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Isolation, Molecular Identification and Influence of Incubation Period On Hemolysine Gene Expression in *Serratia marcescens* Local Isolates

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Abstract
One hundred samples were collected from clinical and environmental sources in Baghdad city . The identification of *S. marcescens* confirmed by using the systems API 20E and VITEK-2. Also the identification of genus was conducted by detection the specific gene *16S rRNA* by polymerase chain reaction (PCR) assay. Twenty eight isolates were found to produce hemolysin as indicated by clear zone around colonies grown on blood agar plates. Four isolates which gave highest absorbability at 405 nm for hemolysin producing isolate (1.09 A) in standardized conditions (pH 7,37 C^o ,24 hours), while highest absorbability for hemolysin producing isolate which gave (1.92A) in 48 hours and (1.04A) in 72 houre . It was found that the highest value of gene expression fold was recorded for the gene *shlA* in conditions at 48 hours was (7.32) and at 72 hours was (2.64) . It was apparent there was a direct proportion between absorbability for hemolysin values and folds of gene expression, therefore the changeover conditions growth of bacteria *Serratia marcescens* leads to changeover of gene expression. *16S rRNA* gene expression results, which was used as reference gene, confirmed that this gene was well suited as housekeeping gene.

Keywords: Gene expression , *Serratia marcescens* ,hemolysin, *16S rRNA* .

INTRODUCTION

Serratia marcescens is widely distributed in natural environments and found in water and soil as well as associated with plants, humans and other animals [1]. *Serratia marcescens* is Gram-negative bacteria belong to the family of Enterobacteriaceae. It is an aerobic and motile bacterium usually described as the primary pathogenic of *Serratia* genus. It can be differentiated from other enteric bacteria due to its characteristic of red pigmentation prodigiosin [2][3].It is becoming widely recognized as an important opportunistic pathogen and is a nosocomial causing respiratory and urinary tract infections, bacteremia, endocarditis, keratitis, arthritis, and meningitis [4][5]. Hemolysins are produced by various pathogenic bacteria and have been proposed to be responsible for their pathogenesis and have also been suggested as virulence factors [6]. *S. marcescens* hemolysin is determid by tow chromosomal genes termed *shlA* and *shlB*. The ShlA (162kDa) polypeptide is the hemolysin itself , whereas ShlB (61 kDa) is required for activation and secretion of ShlA [7]. The hemolysin is secreted across the outer membrane by the ShlB protein, thereby distinguishing this type of secretion from all other known secretion systems [8]. Many hemolysins are pore-forming toxins (PFT), which are capabl to cause the lysis of erythrocytes, leukocytes, and platelets by producing pores on the cytoplasmic membrane [9] [10]. This study was designed to isolation and identificati on of *S. marcescens* from clinical and environmental sources in Baghdad city using conventional and molecular methods. Also this work aims to selection of highest production of hemolysin isolates, studying the role of incubation period factor on hemolysine gene expression.

MATERIALS AND METHODS

Bacterial isolates:

A collection of 100 samples were divided into: Group 1:65 clinical specimens (urine, sputum and wound swabs).Group 2:35 environments specimens (Hospital equipments) The isolates were identified depending on the morphological and biochemical tests [11],then confirmed by the Api 20E test and VITEK-2 test (bioMérieux, France). Samples confirmed as *S. marcescens* .

Primers used in the study are shown in (Table 1) for s*hlA* and *16SrRNA* [12] [13].

Table (1): Primers used in the study

Primers		Primer sequence
16SrRNA	F	5' - AGA GTT TGA TCC TGG CTC AG - 3'
	R	5' - ACG GTC ATA CCT TGT TAC GAC TT - 3'
shlA	F	5'TGGATGAAAAATAATAACTTCAGACTTTCG -3'
	R	5'ATGAATTCCGCGTTATTTGCCGCTGAAC - 3'

DNA extraction

Genomic DNA was extracted by standard DNA Extraction Kit (Dsbio,China) according to the manufacturer's instructions. The purity of DNA was evaluated by calculating the ratio of the absorbance at 260 and 280 nm (A260/A280), DNA concentration and 260/280 ratios are determined using a NanoDrop ND-1000.

Detection of 16s ribosomal RNA gene

Polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA was performed in a DNA thermal cycler,(Applied Biosystem, Singapore) with the following cycling program: Initial denaturation at 94ºC for 5 min, and 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and a final

extension at 72ºC for 10 min using primer sequence are shown in (Table 1).

Liquid hemolysis assay

The hemolysis assay medium contained (200µl) of washed erythrocytes (8%,v/v) in PBS, (200µl) from BHI broth of *Serratia* samples . After the tube contents had been mixed by inversion, This mixture was incubated at 30ºC for 90 min and then centrifuged for 1 min at 13000 rpm, to pellet the red blood cells and bacteria. The amount of free hemoglobin in the supernate (measured by its absorbance at 405 nm against a blank of PBS) [14].

The optimal incubation period that effected on Hemolysin production

A dded 0.1 ml of an overnight nutrient-broth culture of each strain to 10 ml of BHI broth and shaking incubated in different time (24,48,72) hour with stability of temperature and pH (37°C , pH 7),afterwards the hemolysis assay was determined as prepared in above.

Total RNA extraction with TRIzol

Total RNA of all samples was extracted using the TRIzol® LS Reagent following the protocol provided by the manufacturer .

cDNA synthesis for mRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. 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). Thermal cycler steps of conditions cDNA Reverse Transcri ption are show in (Table 2).

Table (2): Conditions of Primers Thermal cycler steps for cDNA Reverse Transcription.

	Step 1	Step 2	Step 3	Step 4
Temperature	25 ºC	42°C	85 ºC	4 ºC
Time	10 min	30 min	5 min	∞

Real Time PCR (qRT–PCR)

QRT-PCR was performed using the QIAGEN Real-time PCR System ((Rotor-Gene Q,Germany) with qPCRsoft software.The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct) employing the 2xqPCR Master Mix Kits components.

Table (3): Component of quantitative real-time PCR used in s*hlA* and *16SrRNA* genes expression experiment.

Component	Volume per 20µl Reaction
qPCR Master Mix	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA Template	4 µl
Nuclease-Free Water	to 20 µl

Housekeeping Gene Amplification

16SrRNA housekeeping gene was used as an internal control to be used in calculating the ΔCT value.

The qPCR Reaction run

The cycling protocol was programmed according to the thermal profile Shown in the (Table 4), for *16SrRNA* and s*hlA* genes.

Table (4): Thermal profile of *16SrRNA* and s*hlA* gene expression

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denature Anneal	95 °C 60 °C	15 seconds 60 seconds	40
Melting curve Analysis	65-95 °C	2-5 seconds/ step	1

Real Time qRT-PCR analysis of *16SrRNA* and s*hlA* genes expression

ΔΔCT

To comparethe transcript levels between different samples the $2^{-\Delta\Delta Ct}$ method was used [15]. The CT of gene of interest was normalized to that of internal control gene. The differencein the cycle threshold (Ct) values between the *16SrRNA* (internal control gene) and *shlA* 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 control samples.

CT values ≥ 38 were considered unreliable and neglected

The ΔCT of the test samples was normalized to the ΔCT of the calibrator:

ΔΔ CT was calculated according to the following equation:

ΔΔ CT=ΔCT (test)- ΔCT(calibrator) Finally, the expression ratio was calculated according to the formula

$2^{-\Delta\Delta Ct}$ = Normalized expression ratio.

RESULTS AND DISCUSSION

The distribution of identified *S. marcescens* isolates (28) according to the type of sample shown in (Table 5).

Table (5): Distribution of *S. marcescens* isolates in clinical and environmental samples.

	Sample type	No. of Sample	No. of *S. marcescens*	(%)
Clinical samples	Urine	32	12	37.5
	Sputum	20	6	30
	Wounds	13	3	23
Environmental samples	hospital equipments	35	7	20
	Total	100	28	28

From urinary tract infections *S. marscecens* isolated was the most frequent, followed by respiratory tract infections and wounds ,these results confirmed these reported by [16]. Wound cultures showed mixed infection with *Pseudomonas spp*., while one of the positive Sputum cultures was S. *liquificans* and has been excluded from this study. As well as [17] who reported that 20 isolates belonging to the *Serratia* species were collected from urine. Study results matched with [18], who could isolate 23 clinical isolates referred to *S.marscecens* from urinary tract infections. On the other hand a recent study in Saudi Arabia produced by [19] found that the *S.marscecens* percentage from sputum (18.5%) and urine culture (8.6%).

Most of the isolates responsible for hospital environment contamination were Gram negative bacilli, the most frequent isolates were *Pseudomonas spp*., Gram positive bacteria were also observed as a source of hospital environment contamination. This result supported by [20] study which showed that the environmental screening yielded 4 strains of *S.marscecens*. That matched with [21], who reported that environmental cultures were positive for *S.marscecens* in 1.4% and confirmed that *S.marscecens* was endemic in neonatal intensive care unit and belonged to one genotype.

The PCR results showed that *16S rRNA* sequence exists in all 28 *Serratia sp.* collected positive samples, and this confirmed the accuracy of biochemical tests. These results may reflect more accurate and more sensitive detection of molecular diagnosis in comparison with biochemical tests, API 20E and VITEK-2. Figure (1) shows the results of PCR of the *16S rRNA* gene.

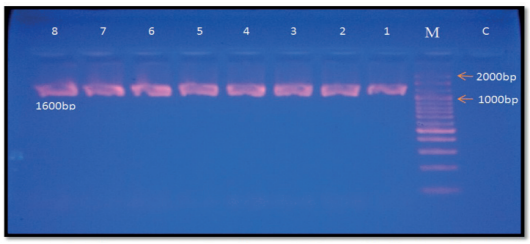

Figure (1): (1.75%) Agarose gel electrophoresis of PCR amplified products for *16S rRNA* gene. Lane (M): l00bp ladder, Lane (1-8): positive result with positive bands of 1600bp *S. marcescens* .

Detection of Hemolysin Primary Screening:

Blood agar plate was used to detect the ability of bacteria to produce hemolysin and as indicated by zone around colonies. Results indicated that all isolates showed a clear zone around the colonies as shown in figure (2).

Figure (2): Hemolysis Caused by *Serratia marcescens* Hemolysin on Blood Agar Plate.

Table (6) indicate that isolate (SMS1,SMS8,SMS23,SMS45)gave the highest absorbance, which means that these isolates have the highest ability to lyse red blood cells due to highest production of hemolysin, and there were selected for further tests. While environmental isolates gave the lowest absorbance because of the hemolysin it is virulent factor .

Table (6): Most efficient bacterial isolation in hemolysin production at(37°C , pH 7 for 24hr).

Bacterial isolate	Ab at 405 nm
SMS1	1.11
SMS5	0.63
SMS8	0.97
SMS9	0.77
SMS13	1.07
SMS14	0.95
SMS16	0.73
SMS25	1.18

Optimal incubation period for Hemolysin production

This study investigates the optimal incubation period that is used in the detection of hemolysis activity by *S.marscecens* and the results of the survy after using varied incubation period (24hr,48hr,72hr) showed that means absorbance of hemolysin for four isolates of *S. marcescens* at 48hr was (1.51A), while Means absorbance of hemolysin at 72hr was (1.18A), as in figure (3). The most efficient incubation period was 48 hour. These results agree with [22]. The hemolysin activity of *S. marcescens* is regulated according to the growth state of the culture. *shlA* and *shlB* expression is maximal in the late logarithmic growth phase [23] [13].[24] who found that the zones of haemolysis were wider after longer periods of incubation.

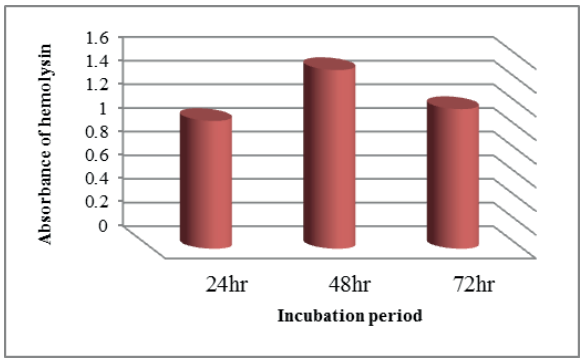

Figure (3): Comparative Means absorbance of hemolysin for four isolates of *S. marcescens* at 405nm with different incubatiom period.

Real time PCR quantification of *16SrRNA* Expression

In bacteria one of the most commonly used housekeeping genes in companion of gene expression data is *16SrRNA* rRNA expression has been shown to be highly dependent on the physiological status of the bacterial cell [25]. The essential assumption in the use of housekeeping genes in molecular studies is that their expression remains constant in the cells or tissue under investigation [26].

To further improve this and although there was a significant difference in the mean Ct value between groups in the present study, the variation of total change in expression of *16SrRNA* was studied in different study groups utilizing

the 2^{-Ct} value and the ratio of 2^{-Ct} of the different study groups to that of control group, as shown in (Table 7).

Table (7): Fold expression of *16S rRNA* for Incubation period factor.

Group (Factors)	Means Ct of 16S rRNA	2^{-Ct}	experimental group/ Control group	Fold of gene expression
Control (24hr)	17.45	5.5 E-6	5.5 E-6/5.5 E-6	1.00
48hr	17.60	5.0 E-6	5.0 E-6/5.5 E-6	0.90
72hr	17.48	5.4 E-6	4.45 E-6/5.5 E-6	0.98

The computed ratio for gene fold expression was 1.00,0.90 and 0.98 respectively. These small variations in gene fold expression between the study groups renders *16SrRNA* gene a useful control gene. Figure (4) shows the amplification plots and dissociation curves *for16S rRNA*.

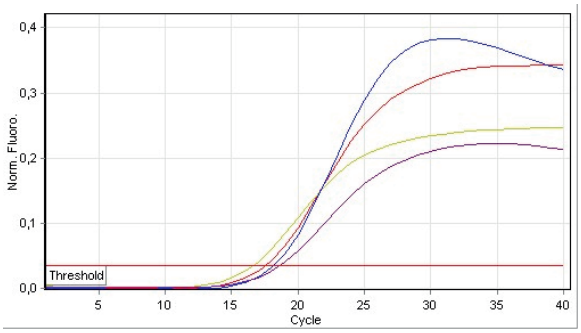

Figure (4): *16SrRNA* amplification plots by qPCR.Ct values ranged from 16.65 to 17.20. The photograph was taken directly from Rotor-Gene qPCR machine.

Real time PCR quantification of *shlA* Expression

In incubation period factor The mean Ct value of *shlA* cDNA amplification was **(16.09)** in the control. The Ct values in 48 hours was a mean **(13.37)**. While Ct values in 72hours was mean **(14.72)**, there was a significant difference in the mean Ct values between the different incubation period factor.

Expression of the *shlA* gene was not equal in both 48hr and 72hr and The fold of gene expression of *shlA* gene for the four isolates in 48hr mean value(**7.32**), this result was higher than the fold of *shlA* gene in 72hr with mean value (**2.64**). This is important evidence that *shlA* gene expression increases in 48hr. When we compared the absorbance of hemolysin at 405 nm values of the isolates used in the gene expression with the results of fold, it was found that there was a parallel elevation in the two parameters and a significant correlation was demonstrated for the gene *ShlA* and it was obvious that the highest values of fold was recorded in 48hr with the highest absorbance of hemolysin value (1.65A) for the gene. The results are shown in Table(8). The hemolysin activity of *S. marcescens* is regulated according to the growth state of the culture. *shlA* and *shlB* expression is maximal in the late logarithmic growth phase[23]. The hemolysin production decreased in the stationary phase of cell growth after 84 hr. This loss of activity might be due to the hemolysin autolysis[27]. In fact, maximum hemolysin production was detected at the 48 hr. Figure (5)(6) shows the amplification plots and dissociation curves for *shlA*.

Table (8): Fold of *shlA* expression Depending on $2^{-\Delta\Delta Ct}$ Method for Incubation period factor

Groups	Means Ct of *ShlA*	Means Ct of 16SrRNA	ΔCt (Means Ct of *ShlA* - Means Ct of 16SrRNA)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Control (24hr)	16.09	17.45	-1.36	2.56	2.56/2.56	1
48hr	13.37	17.60	-4.23	18.76	18.76/2.56	7.32
72hr	14.72	17.48	-2.76	6.77	6.77/2.56	2.64

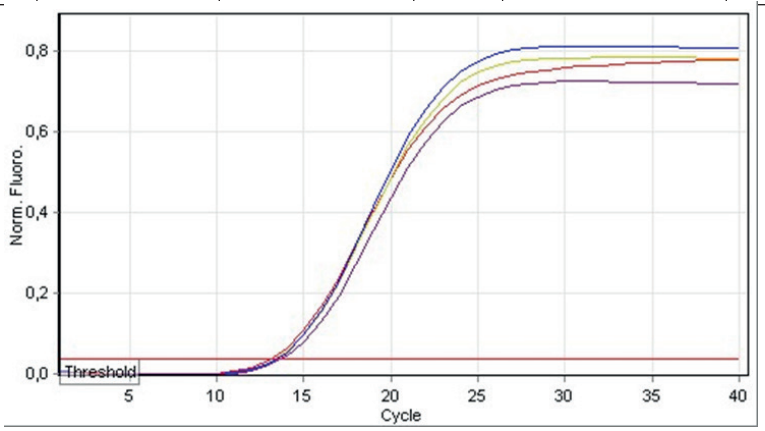

Figure (5): *shlA* amplification plots by qPCR Samples included 48hr factor .Ct values ranged from 13.10 to 13.75. The photograph was taken directly from Rotor-Gene qPCR machine.

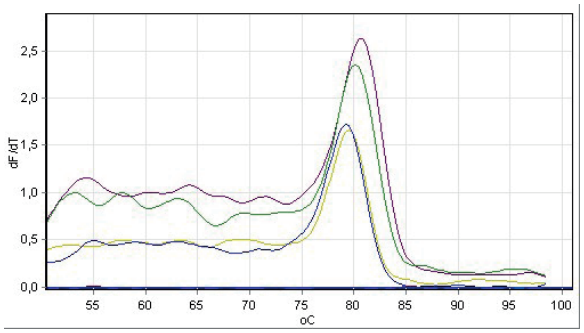

Figure (6): *shlA* dissociation curves by qPCR Samples included all study groups. Melting temperature ranged from 87°C to 89°C, No primer dimer could be seen. The photograph was taken directly from Rotor-Gene qPCR machine.

The ability of bacteria to persist in the human host depends on prompt adaptation to changing environmental conditions such as temperature, pH, osmolarity, Incubation period ,oxygen tension , nutrients and hemolysin virulence factors was strongly enhanced by iron depletion [5] Previous studies have suggested that certain environmental factors on the nucleoid modulate the expression of several virulence genes [10] [28].

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