii* were collected during 2013 from the main hospital (Imam Reza) in Kermanshah (west of Iran). This is a major local hospital in the area and patients are referred to this center from cities and neighboring provinces. All isolates were from hospitalized patients. The clinical samples were included urine (n=24, 48%), blood (n=11, 22%), stool (n=8, 16%), and others (respiratory tract and wound secretions, ascitic fluid and sputum) (n=7, 14%). The patients were 23 females (46%) and 27 males (54%) with the average age of 36.8 year old. All bacterial isolates were identified by bacteriological and biochemical tests followed by confirming using API20E Kit (bio-

Merieux, France).

**Antibiotic susceptibility testing.** Antimicrobial susceptibility testing for six  $\beta$ -lactam antibiotics was carried out using disk diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI) (11). The antibiotics were penicillins (ampicillin, piperacilin-tazobactam), cephalosporins (cefazolin, cefotaxim), monobactam (azetronam) and carbapenem (meropenem) (Mast, England).

**ESBL phenotypic confirmatory test.** Phenotypic confirmatory test (PCT) of ESBL was performed using the combined disk method on Mueller Hinton Agar (Merck, Germany) according to the CLSI recommendations. In this method, cefotaxime and ceftazidime alone and in combination with clavulanic acid were used. If the inhibited zone diameter increased  $\geq 5$  mm for either antimicrobial agents in combination with clavulanic acid it was considered phenotypic positive for ESBL (11).

**Molecular typing by PFGE.** All fifty isolates were used for genotyping by PFGE according to a previously described protocol (12) with some modifications. *C. freundii* isolates and *Salmonella enterica* serovar Braenderup H9812 (As DNA marker) genome were digested with 20U *Xba*I (Fermentas, Lithuania). Following *Xba*I digestion, the genomes were loaded into a 1% Low electroendoosmosis agarose (Merck, Germany). Electrophoresis was performed using a CHEF MAPPER apparatus (Bio-Rad, USA) at 14 °C for 22 h under the following conditions: initial switch time, 6.8 s; final switch time, 34.8 s; included angle, 120°; voltage gradient, 6 V/cm; ramping factor, linear. Gels were stained with ethidium bromide and visualized under UV light using Gel Doc apparatus (Bio-Rad, USA).

**Software analysis.** The DNA fragment patterns were analysed using Gelcompar II version 6.6 software (Applied maths, Belgium). The Dice coefficient was used to calculate similarities for cluster analysis. A cluster of isolates was defined to include all isolates with 80% cutoff for similarity of their DNA patterns according to the Tenover's criteria (13).

**Statistical Analysis.** Data were recorded and entered into an Excel file. Statistical analyses were per-

formed using SPSS software (version 16). Variables were analysed by chi-square test. A p-value of < 0.05 was set as the statistical significance of all analyses. Simpson's Index of Diversity (*D* value) was calculated using  $D = 1 - \left( \frac{\sum n(n-1)}{N(N-1)} \right)$  equation.

**RESULTS**

The antibiotic susceptibility of isolates presented in Table 1. Phenotypic ESBL production was detected in 12 (24%) isolates. PFGE showed the majority of isolates had 11 to 13 DNA bands (Figs 1, 2). According to the DNA fingerprints, 39 clusters (*X*<sub>1-39</sub>) were differentiated among isolates. The *X*<sub>1</sub> and *X*<sub>2</sub> clusters were the major clones, each with 3 isolates that were mostly urinary isolates. The next cluster (*X*<sub>3</sub>) with 2 isolates included 1 ESBL positive isolates resistant to all antibiotics tested, except carbapenems. The cluster *X*<sub>4</sub> to *X*<sub>7</sub> each with 2 isolates were from ICU and infectious wards (Table 2). The *X*<sub>8</sub> to *X*<sub>39</sub> clusters, each had a unique genotype and they were mostly from urine and blood samples with 8 (16%) ESBL positive isolates. They showed highly variable levels of resistance with diversely genotypic patterns. Finally, two strains of bacteria were nontypeable. The *D*

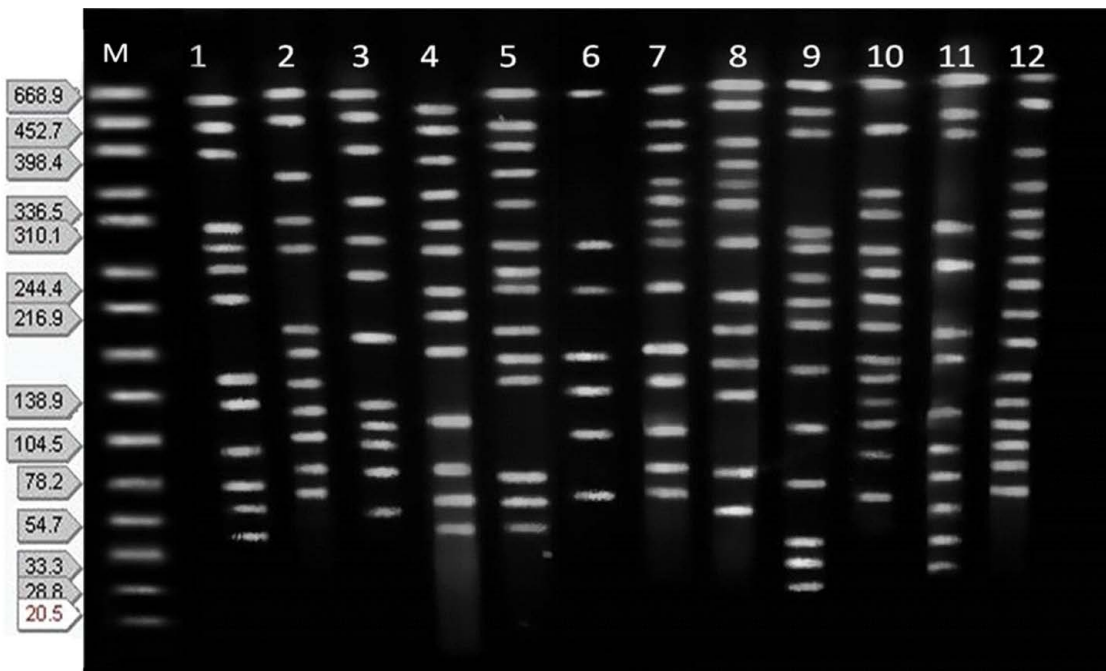
value was 0.9902.

**Table 1.** The antibiotic susceptibility of *C. freundii* isolates to β-lactam antibiotics (%)

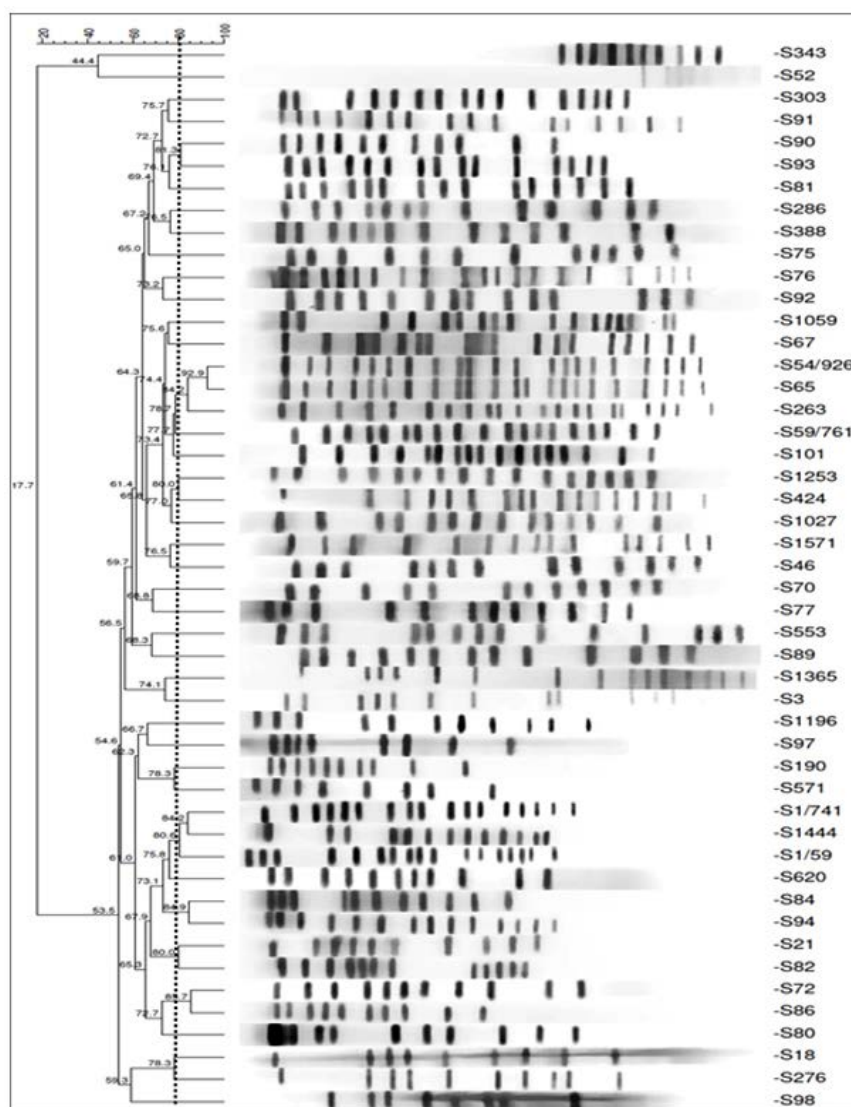
Antibiotics	Resistance	Intermedite	Sensetive
Ampicillin	38(76)	2(4)	10(20)
Pipracilin-tazobactam	3(6)	8(16)	39(78)
Cefazolin	44(88)	6(12)	0
Cefotaxim	13(26)	6(12)	31(62)
Meropenem	2(4)	2(4)	46(92)
Azetronam	9(18)	2(4)	39(78)

**Table 2.** The distribution of clusters isolated among hospital wards

Cluster number	Number of isolates	The number of isolates from hospital wards		
		ICU	Infectious	Surgery
X1	3	1	1	1
X2	3	1	1	1
X3	2	-	1	1
X4	2	1	1	-
X5	2	1	1	-
X6	2	1	1	-
X7	2	1	1	-



**Fig 1.** CHEF profiles of 12 *C. freundii* isolates. M: *Salmonella* serotype Braenderup strain H9812 as a molecular size marker. Number 1 to 12 the profiles of isolates.



**Fig 2.** Dendrogram for phylogenetic relationship among *C. freundii* isolates using PFGE data. The dotted line shows 80% cut off.

## DISCUSSION

Over recent years, the rate of nosocomial infections caused by *C. freundii* has been increased worldwide (4, 14). Research found the most frequent isolates of nosocomial infections among hospitalized neonates are *Citrobacter* spp. indicated the emergence of this bacteria as important nosocomial pathogens (4). In our study, resistance to cephalosporins, aztreonam and piperacillin was lower than some other studies. For example, a study in India has reported the resistance for cefotaxime 89%, ceftazidime 81.5% and aztreonam 74.7% (15). A research in Japan also showed

17.9% resistant to piperacillin(16). The results of previous studies, as well as our study, are compatible for the lowest resistance of *C. freundii* to carbapenems (16, 17).

The rate of ESBL production among our isolates was consistent with the findings of some previous studies. For instance, the prevalence of ESBLs in *C. freundii* isolates has been reported from 4.9 to 20.6 percent in South Korea (18, 19), 10.5 percent in Pakistan, 12.5 percent in Jordan and 29.5 percent in Poland (20-22). However, some other studies have reported lower or higher rates for ESBL in *C. freundii*. For example, the rates of 1.6 percent in France and 0.2 to 4.6% in Japan

have reported which are lower than our results (15, 23). On the other hand, studies have reported higher rates including a study in India with 41.1% of ESBL (24). These variations may reflect the diversity of regional resistant strains and suggest that continuous surveillance is essential to detect the emergence and spread of ESBL-producing strains for this bacterium. Excessive antibiotic exposure (especially the extended spectrum cephalosporins) and extended hospital stay has been identified as the risk factors for the selection of the ESBL producing strains (25).

The molecular typing of *C. freundii* isolates revealed two features among genotypic patterns; the first was the isolates with genotypic diversity and the second, the isolates with more than 80% similar genotypes. The genotypic polymorphism can reflect the various origins of isolates. Given the spread of *C. freundii* in hospital environment, it is not surprising that the isolates may be acquired by patients from different sources. Similarly, the results of several studies in various countries on *Citrobacter* spp. indicated the genotype diversity among isolates. For example, a study on 57 strains of *Citrobacter* spp. found 36 different molecular types among the isolates (4). Research on 38, 26 and 12 of *C. freundii* isolates using PFGE, have reported 23, 20 and 11 genotypic patterns, respectively (26-28). A study in China on 51 *Citrobacter* strains (including 43 *C. freundii*, 4 *C. youngae* and 4 *C. braakii*) from different sources, revealed 48 different clusters by PFGE with similarity between 43.8% and 100% (29). Another study in Japan on 57 ESBL-producing isolates found 13 different clusters and 18 unique PFGE types (23). The results of all above studies are compatible with our study for genotypic diversity of isolates.

On the other hand, a number of isolates with similar genotype in our study may indicate the dissemination of strains inside hospital environment, in particular in infectious ward and intensive care units. The spread of bacteria can happen in different ways, including by hospital staff or contaminated devices and equipments (8). Resistant isolates, in particular ESBL positive strains that cause nosocomial infections can be dominated population over time and this may limit the choice of effective antibiotics for their treatment (4).

## CONCLUSION

Based on the results, the diversity patterns of *C. fre-*

*undii* genotypes indicate the various origins of strains. However, the presence of strains with similar genotypes in clusters indicating a common origin for these strains. This fact may reflect the spread through hospital environments, especially in intensive care unit of the hospital. Given the clinical significance of ESBLs producing isolates, identification of these organisms is critical in order to get a better therapeutic response and reduce the risk of nosocomial outbreaks in the future.

## REFERENCES

1. Smith A, Saiman L, Zhou J, Della-Latta P, Jia H, Graham PL 3rd. Concordance of gastrointestinal tract colonization and subsequent bloodstream infections with gram-negative bacilli in very low birth weight infants in the neonatal intensive care unit. *Pediatr Infect Dis J* 2010; 29:831-835.
2. Whalen JG, Mully TW, English JC 3rd. Spontaneous *Citrobacter freundii* infection in an immunocompetent patient. *Arch Dermatol* 2007; 143:124-125.
3. Gupta R, Rauf SJ, Singh S, Smith J, Agraharkar ML. Sepsis in a renal transplant recipient due to *Citrobacter braakii*. *South Med J* 2003; 96:796-798.
4. Pepperell C, Kus JV, Gardam MA, Humar A, Burrows LL. Low-virulence *Citrobacter* species encode resistance to multiple antimicrobials. *Antimicrob Agents Chemother* 2002; 46:3555-3560.
5. Iredell J, Brown J, Tagg K. Antibiotic resistance in *Enterobacteriaceae*: mechanisms and clinical implications. *BMJ* 2016; 352:h6420.
6. Cantón R, Oliver A, Coque TM, Varela Mdel C, Pérez-Díaz JC, Baquero F. Epidemiology of extended-spectrum beta-lactamase-producing *Enterobacter* isolates in a Spanish hospital during a 12-year period. *J Clin Microbiol* 2002; 40:1237-1243.
7. Jacoby GA, Muñoz-Price LS. The new beta-lactamases. *N Engl J Med* 2005; 352:380-391.
8. Garcia-Cruz CP, Arguilar MJN, Arroyo-Helguera OE. Fungal and bacterial contamination on indoor surfaces of a hospital in Mexico. *Jundishapur J Microbiol* 2012; 5:460-464.
9. Hyytiä-Trees EK, Cooper K, Ribot EM, Gerner-Smidt P. Recent developments and future prospects in subtyping of foodborne bacterial pathogens. *Future microbiol* 2007; 2:175-185.
10. Foley SL, Lynne AM, Nayak R. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infect Genet Evol* 2009; 9:430-

- 440.
11. CLSI. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. CLSI document M100-S21. Wayne, PA: Clinical and Laboratory Standards Institute; 2011. 31:M100-S21.
  12. Durmaz R, Otlu B, Koksall F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella* spp. *Jpn J Infect Dis* 2009; 62:372-377.
  13. Tenover FC, Gay EA, Frye S, Eells SJ, Healy M, McGowan JE Jr. Comparison of typing results obtained for methicillin-resistant *Staphylococcus aureus* isolates with the DiversiLab system and pulsed-field gel electrophoresis. *J Clin Microbiol* 2009; 47:2452-2457.
  14. Kim PW, Harris AD, Roghmann MC, Morris JG Jr, Strinivasan A, Perencevich EN. Epidemiological risk factors for isolation of ceftriaxone-resistant versus -susceptible *Citrobacter freundii* in hospitalized patients. *Antimicrob Agents Chemother* 2003; 47:2882-2887.
  15. Mohan S, Agarwal J, Srivastava R, Singh M. Observations on *Citrobacter* species from a tertiary care health center with special reference to multi-drug resistance and presence of CTX-M gene. *Indian J Pathol Microbiol* 2014; 57:439-441.
  16. Ishii Y, Tateda K, Yamaguchi K, Japan Antimicrobial Resistance Surveillance Participants Group (JARS). Evaluation of antimicrobial susceptibility for beta-lactams using the E-test method against clinical isolates from 100 medical centers in Japan (2006). *Diagn Microbiol Infect Dis* 2008; 60:177-183.
  17. Ishii Y, Ueda C, Kouyama Y, Tateda K, Yamaguchi K. Evaluation of antimicrobial susceptibility for  $\beta$ -lactams against clinical isolates from 51 medical centers in Japan (2008). *Diagn Microbiol Infect Dis* 2011; 69:443-448.
  18. Choi SH, Lee JE, Park SJ, Kim MN, Choo EJ, Kwak YG, et al. Prevalence, microbiology, and clinical characteristics of extended-spectrum beta-lactamase-producing *Enterobacter* spp., *Serratia marcescens*, *Citrobacter freundii*, and *Morganella morganii* in Korea. *Eur J Clin Microbiol Infect Dis* 2007; 26:557-561.
  19. Park YJ, Park SY, Oh EJ, Park JJ, Lee KY, Woo GJ, et al. Occurrence of extended-spectrum beta-lactamases among chromosomal AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens* in Korea and investigation of screening criteria. *Diagn Microbiol Infect Dis* 2005; 51:265-269.
  20. Lartigue MF, Fortineau N, Nordmann P. Spread of novel expanded-spectrum beta-lactamases in *Enterobacteriaceae* in a university hospital in the Paris area, France. *Clin Microbiol Infect* 2005; 11:588-591.
  21. Hafeez R, Aslam M, Mir F, Tahir M, Javaid I, Ajmal AN. Frequency of extended spectrum beta lactamase producing gram negative bacilli among clinical isolates. *Biomedica* 2009; 25:112-115.
  22. Empel J, Baraniak A, Literacka E, Mrówka A, Fiett J, Sadowy E, et al. Molecular survey of beta-lactamases conferring resistance to newer beta-lactams in *Enterobacteriaceae* isolates from Polish hospitals. *Antimicrob Agents Chemother* 2008; 52:2449-2254.
  23. Kanamori H, Yano H, Hirakata Y, Endo S, Arai K, Ogawa M, et al. High prevalence of extended-spectrum  $\beta$ -lactamases and qnr determinants in *Citrobacter* species from Japan: dissemination of CTX-M-2. *J Antimicrob Chemother* 2011; 66:2255-2262.
  24. Batchoun RG, Swedan SF, Shurman AM. Extended spectrum beta-lactamases among gram-negative bacterial isolates from clinical specimens in three major hospitals in northern Jordan. *Int J Microbiol* 2009; 2009:513874.
  25. Shaikh S, Fatima J, Shakil S, Rizvi SM, Kamal MA. Risk factors for acquisition of extended spectrum beta lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* in North-Indian hospitals. *Saudi J Biol Sci* 2015; 22:37-41.
  26. Nawaz M, Khan AA, Khan S, Sung K, Steele R. Isolation and characterization of tetracycline-resistant *Citrobacter* spp. from catfish. *Food Microbiol* 2008; 25:85-91.
  27. Bai L, Xia S, Lan R, Liu L, Ye C, Wang Y, et al. Isolation and characterization of cytotoxic, aggregative *Citrobacter freundii*. *PLoS One* 2012; 7:e333054.
  28. Liu CP, Weng LC, Tseng HK, Wang NY, Lee CM. Cefotaxime-resistant *Citrobacter freundii* in isolates from blood in a tertiary teaching hospital in Northern Taiwan. *J Infect* 2007; 55:363-368.
  29. Zhang XA, Bai XM, Ye CY, Ren ZH, Xu JG. Establishment and comparison of pulsed-field gel electrophoresis, multiple-locus variable number tandem repeat analysis and automated ribotyping methods for subtyping of *Citrobacter* strains. *Biomed Environ Sci* 2012; 25: 653-662.