Research article

Molecular study of *Enterobacter cloacae* isolated from leukemia patients

Inas S. Mohammed[1], Sussain S. Hussain[2], Rajwa H. Essa.

[1]Department of Biomedical Engineering, Biomechanical Branch, University of Technology, Baghdad, Iraq
[2]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

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Corresponding author: **Inas S. Mohammed** Email: inassmohammed1@gmail.com

ABSTRACT

Introduction and Aim: Polymerase chain reaction (PCR) of 16S rRNA and virulence genes associated with Type Three Secretion System (TTSS) has been used as a rapid method for the identification of the pathogen *Enterobacter cloacae* in leukemia patients. Rapid diagnosis of this pathogen becomes necessary for starting a proper treatment in these patients. In the current study, we aimed to isolate *Enterobacter cloacae* from leukemia patients and study the TSSS genes associated with these isolates using molecular methods.

Materials and Methods: *E. cloacae* isolates identified using biochemical tests was molecular confirmed by 16S rRNA polymerase chain reaction (PCR)**.** Genomic DNA extracted was also subjected to the Type Three Secretion System **(**TTSS) associated virulence genes *esc*V and *asc*V using specific primers.

Results: 30 (23.07%) out of the 130 blood samples tested in this study were identified as *E. cloaca* by biochemical tests. Further confirmation using molecular methods showed only 11 of these isolates to be 16S rRNA positive. Few of these isolates were positive for the TTSS associated ASCV gene of the samples positive for presence of the *asc*V gene. All strains were negative for the *esc*V gene.

Conclusion: PCR is the best technique in comparison with other conventional methods for the diagnosis of *E. cloacae* in leukemia patients because of its safety, high sensitivity, specificity, and speed.

Keywords: PCR; 16S rRNA; Type III secretion system; *Enterobacter cloacae*; leukemia patients.

INTRODUCTION

Leukemia is known to affect men and women of all ages. According to the World Health Organization report (WHO 2014), leukemia is reported to be the second major cause of mortality in women and the third in men of Iraq (1). Leukemia patients are highly immunosuppressed and at a risk of developing infections. The genus *Enterobacter* of the Enterobacteriaceae family has been shown to be associated with leukemia-related infections. *Enterobacter* spp. are Gram-negative facultative anaerobic rods of 2 mm length and motile via peritrichous flagella (2). Despite the fact that an *E. cloaca is* the major prevalent *Enterobacter species* responsible for nosocomial infections, not much is identified regarding the factors that influence its virulence and pathogenicity (3). Clinically, the diagnosis of *Enterobacter* spp. is based on culture method and biochemical tests, while molecular identification to the species level uses the 16S rRNA Polymerase Chain Reaction (PCR) technique (4). For the bacterial infections' diagnosis, sequencing analysis was proposed as an alternate way to overcome the drawbacks of standard culture-based bacterial identification (5). 16S ribosomal RNA (rRNA) genes are the most commonly employed molecular marker for bacterial classification in sequence-based studies (6). Although 16S rRNA gene has been generally used in the identification of *E. cloacae*, the presence of ASCV and ESCV genes of the Type III secretion system (TTSS) have been used as a marker in diagnosing pathogenic *E. cloacae* associated with leukemia patients (7).

In this study we aimed to identify *E. cloacae* in patients with Leukemia. Further, to ascertain whether these strains are pathogenic, we investigated into the presence of *E. cloacae* TTSS associated genes which are used as a general indicator of bacterial virulence

MATERIALS AND METHODS

Sample collection and bacterial isolation

A total of 200 samples consisting of urine (50) and peripheral blood (130) were isolated from Leukemia patients. These samples were divided into two categories: female (105) samples and male (75) samples and (20) controls were received from Medical city/ Baghdad Teaching Hospital/Hematology Center, Iraq during the period April 2020 to April 2021. Blood (2-3 ml) drawn from each leukemia patient was transferred to test tube containing sterile Brain-Heart Infusion medium and incubated at 37˚C for 24h for enhancing bacterial growth (8). After 24h, a loopful of culture was sub-cultured onto Trypticase soy agar and MacConky agar medium and further incubated for 24 h at 37˚C. Colonies developed were picked and

subjected to biochemical tests for the identification of the bacteria grown (9, 10). Molecular identification for further confirmation was done using PCR (11)

Biochemical tests for *Enterobacter cloacae*

Biochemical identification for cultures was done using the universal identification GN24 kit (DIAGNOSTICS i.n.c, Galanta, Slovakn Republic) capable of identifying >200 species of glucose fermenting /nonfermenting Gram negative bacteria. All tests were carried out according to manufacturer's instructions. Briefly, an 18-24 old culture of the pure colony developed on agar plates was picked and suspended in 0.85% saline solution. 100 µl of well-homogenized suspension was transferred to the 24 wells strip of the microtitration plate of the GN 24 kit containing the dehydrated substrates. The microtitration plate were incubated for (18-24) hours at (37˚C) after which the color change in each well was visually recorded and evaluated by color description stated in the kit leaflet. The confirmed isolates were also subjected to a catalase test (CAT) not included in the kit.

Genomic DNA extraction

Genomic DNA from pure cultures of *E. cloaca* was extracted based on the ABIO pure extraction protocol involving the following steps: About 1 ml of an overnight grown culture of *E. cloaca* was taken and centrifuged at 13,000 rpm for 2 mins. Cell pellet has been entirely re-suspended in (200µl) of the Buffer CL. For the cell lysis and protein digestion, (20µl) of the Proteinase K solution (20 mg/ml) was added to (200µl) of Buffer CL and cell pellet, after that, the tube has been vigorously mixed with the vortexing and incubated at a temperature of (56˚C for 30 min), for further lysis incubated at a temperature of (70˚C for 30 min). After incubation, (200µl) of the Buffer BL has been added into sample, after that, the tube has been thoroughly mixed with the use of the vortex and after that, incubated at a temperature of (70˚C for 30 min).

16S rRNA Polymerase Chain Reaction (PCR)

For molecular detection of *E. cloaca*, the DNA extracted was subjected to 16S rRNA PCR using the primers 27 F:5'-AGAGTTTGATC CTGGCTCA G-3' and 1492R: 5'-TACGGTTACCTTGTTACGACTT-3' to yield an amplicon of size ~1500bp. The lyophilized primers obtained was resuspended in nuclease free water to a final concentration of 100 pmol/ml. Aliquots of 10ul of the stock solution were made and stored at -20ºC until further use.

The PCR was carried out in a mixture consisting of 23 µl master mix, 1 µl each of forward and reserve primers, 2 µl of template DNA and 8.5 µl nuclease free water. The amplifications were carried out in a programmable thermocycler (ABIOpure, USA), with the following steps: initial denaturation at 95˚C for 5 min followed by 1 cycle, denaturation at 95˚C for 30 second by 30 cycles, annealing at 60˚C for 30 second and extension at 72˚C for 1 min by 30 cycles with a final extension at 72˚C for 7 min and hold at 10˚C for 10 min by 1 cycle. The PCR products were separated by electrophoresis performed in 1% agarose gel (100 v/m AMP for 75 min), followed by staining with ethidium bromide and visualized using a UV transilluminator (320 nm) and photographed. Positive and negative controls of PCR were included in each experiment. The PCR products were purified and outsourced for sequencing (Macrogen Corporation, Korea). The results obtained analyzed were analyzed using the Genious software (Macrogen corporation, Korea).

PCR for TTSS genes

PCR was carried out to determine the presence of genes (*esc*V and *asc*V) associated with *E. cloaca* TTSS as mentioned previously (12). The primers used were as follows: ESCV2 5′-TAACTTCTTTCCCCA CAATC-3′, ESCV2(5′-TATCCCCAACAGGCAAAC -3′ which targets the *esc*V gene and ASCV-F (5′-GTAARCAGATGAGTATCGATGG-3′) and ASCV-R (5′-GAGACSCGGGTGACGATAAT-3′) targeting the *asc*V gene. PCR assay was performed in a final volume of (10 µl) master mix, (1 µl) forward and reserve primer, (6 µl) nuclease free water and (2 µl) of samples DNA. The PCR amplifications was carried using the following steps: initial denaturation at 95˚C for 5 min followed by 1 cycle, denaturation at (95˚C) for 30 seconds by 30 cycles, annealing at 55˚C for 30 seconds and extension at 72˚C for 30 seconds by 30 cycles with a final extension at (72˚C) for 7 min and hold at 10˚C for 10 min by 1 cycle. Electrophoresis, sequencing, and analysis of the genes was done as described for 16s rRNA gene.

RESULTS

Identification of *E. cloacae* by biochemical tests

The result shows that 30 out of 130 blood samples were positive. The isolates were identified to be *E. cloaca* based upon their typical biochemical reactions (13) as given in Table 1. *E. cloaca* strains are motile, negative for tests oxidase, H_2S, urease and indole, while positive for citrate and catalase tests.

Typical red to pink colonies of *E. cloaca* developed in MacConkey agar are shown in Fig.1. Phenotypically, 30 (23.07%) blood samples were positive for the presence of *E. cloaca*.

Table 1: Biochemical characters of *Enterobacter cloacae* isolates

No	Test	Biochemical result
1.	Morphology shape	rod
2.	Indole test	-
3.	M R test	-
4.	V-P test	+
5.	Citrate test	+
6.	Catalase test	+
7.	Oxidase test	-
8.	Urease test	-
9.	Motility	+
10	Production of H_2S	-

+ = Positive, - = Negative, Methyl red test, V-P: Voges-Proskauer test

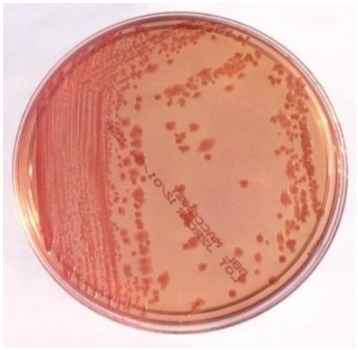

Fig. 1: *E. cloacae* colonies on MacConkey agar

Genotypic detection of *E. cloacae*

The 30 *E. cloacae* isolates identified by biochemical tests were further subjected to molecular identification using16S rRNA PCR. Results indicated only 11 of the 30 isolates to molecularly confirm as *E. cloacae* yielded an amplicon size of approximately 1500bp for 16S rRNA (Fig 2). Reference strain of *E. cloacae* was positive for 16S rRNA PCR.

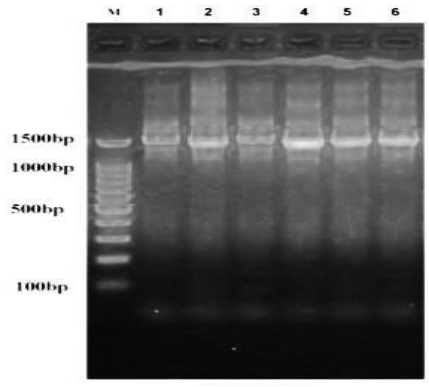

Fig.2. Detection of *Enterobacter cloacae* using 16S rRNA PCR Lanes 1-6: positive samples with an amplicon size of 1500bp; M: 100bp DNA marker

PCR detection of TTSS genes

Thirty samples tested positive for *E. cloacae* in this study were analyzed for the presence of the TTSS genes *esc*V and *asc*V using specific oligonucleotide primers. The gene *esc*V was seen to be absent in all strains tested. However, the *asc*V gene was seen to be present in 11 strains (36.7%) of the strains yielding an amplicon of size 331bp by PCR (Fig. 3).

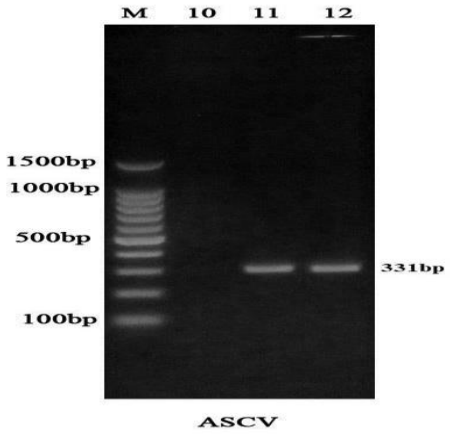

Fig. 3: PCR for the TTSS associated *asc*V gene. Lane 10: Negative for *asc*V gene; Lanes:11-12: Positive for *asc*V gene (331bp size) M lane: 100bp DNA Ladder.

DISCUSSION

In the present study, blood samples isolated from leukemia patients showed the presence of *Enterobacter cloacae.* A malignant condition known as leukemia results from the abnormal proliferation of the blood cells in blood-forming organs and bone marrow, and it may be classed based on the pace of the progression. The development of bacteremia in cancer patients due to *Enterobacter* spp. has been previously reported (14). The identification of *E. cloacae* strains in 11 of the leukemia patients in this study therefore assumes significance as the presence of this bacterium could lead to bacteremia among these patients. Moreover, this study also noted that routine biochemical tests in the identification of this bacterium can give false positive results, demonstrating the need for molecular techniques for rapid and accurate identification and being clinically relevant for the implementation of an appropriate treatment plan.

Many Gram negative bacterial pathogens are known to possess the Type III secretion systems (T3SSs), known as “injectosomes” (15) which are unique virulence mechanism through which the pathogen inject effector proteins directly into the infected host cells to promote adherence, invasion, and colonization. The TTSS consists of several proteins which have a significantly impact on the virulence of the organism. A previous study has shown the presence of TTSS in clinical isolates of *E. cloacae* having cytotoxic activity (12). An analysis for the presence of *asc*V and *esc*V TTSS genes among *E. cloacae* in this study revealed few strains to possess the *asc*V gene. None of the *E. cloacae* strains

possessed the *esc*V gene which is consistent with previous study (12). The *asc*V gene, a homologue of the *Ysc* gene family encodes inner membrane proteins in *Yersinia* spp, *Aeromonas* spp and *Pseudomonas aeruginosa* (16;17) playing a crucial role in the pathogenesis of the bacterium. Hence, the presence of the *asc*V gene among leukemia *associated E. cloacae* strains indicates that this bacterium could be pathogenic causing cytotoxic to the host cells (12).

CONCLUSION

In patients with leukemia, diagnoses of *Enterobacter cloacae* infection should be based on molecular techniques such as 16S rRNA PCR rather than biochemical tests. Presence of TTSS genes within *E. cloacae* could be used as a marker for knowing the pathogenic nature of the bacterium.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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