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Study the role of pH in transcriptional activator (*csg*D) gene expression in *Enterobacter cloacae* locali isolates.

Basima Qasim Hasan AL-Saadi, Marrib Nazih Rasheed, Hiba Hazim Hamid ALYousif, Noor Yahyaa Salman and Sara Faisal Hasan

Institute of genetic engineering and biotechnology for post graduate studies / University of Baghdad, Baghdad / **Iraq**

***Correspondence:** basima22000@yahoo.co.uk **Accepted:** 31 Jan.2019 **Published online:** 11 Apr. 2019

Enterobacter cloacae is the most commonly isolated species of genus Enterobacter which has been accepted as the etiological agent of many infections in hospitalized and enfeebled patients and has been known as significant bacterial pathogen in recent years. *E. cloacae* are common gram negative opportunistic type of bacteria that cause disease after the host immune system has been weakened by another infection or injury and is associated with nosocomial infections. The infection may be contracted through the skin GIT,UTI or cross contamination .Aims of present study were to the presence of transcriptional activator of the csg BAC operon gene (*csgD)*and study the role of pH as environmental factor in gene expression of (csgD) .In this study urine samples from 75 patients clinically diagnosed with urinary tract infection in Baghdad city , for gram staining ,culture, Api20 Esystem and gene expression of(csgD) gene in standardized pH7 and acidic pH4was done by RT-PCR using specific primers .Conventional methods of gram staining ,culture and API 20E system showed positive results for *E. cloacae in* 10(13.3%)out of 75patients.The results found the highest values of gene expression fold for the csgd gene in pH7 (1) while lowest value of fold for (*csgD*)at acidic pH4(0.076) therefore the change conditions growth such as pH of bacteria *E. cloacae* leads to change of gene expression *of (csgD).rpoB* gene expression results, Which was used as reference gene ,confirmed that this gene was well suited as housekeeping gene .

Keywords: E. cloacae, gene expression ,transcriptional activator(csgD) gene

INTRODUCTION

Enterobacter cloacae are nosocomial pathogens that are responsible for various infections including respiratory tract infections, intra- abdominal infections, skin and soft tissue infections and urinary tract infection (Akbari, et al., 2016).The most important characteristic of this *m.o* is a gram negative facultative –anaerobic , rode –shaped and due to their prevalence in the body , this organism affects mostly vulnerable age groups such as the elderly and the young and can cause prolonged hospitalization (Sedberry-Ross and Pohl,2008).Many people suffering from urinary tract infection with *E. cloacae* complain of frequenturination, pain or burning while urination and reduce urine flow (Ko¨vesandaWullt,2016) .Curli is a new class of bacterial surface structures which is express in *E. cloacae* , *Escherichia coli* , and *Salmonella* spp. Curli fibers (also known as a thin aggregation fimbriae) are a major factor in adhesion to surface ,cell aggregation and biofilm formation (Neem et al., 2003) .Expression of Curli is linked to cellulose biosynthesis which lead to the production of an extracellular matrix and resultin acell- cell and cell-surface interactions .

Curli expression is dependent on different environmental and physiological cues such as alow growth temperature < 30C°, low osmolarity and slow growth or starvation (i.e., conditions usually encountered by the bacteria outside the mammalian host)(Prigent-Combaret et al., 2001) . curli are an important virulence elements as it interact with range of host proteins , which are suggested to help bacteria spreading in the host (Olsen, et al., 1993) , however , expression of *csgD* increases in late exponential phase and is influenced by temperature , osmolarity and availability of nutrients ,oxygen and ironin addition production of curli is subject to complex regulation, which affects both the *csg-DEFG* operon (encoding the *csgD*transcription regulator and the *csgEFG* curli-specific transport system) and the *csgBAC*operon (encoding curli structural subunits) .Curli production is dependent on the *csgD* transcription activator, which also promotes cellulose biosynthesis (Brombacher et al.,2003) .

This study was design to detect the presence of *Enterobacter cloacae* in urine culture of Iraqi patient with urinary tract infection and to investigate the effect of PH on expression of Curli biogenesis gene, *csgD.*

MATERIALS AND METHODS

Subjects:

In this study a total of 10 isolates were collected from urine cultures of 75 in patients suffering from UTI admitted to AL-Kathumia pediatric governmental hospitals in Baghdad , these isolates were obtained between April to September /2017.

The sample were collected and stored. Bacterial identification as *E. cloacae* were performed by standard biochemical methods using the (API20 E, Biomerieux, France) .Bacterial isolates were preserved by inoculation of single pure isolated colony to brain heart infusion agar (BHIA) slant incubated over night at 37C°then stored at 4 C°for few weeks for further study (WHO,2003)

Molecular study:

*csgD*Gene Expression using RT-qPCR Technique

The expression levels of *csgD* gene were estimated by Quantitative reverse transcription PCR (q RT- PCR). The oligonucleotide primers *csgD* - F(TGAAARYTGGCCGCATATCAATG) and csgD- R(ACGCCTGAGGTTATCGTTTGCC)*csgD*gene sequence for detection of the gene expression , as PCR product 355 bp**(**Akbari et al.,2015). , as well as Specific primers *rpoB*gene sequence were used to detect *Enterobacter cloaecea*as housekeeping gene *rpoB*-F(GCA ACT TGT TGT CGC GGA TT) and *rpoB*- R (TCG ACC GTC GTC GTA AGC T)They were prepared according to the information of the supplying company.

The expression levels of *csgD* gene were estimated by Quantitative reverse transcription PCR (qRT-PCR) . To confirm the expression of target gene, RT-qPCR SYBR Green assay was used .The mRNA level of endogenous control gene *RpoB* were amplified and used to normalize the mRNA levels of the csgD gene.

QRT-PCR was performed by using the QIAGEN Real-time PCR System with qPCRsoftware (Rotor-Gene Q,Germany). The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct) employing the 2xqPCR Master Mix Kits components. The reaction was done in a duplicate and included a non-template control (NTC).

The measurement of gene expression of the *csgD* gene in these isolates was done in standardized incubation conditions (pH 7, 37 Cº, 24 hrs.) Andunder variable incubation pH conditions

*csgD*Gene Expression in Standardized Incubation Conditions

The total RNA of samples was extracted using General RNA extraction kit (Dsbio, China) following the protocol provided by the manufacturer then Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit (Wizbiosolution). The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl).Synthesized cDNA was immediately used as template for PCR

Housekeeping Gene Amplification

RpoB housekeeping gene was used as an internal control to be used in calculating the ΔCT value. A qPCR reaction of amplification of *RpoB* was done with the Thermal profile shown in table (2).

Real Time qRT-PCR Analysisof *shlA*Gene Expression

1. ΔCT

The expression ratio was calculated without a calibrator sample 2-ΔCt according to the following equation (Schmittgen et al., 2008):

ΔCT (test) = CT gene of interest (target gene) – CT internal control (housekeeping gene).

Finally, the expression ratio was calculated according to the formula

2−ΔCt = Normalized expression ratio.

2- ΔCT

To compare the transcript levels between different samples the 2-ΔΔCt method was used (Livak and Schmittgen, 2001).

The CT of gene of interest was normalized to that of internal control gene. The and *(csgD*gene) interest gene was calculated as the following formula:

ΔCT (test) = CT gene of interest (target, test) – CT internal control.

ΔCT (calibrator) = CT gene of interest (target, calibrator) – CT internal control.

The calibrator was chosen from the control samples.CT values ≥ 38 were considered unreliable and neglected .The ΔCT of the test sampleswas normalized to the ΔCT of the calibrator. ΔΔ CT was calculated according to the following equation:

ΔΔ CT=ΔCT (test)-□CT (calibrator)

Finally, the expression ratio was calculated according to the formula

2-ΔΔCt = Normalized expression ratio.

***csgD* Gene Expression in Different Environmental Factors**

Volume equal 0.1 ml of an overnight nutrient-broth culture of each strain was added to 10 ml of BHI broth and shaking incubated in different pH. Factors after growth, RNA extraction was done by the same steps by total RNA Isolation System kit, then done c DNA synthesis for mRNA. , RT master mixed and programs that were used in standardized conditions.

RESULTS AND DISCUSSION

Enterobacter species, particularly *Enterobacter cloacae,* are important nosocomial pathogens responsible for about 13.20 % inUTI Iraqi patients according to previous study conducted by(Basima et al., 2017*) Enterobacter cloacae* exhibited the highest biofilm production (87.5%) among isolated pathogens (Clark, 2015**)**

In the present study RT-qPCR applied utilizing SYBR green, fluorescent dye, the amplification has been recorded as Ct value (threshold cycle), is that high Ct values indicate low gene expression and low Ct value indicates a high gene expression (livak and Schmittgen , 2008). The reason of using of *RpoB* gene as refrence gene is that its expression remains constant in the cells even the conditions are change. According to the 2–Δct value , the fold of expression for rpoB gene was 1, 1.09 under varial PH conditions, pH7 and pH 4 respectively. Little variation in the fold of gene expression make *RpoB* gene a useful reference gene as represent in(Table 1and Figure1)

Expression for *csgD* gene by RT-qPCR

Expression of *csgD* is highly regulated. A variety of environmental factors including nutrient limitation, low osmotic strength, low temperature, microaerophilic conditions and iron limitation increase expression *of csgD* **(**Neema*,* et al.,2003)as shown in (Table2 and Figure 2)

The result showed the fold of gene expression for the *csgD* gene. In the present study, quantitative RT- PCR assay analyzed the mRNA expression of *csgD* genes by comparing bacterial growth with different pH factors as variable factor foreach sample. The Ct values of gene amplification were recorded from the software of quantitative RT PCR. The calculation of gene expression fold change was done by using relative quantification (Livak and Schmittgen, 2008). This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *csg D*cDNA amplification of each single case and that of the *rpoB* gene

The fold of gene expression in pH4 was lower than control in (0. 076) times. These results indicate a significantly decrease expression of *csgD* gene at pH 4 . Acid adaptation is likely to be an important variable in bacterial pathogenicity since pH has been identified as a regulator of the expression of genes involved in virulence,and probably are able to express curli and suggest that this virulent factor may play a significant role in invasiveness and pathogenesis of *E. cloacae*. Astudy condected by(Akbari et al., 2015) reveal that most of the *E. cloacae* strains isolated from blood cultures have *csgD* 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*

Table (1) Fold expression of rpoB gene for pH factor

factor	Mean ct of rpoB	Factor 2 –Δct	Experimental /control	Fold
Control temp.37c°, pH7	30.83	5.2 E.q	5.2Eq/ 5.2 E.q	1
pH 4	30.7	5.7 Eq	5.7 E.q/5.2 E.q	1.09

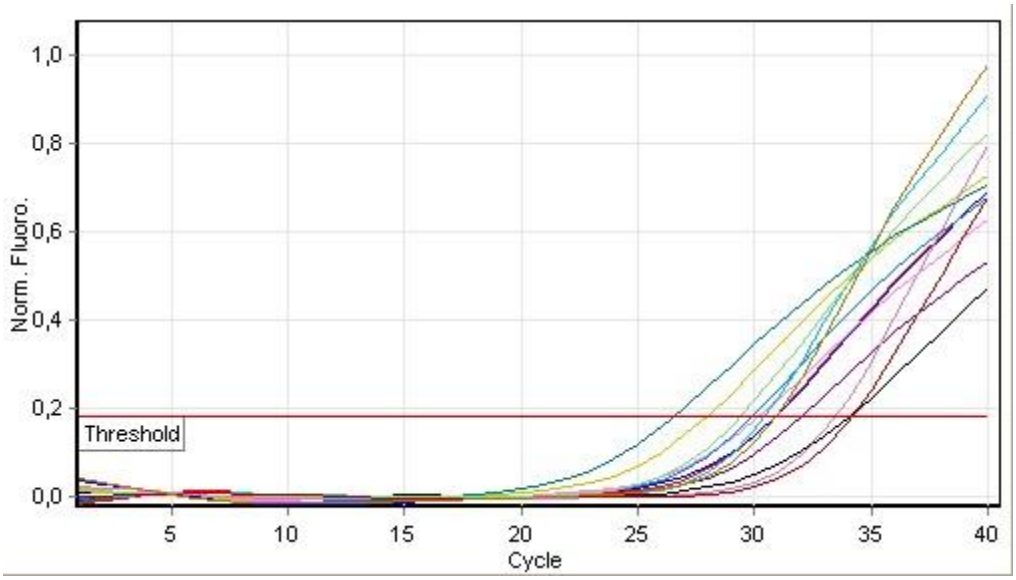

Figure (1) :*rpoB gene*amplification plots by qPCR. The photograph was taken directly from Rotor-Gene qPCR machine.

Table (2) fold of *csgD* expression depending on 2 –Δct method for pH4

groups	Means of csgD	Means of rpoB	Δ ct	2- Δct	Experimental/controls	fold
control	29.9	30.8	-0.9	1.86	1.86 /1.86	1
pH4	33.5	30.7	2.8	0.143	0.143 / 1.86	0.076

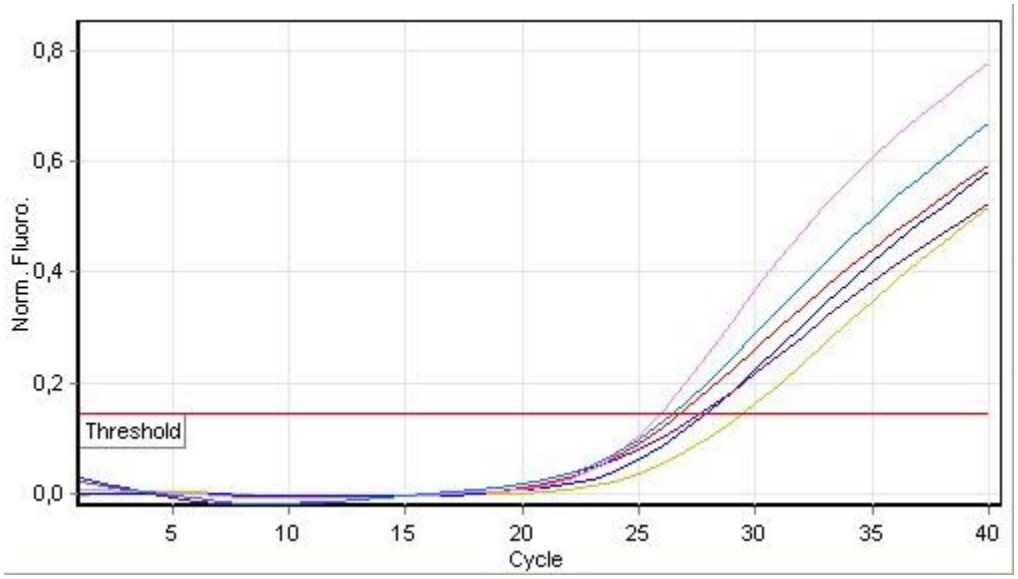

Figure (2) *csgD*amplification plots by qPCR Samples included pH4 factor.

CONCLUSION

The change of growth conditions such as pH of bacteria *E. cloacae* leads to change of gene expression *of (csgD), also rpoB* gene expression results, Which was used as reference gene ,confirmed that this gene was well suited as housekeeping gene.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

BQHA and MNR contributed to the design of the experiments and performed the experimental work. All authors carried out laboratory tests. BQHA wrote the manuscript, all authors revised and approval the final version.

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