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Detection of Curli Biogenesis Genes Among *Enterobacter cloacae* Isolated From Blood Cultures

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Background: *Enterobacter cloacae* bacteremia infection is an significant cause of morbidity and mortality in both developing and developed countries. Curli fibrils are considered the most important virulence factor in these bacteria.
Objectives: The purpose of this research was to detect curli biogenesis genes among *Enterobacter cloacae* isolated from blood cultures.
Materials and Methods: Nine *E. cloacae* isolates were collected from blood cultures of inpatients admitted to three hospitals in the Tehran, Iran during December 2012 to November 2013. Confirmation of identity of the infecting organism as *E. cloacae* was performed by API20E system and the presence of a *csgA* and *csgD* genes by PCR using *csgA* and *csgD* specific primers.
Results: All of *E. cloacae* bloodstream strains (100%) harbored *csgD* gene (curli biogenesis activator) and seven (77.75%) carried *csgA* gene (curli major subunit) which is indicative of wide distribution of this virulence factor among our isolates.
Conclusions: Extensive presence of curli biogenesis genes (*csgD* and *csgA*) among *E.cloacae* strains and probably expression of those crucial role of this virulence factor in *E. cloacae* pathogenesis and invasiveness.

Keywords: Enterobacter cloacae, Bacteremia, Bacterial Fimbriae

1. Background

Enterobacter cloacae is the most commonly isolated species of genus *Enterobacter*, which has been accepted as the etiologic agent of many infections in hospitalized and enfeebled patients and has been known as a significant bacterial pathogen in recent years (1). *E. cloacae* are often isolated from nosocomial infections, including pneumonia, urinary tract and bloodstream infections. Particularly, *E. cloacae* are responsible for 3% - 6% of bloodstream infections, with approximate mortality rates ranging from 27% to 61% (2). Curli is a new class of bacterial surface structures which is expressed in *E. cloacae*, *Escherichia* coli and *Salmonella* spp. and is specified by its ability to bind to serum protein fibronectin (3). Curli fimbriae is involved in bacterial adherence to surfaces, cell accumulation and is a significant part of the extracellular matrix essential for the establishment of developed biofilms. Curli fimbriae is also regarded as significant virulence elements as it interact with a wide range of host proteins, which are suggested to help bacterial spreading in the host. These include extracellular matrix proteins and contact-phase proteins. Curli is known by toll-like receptors, leading to activation of the innate immune system. Curli is so regarded pathogen-associated molecular patterns (PAMPs) (4).

An extremely control pathway involving two divergently expressed operons is essential for curli biosynthesis. The *csgBAC* operon encodes the major curli subunit, *csgA*, and its homolog *csgB*. The *csgDEFG* operon encodes *csgD*, a transcriptional activator of the *csgBAC* operon, together with *csgE* and *csgF*, which act as chaperones and are needed for efficient Curli construction (5, 6). Curli homologous have formerly been recognized in some, but far from all genera of the *Enterobacter*iales (*Escherichia*, *Shigella*, *Salmonella*, *Citrobacter* and *Enterobacter*) (4).

2. Objectives

In the present investigation, we intended to detect the *E. cloacae* isolates in blood cultures of patients with sepsis and to study the presence of major Curli biogenesis genes; *csgD*, *csgA*.

3. Materials and Methods

3.1. Bacterial Strains

Nine *E. cloacae* isolates were collected from blood cultures of inpatients admitted to three major academic and governmental hospitals in the Tehran, Iran during December 2012 to November 2013. Samples were systematically and prospectively collected and stored. Confirmation of identity of the infecting organism as *E. cloacae*

was performed with phenotypic identification systems; API20E (BioMerieux, Marcy l'Etoile,France). The results were interpreted with the Analytical Profile Index (API) database of the ApiLab Plus software (api web stand alone V 1.2.1; BioMerieux, Marcy l'Etoile, France). The *E. cloacae* PTCC 1003 was used as positive control.

3.2. Molecular Confirmation of E. cloacae Isolates and Detection of Curli Biogenesis Genes

The oligonucleotide primers *Hsp60*-F (5'-GGTAGAAGAAGGCGTGGTTGC-3') and *Hsp60*-R (5'-ATGCATTCGGTGGTGATCATCAG-3') were used for genomic amplification of a 341bp fragment of the *hsp60* gene (7). Forward strand of the amplified DNA fragment was used for direct sequencing using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea).

The presence of a *csgA* and *csgD* genes was determined by PCR using *csgA* and *csgD*-specific primers listed in Table 1. Chromosomal DNA was prepared for PCR analysis by boiling method in which fresh bacteria colonies were suspended in distilled water molecular grade and boiled for 10 minutes. The suspension was centrifuged (10 minutes at 10,000 rpm) and the supernatant transferred into a new tube and used directly for PCR assay.

Reactions were performed in a total volume of 20 μL under standard conditions: TaqDNA *Polymerase* 2x Master Mix (Taq DNA *polymerase* 2x Master Mix RED 1.5 mM MgCl2, Cat. No. A180301, Ampliqon A/S, Stenhuggervej 22, 5230 Odense M, Denmark) 8 μL, primer-F 0.5 μL (2.5pM), primer-R 0.5 μL (2.5pM), DNA template 1 μL and distilled water molecular grade 10 μL. PCR amplifications were performed in a DNA Thermal Cycler with the following parameters: denaturation for 5 minutes at 95°C; 35 cycles of 95°C for 15 second, 60°C for 15 second, and 72°C for 15 second; extension for 5 minutes at 72°C.PCR products were visualized after electrophoresis in 1% agarose gels in 0.5x TBE buffer, staining with CinnaGen DNA Safe Stain (CinnaGen DNA safe Stain, Cat. No: PR881603, SinaClonBioScience), and exposure to UV light.

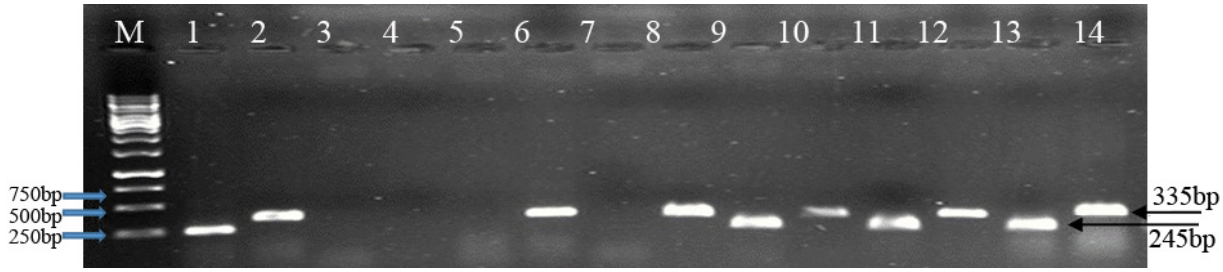
4. Results

All of *E. cloacae* strains which were identified by API 20E had the code of 3.305.573.57, and at least 95% identity with *E. cloacae* species. Sequencing of *hsp60* gene and alignment of 341 bp sequence with BLAST software of GenBank database confirmed the identity of isolates. The *csgD* gene showed a band of expected size (355bp) in all 9 *E. cloacae* under study (100%). Seven of nine *E. cloacae* isolates produced 245bp amplification band of *csgA* gene (77.75%).The result of PCR analyses for *csgA* and *csgD* genes is shown in Figure 1.

Table 1. Primers Used In This Study

Primer	Sequence (5 - 3)	Target Gene	PCR Product (bp)	Reference
Hsp60		*hsp60*	341	(7)
F	GGTAGAAGAAGGCGTGGTTGC			
R	ATGCATTCGGTGGTGATCATCAG			
csgA		*csgA*	245	This study
F	ATTGCAGCAATCGTAGTTTCTGG			
R	ATWGAYCTGTCATCAGAGCCCTGG			
csgD		*csgD*	355	This study
F	TGAAARYTGGCCGCATATCAATG			
R	ACGCCTGAGGTTATCGTTTGCC			

Figure 1. PCR Amplification of *csgA* and asgD Genes



Lane. M: 1 kb ladder, lane. 1, positive control of *csgA* gene and lane 2 positive control of *csgD* gene, lane3 and 4 negative control of *csgA* and *csgD* genes, lanes 6, 8, 10, 12 and 14 *csgA* gene of strains that isolated from blood culture. Lanes 7, 9, 11 and 13 *csgD* gene of strains that isolated from blood culture.

5. Discussion

E. cloacae bacteremia infection is a serious cause of morbidity and mortality in both developing and developed countries, especially Iran (1, 8). In a prospective study of Karambin et al. (2010) on 611 newborns admitted with the probable diagnosis of septicemia, 64 (10.6%) cases had positive blood culture and commonest pathogens identified were *Enterobacter* spp. (78.1%) and *Klebsiella* spp. (6.2%) (9). In a 2-year retrospective study of Rahbar et al. (2005) on 6,492 patients in various wards, 593 (9.1%) had positive blood cultures, that among which gram-negative bacilli were responsible for 42.3% of isolates and *Enterobacter* spp. was in the second (10).

All of *E. cloacae* bloodstream strains in this study harbored *csgD* gene which shows the wide distribution of this adhesin among blood isolates. Seven of isolates (77.75%) carried *csgA* gene and its absence in remaining 32.25% of isolates may be probably due to the absence of entire *csgA* gene or point mutations within its coding sequence which correlates with the primer annealing site. Studies of Barnhart et al. (2006) have shown that mutants of *csgA* show non-curliated phenotype (11). Bian et al. (2000) showed evidence that curli is expressed in vivo in human sepsis and suggested a possible role for curli and *csgA* in the induction of proinflammatory cytokines during E.coli sepsis (3). Biesecker (2012) showed a rise within the survival of curli-producing E. coli in vivo, and advised that curli defends against complement killing (12). Therefore, probably isolates of *E. cloacae* that produce the *csgA* are more virulant than those with no *csgA* synthesis and curli product. This may the reason for the extensive presence of *csgA* and *csgD* in *E. cloacae* strains isolated from bloodstream.

In conclusion, this study showed that most of the *E. cloacae* strains isolated from blood cultures have csg genes and probably are able to express curli and suggest that this virulant factor may play a significant role in invasiveness and pathogenesis of *E. cloacae*.

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Footnote

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