Jundishapur J Microbiol. 2019 January; 12(1):e67663. doi: 10.5812/jjm.67663.

Published online 2019 January 12. **Research Article**

Cluster Analysis and Genetic Characterization of *Enterobacter cloacae* Complex from Blood Cultures

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Received 2018 February 18; **Revised** 2018 December 10; **Accepted** 2018 December 14.

Abstract

Background: *Enterobacter cloacae* bacteremia is reported as an important cause of morbidity and mortality. The members of *E. cloacae* complex are clinically involved in nosocomial infections.
Objectives: The purpose of this study was to investigate the prevalence of *E. cloacae* complex and its members in blood samples and conduct the *hsp60* cluster analysis and genotyping of the isolates.
Methods: Eight isolates of *E. cloacae* complex were collected from blood cultures of hospitalized patients during the study period (December 2012 to November 2013). The *hsp60* sequencing was done for the genetic classification. Pulsed-field gel electrophoresis (PFGE) was used for genotyping of the isolates.
Results: Fifty percent of the isolates belonged to two *E. hormaechei* subspecies. Three isolates (37.5%) clustered within genotype III while only one isolate fitted cluster XIII genotype (12.5%). Pulsed-field gel electrophoresis analysis revealed four different pulsotypes.
Conclusions: Different *E. cloacae* complex species and subspecies unequally contribute to the pathogenesis of blood infections and the subspecies of *E. hormaechei* were found to be most prevalent. Moreover, the common *E. cloacae* pulsotypes were observed to essentially produce identical *hsp60* sequence types, indicating the probable clonality of isolates with identical pulsotypes.

Keywords: *Enterobacter cloacae*, Heat-Shock Proteins, Pulsed-Field Gel Electrophoresis

1. Background

Enterobacter cloacae is related to *Enterobacter* species (1). It has been estimated that *E. cloacae* complex is responsible for 5% of bloodstream infections; hence, it can be considered an important nosocomial pathogen (2). *Enterobacter cloacae* complex (ECC) has been found in various environmental niches (3) and it displays broad phenotypic and genotypic profiles as reported all over the world. According to the results of recent studies, it has been demonstrated that the members of ECC are opportunistic pathogens that can cause nosocomial outbreaks. A variety of infections has related to *E. cloacae* including urinary tract, lung, blood, skin, and wound infections and pneumonia (4).

Bloodstream infection caused by ECC has become a serious threat in nosocomial infection control in the last decade. Regarding previous studies, many risk factors can induce the septicemia related to ECC including invasive procedures, antibiotic therapy diet, prolonged hospitalization, and immunosuppressive therapies (5, 6). Although the classification of ECC species has been performed based on phenotypic methods, molecular approaches have played critical roles in the delineation of ECC in recent decades. According to a study by Hoffmann and Roggenkamp, the ECC can be classified into 13 genetic clusters based on the sequence analysis of the *hsp60* gene sequence (5). Almost 10 different species in ECC have been demonstrated to cause nosocomial infections in the wound, lung, and urinary tract, especially in intensive care units. The phylogenetical relationship between different species among ECC plays a critical role in the determination of the infection source and nosocomial infection control. Earlier studies have revealed that the prevalence of *E. hormaechei* and cluster III is higher than that of other strains or clusters of ECC in clinical samples (5, 7-9).

The species type and related genetic cluster of ECC can vary in different clinical samples; for instance, an analysis of infections pertaining to orthopedic implants revealed

that the most prevalent strains of ECC were *E. hormaechei* subsp. *Steigerwaltii* and *oharae*, while the members of cluster III were more common in other anatomical samples (10). Moreover, the analysis of *E. hormaechei* strains involved in a nationwide outbreak revealed that these strains harbored the genes associated with resistance or putative virulence located on mobile genetic elements. These properties might contribute to high genomic plasticity and epidemicity (11).

Previous studies demonstrated a lateral gene transfer between *E. cloacae* and other *Enterobacteriaceae*, which is caused by mobile genetic elements (12). These findings emphasize the evolutionary potential of the members of ECC for the acquisition of virulence and drug resistance determinants (13). Since *E. cloacae* bacteremia can cause mortality and morbidity in different parts of the world, evenly in both developed and developing countries, particularly Iran, acquisition of information about this organism and its genetic characterization can play a critical role in the prevention and treatment of the related infection.

2. Objectives

The study was designed to determine the prevalence of *E. cloacae* complex and its members in blood samples, as well as to classify the isolates via analyzing the distribution of *hsp60* clusters and genotyping of the isolates by the pulsed-field gel electrophoresis (PFGE) technique.

3. Methods

3.1. Ethics Statement

The present study was approved by the ethics and research committee of Iran University of Medical Sciences (IR.IUMS.REC.1393.24894).

3.2. Bacterial Strains and Epidemiological Data

The sample size was calculated according to the sample size calculating formula by the statistical advisor of the project considering the prevalence of *E. cloacae* isolated from blood culture specimens. In this study, eight *E. cloacae* species were isolated from 4986 blood cultures of hospitalized patients from three large teaching hospitals in Tehran, Iran, from December 2012 to November 2013. In total, 10% of all the samples were positive for bacterial pathogens among which, eight belonged to ECC. The blind blood culture method was used. In brief, blood culture bottles were incubated at 37°C for up to seven days until the microbial growth appeared. The blood culture bottles were subcultured onto blood agar, chocolate agar, and MacConkey agar at 37°C for at least 48 hours. The phenotypic identification of *E. cloacae* strains was performed using an API 20E system (BioMerieux, France). The results were interpreted with the analytical profile index database of the API Lab Plus software (API web stand-alone V 1.2.1; BioMerieux, Marcy l'Etoile, France) (14). *Enterobacter cloacae* PTCC 1003 (Persian Type Culture Collection) and *E. cloacae* PTCC 1798 were kindly provided by the IROST (Iranian Research Organization for Science and Technology) and used as positive controls. All of the *E. cloacae* strains showed at least 95% identity with *E. cloacae* by API 20E and they were assigned the code number 3.305.573.57.

3.3. Cluster Analysis of Isolates Using Sequencing Method

The boiling method was used for the preparation of bacterial DNA for PCR analysis. Fresh bacterial colonies were suspended in distilled RNase/DNase free water and boiled for 10 minutes, followed by centrifugation at 10,000 rpm for 10 minutes. The partial sequencing of the *hsp60* gene was done as described previously (5). Briefly, the *hsp60* gene (341 bp) was amplified using two primers *hsp60*-F (5'GTAGAAGAAGGCGTGGTTGC3') and *hsp60*-R (5'ATGCATTCGGTGGTGATCATCAG3'). The PCR was performed in a reaction mixture (total volume 25 μL) containing 15.6 μL sterile RNase/DNase free water, 2.5 μL 10X Taq polymerase buffer, 0.3 μL dNTPs (10 mmol L^{-1}), 0.5 U Taq DNA polymerase, 25 pmol of each primer, 0.6 μL of $MgCl_2$ (500 mM), and 5 μL of template DNA. Amplification was performed with the following program: Initial denaturation at 94°C for 5 minutes, followed by 30 cycles consisting of denaturation (94°C for 30 seconds), annealing (57°C for 30 seconds), and extension (72°C for 60 seconds). PCR was performed on a Bio-Rad MJ mini gradient thermal cycler. The PCR products were subjected to 1% agarose gels electrophoresis. Direct sequencing was performed with an ABI 3730X capillary sequencer (Genfanavaran; Macrogen, Seoul, Korea) using the forward strand of the amplified *hsp60* gene.

3.4. Nucleotide Sequence Analysis and Accession Numbers

The 341-bp sequences of the *hsp60* gene from the eight strains were deposited in GenBank under the accession numbers: KY549335, KY549336, KY549337, KY549338, KY549339, KY549340, KY549341, and KY746355. The sequences were compared with 46 reference sequences from the previously described strains in taxonomic studies, 10 type sequences strains (5, 10), and two positive controls (PTCC 1798 and PTCC 1003) using the MEGA software.

3.5. Genotyping of Isolates by Pulsed-Field Gel Electrophoresis (PFGE)

The PulseNet standardized protocol was used for subtyping of *Enterobacter* spp. (15). In brief, the bacterial sus-

pensions were prepared using the overnight grown cultures of bacteria and cell suspension buffer (100 mmol L^{-1} Tris, 100 mmol L^{-1} EDTA, pH 8.0). The bacterial suspensions were adjusted to a concentration of equal to the optimal density of 0.8 - 1.0 at 610 nm wavelength. Agarose plugs were made by cell suspensions, SeaKem Gold agarose (Lonza, Rockland, ME, USA), and proteinase K, treated with lysis buffer (50 mmol L^{-1} Tris, 50 mmol L^{-1} EDTA (pH 8.0), 1% sarcosine, and 0.5 mg proteinase K) at 54°C for 1 hours. Washing steps were done with sterile ultrapure water and TE buffer (10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA, pH 8.0) twice and four times, respectively. After washing, 40 units of *Xba*I restriction enzyme (Roche Diagnostic, Mannheim, Germany) were added to the digestion of plug embedded DNA.

Salmonella enterica serotype Braenderup H9812 was used as a DNA size marker. A CHEF Mapper XA System (Bio-Rad) was applied for electrophoresis with 200 V at 14°C for 18 hours with the increasing pulsed time from 2.16 to 54.17 seconds. The Gel Compare II version 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze the PFGE patterns. The patterns were compared using the Dice coefficient and unweighted pair-group method using arithmetic averages (UPGMA) clustering. A dendrogram was constructed using an optimization value of 0.5% and a position tolerance of 1.0%.

4. Results

4.1. Prevalence of Species and Genotypes

Each isolate was allocated to its individual species, subspecies, or genotypes by the sequence analysis of the 341-bp fragment of the *hsp60* gene. A neighbor-joining tree was constructed including all clinical type and reference strains of the ECC, as well as additional type strains of the *Enterobacter* genus as out-group (Figure 1). Four of the 13 genotypes and species reported so far were also found in our study. Four of the eight isolates (50%) were found belonging to two *E. hormaechei* subspecies, three of which (37.5%) were identified as *E. hormaechei* subsp. *Steigerwaltii*, while one isolate (12.5%) clustered with the *E. hormaechei* type strain (*E. hormaechei* subsp. *oharae*), suggesting that *E. hormaechei* was the most eminent in our collection of bloodstream infections. Three isolates (37.5%) clustered with the strain of genotype III and were the second most frequent genotypes of the ECC. Cluster XIII (sequence crowd cluster) was found in one isolate (12.5%) and the other clusters were absent among the isolates in this study. All frequencies are summarized in Table 1.

Table 1. Distribution of Clinical Strains Within the Genetic Clusters of *E. cloacae* Complex Derived From the *hsp60* Sequencing.

Name of Strain	Cluster	Number of Strains (%)
***E. cloacae* III**	III	3 (37.5)
E. hormaechei* subsp. *oharae	VI	1 (12.5)
E. hormaechei* subsp. *steigerwaltii	VIII	3 (37.5)
***E. cloacae* sequence crowd**	XIII	1 (12.5)
Total	4	8 (100)

4.2. Pulsed-Field Gel Electrophoresis

Fingerprints with approximately 7 to 12 bands per isolate were detected as the results of PFGE (Figure 1). According to the criteria of Tenover and colleagues the isolates would be considered belonging to the same clone if PFGE patterns differed by fewer than two restriction sites (16). One of eight strains was not typable and did not produce a distinguishable pattern even after multiple attempts (*E. cloacae* sequence crowed XIII). The PFGE analysis revealed four different genotypes, among which two were common types and two were single types. The common type A comprised two strains and the common type B involved three strains. The remaining two strains (single clonal type) produced distinguishable PFGE patterns that indicated the absence of any clonal relationship with the common types (Figure 2).

4.3. Statistical Analysis

Data were statistically analyzed by the chi-square test using SPSS software V.22. A significant correlation ($P < 0.05$) was observed between the *hsp60* sequence type of the isolates and related pulsotypes. The isolates were assigned to common *hsp60* subspecies and clusters were uniformly allocated within PFGE common types. This phenomenon strongly suggests the clonality of *E. cloacae* isolates with identical PFGE profiles.

5. Discussion

Due to the high level of similarity in phenotypic features of the strains belonging to different species and subspecies of ECC, the sequencing of the *hsp60* gene was introduced to distinguish between different strains with similar phenotypic profiles. This approach has been shown extremely high resolution in differentiating the species, sub-species, and genotypes of the studied complex. In the present study, the majority of the isolates belonged to the subspecies of *E. hormaechei*, followed by *E. hormaechei* subsp. *Steigerwaltii*, and *E. hormaechei* subsp. *oharae*. This is in agreement with a study by Kremer and Hoffman

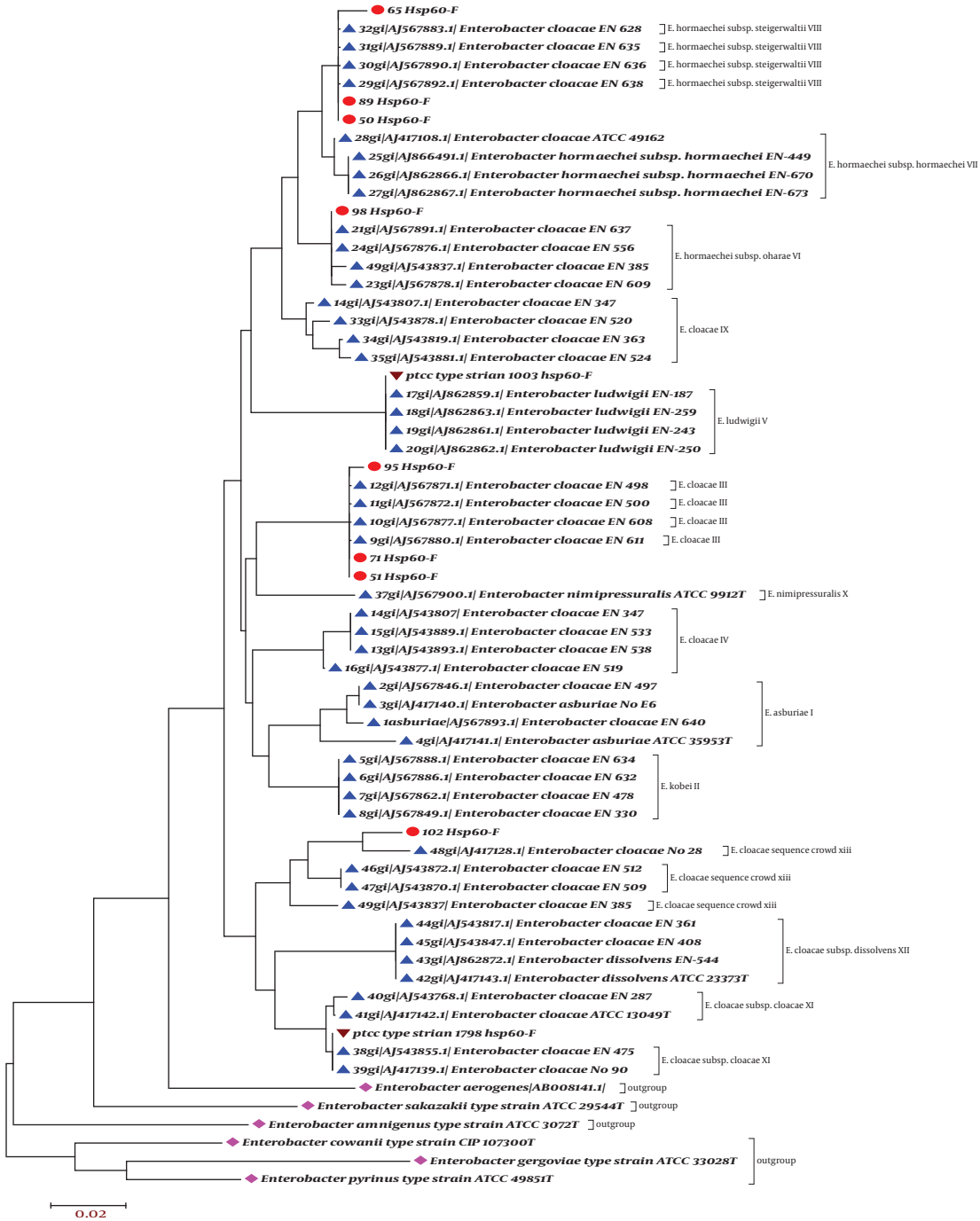
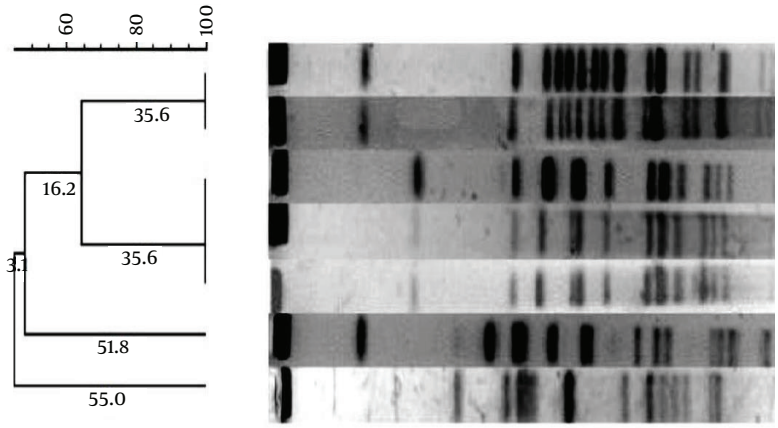


Figure 1. Neighbor-joining (unrooted tree) resulting from the analysis of 341 nucleotides (78 variables) of the *hsp60* gene sequences of nine clinical strains from blood cultures and 46 references and 11 type strains of the genus *Enterobacter*. Reference strain outgroup strains Persian type culture collection.

who showed that the subspecies of *E. hormaechei* are the most common ones in *E. cloacae* complex isolated from blood cultures, while the second most prevalent one was reported to be *E. cloacae* III in their study (9). Moreover, Stumpf and colleagues showed that the frequency of *E. cloacae* III and *E. hormaechei* subsp. *oharae* was 78% and 22%,



Types	Cluster	Hospital No.
A	III	1
	III	1
B	VIII	2
	VIII	2
	VIII	2
C	III	2
D	VI	3
Non-typable	XIII	3

Figure 2. Dendrogram drawn on the basis of the results of PFGE patterns of *Enterobacter cloacae* isolates from the blood infections compared to the results of *hsp60* gene sequencing

respectively, among the 23 members of *E. cloacae* complex isolated from blood cultures (17).

Our previously published data indicated that the majority (64%) of *E. cloacae* isolates originating from urinary tract infections (18) belonged to three *E. hormaechei* taxa, which comprised the foremost dominant species of our collection with the best clinical relevance to UTI (19). Moreover, Morand and colleagues demonstrated that clusters III, VI, and VIII in non-orthopedic clinical samples and clusters VI and VIII in orthopedic samples were the most common clusters (10). In other studies, it has been confirmed that the subspecies of *E. hormaechei* including subsp. *Steigerwaltii* and *E. cloacae* III are the most prevalent subspecies in *E. cloacae* complex isolated from clinical samples (5, 9, 10). Interestingly, the majority of multi-drug resistant strains are related to the strains within cluster VIII (20, 21).

The fingerprinting profile of all isolates was determined using the PFGE technique and the related dendrogram was generated to assess the clonal relatedness of the isolates. Accordingly, four pulsotypes (A to D) were obtained from seven strains, two of which (pulsotypes A and B) were assigned as common types (Figure 2). The most dominant one was pulsotype B, which was identified in three strains. The isolates with identical *hsp60* subspecies and clusters uniformly distributed within PFGE common types. It was elucidated in our previous study that the *E. cloacae* isolates obtained from UTI with common PFGE genotypes belong to identical *hsp60* clusters but similar to the present one. There were particular individual pulsotypes that could be assigned in common *hsp60* clusters (5). This suggests (i) the PFGE has more discriminatory power than *hsp60* genotyping and (ii) the circulation of common *hsp60* genotypes among different hosts might lead to the occurrence of multiple point mutations and affect the related PFGE genotypes. Stumpf and colleagues also reported the high discriminatory power of PFGE method than *hsp60* sequencing in distinguishing the clonality of clinical isolates (17).

5.1. Conclusions

In conclusion, this is the first report from Iran concerning *hsp60* cluster analysis and PFGE genotyping of *E. cloacae* isolates of blood cultures origin. No previous study has reported genotypes and subspecies/clusters of *E. cloacae* isolates from blood origin. The subspecies of *E. hormaechei* and genotype III are the most prevalent genotypes of the *E. cloacae* complex in blood cultures. This confirms that *E. cloacae* genotypes and clusters have an unequal contribution to the pathogenesis of blood infections. Moreover, common *E. cloacae* pulsotypes essentially produce identical *hsp60* sequence types, indicating the probable clonality of isolates with identical pulsotypes and the higher discriminatory power of PFGE compared to *hsp60* sequence typing.

Acknowledgments

This work was supported by the Antimicrobial Resistance Research Center, Iran University of Medical Sciences, Tehran, Iran.

Footnotes

Authors' Contribution: Bita Bakhshi and Mina Boustanshenas designed the study and drafted the manuscript; Majid Akbari performed the experimental methods on bacterial strains; Ali Majidpour consulted the study as the infectious diseases specialist.

Conflict of Interests: None declared.

Financial Disclosure: The authors declare that no competing financial interests exist.

Funding/Support: This study was financially supported by the Antimicrobial Resistance Research Center, Iran University of Medical Sciences, Tehran, Iran.

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