Gene Expression of Biofilm Regulatory Protein A (*Brp A*) in *Streptococcus mutans* Isolated from Oral Cavity

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الخلاصة

جمعت 100 مسحة فموية من مرضى تسوس الاسنان وامراض ما حول الاسنان الذين تراوحت اعمارهم ما بين 15-65 سنة ومن كلا الجنسين والذين راجعوا المركز التخصصي لطب الاسنان في الديوانية للمدة من كانون الاول 2012 الى اذار 2013 لغرض قياس التعبير الجيني لمورثة البروتين المنظم للغشاء الحيوي (*brp A*) من عزلات السبحيات (*S.mutans*) باستخدام تقنية تفاعل سلسلة انزيم البلمرة بالوقت الحقيقي. تم قياس التعبير الجيني لمورثة البروتين المنظم للغشاء الحيوي (*brp A*) بالمقارنة مع المورثة (*16s rRNA*) كمورث مرجعي لعزلات ال(*S.mutans*) باستخدام تقنية تفاعل سلسلة انزيم البلمرة بالوقت الحقيقي (RT-PCR) ،اذ اظهرت نتائج التعبير الجيني (النسبي والمطلق) في معاملات الاختبار لمورث ال (*brp A* gene) وجود تناقص في تعبيره الجيني في معاملات الاختبار (*S.mutans* النامية مع 1% كلوكوز و50% خلاصة الكمون) بالمقارنة مع معاملة السيطرة (عزلات *S.mutans* النامية في 1% كلوكوز فقط). استنتجت الدراسة الحالية اهمية المورث (*brp A* gene) في تكوين الغشاء الحيوي لعزلات ال *S.mutans* المقاومة للمضادات الحيوية ، وتوصي الدراسة باستخدام نبات الكمون كمثبط لنمو واختزال الغشاء الحيوي من خلال ادخاله في تركيبة معاجين الاسنان .

Abstract:

A total of 100 oral swabs were collected from patients suffering from dental caries and priodontal infections, ranging from 15-65 years old of both genders whom admitted to the Al-Diwaniyah Teaching Special Center of Dentistry within the period from December 2012 to March 2013, in order to quantify the gene expression of biofim regulatory protein (*Brp A*) in *Streptococcus mutans* (*S. mutans*) isolates by using the Real Time polymerase chain reaction. The gene expression of biofilm regulatory protein A(*Brp A*) was quantified in comparison with housekeeping gene *16s rRNA* as a reference gene of biofilm *S.mutans* by using reverse transcriptase polymerase chain reaction (RT-PCR).The results revealed that the expression (absolute) of *BrpA* gene is decreased in the test treatment (*S.mutans* with 1% glucose+ 50% curcumine) in comparison with control (*S.mutans* grown in 1% glucose only).

In conclusion, the present study established the role of *Brp A* gene in biofilm formation and recommended to possibility of using the curcumine as adddative material in composition of toothpaste due to their inhibitory and reduction effect on biofilm *S.mutans* isolates.

Introduction:

Dental plaque is a complex microbial biofilm , it is considered as the primary etiologic in development of dental caries and periodontal disease . The bacteria form (60% - 70%) of its volume, may reach to 300 – 500 cell. These accumulation subjected teeth and the periodontium to high concentration of bacterial metabolites , which result in dental infection . Plaque bacteria produce two forms of toxin which work as extracellular enzymes and endotoxin (1). *S.mutans* are primary colonizer of oral hard and soft tissue . It is highly cariogenic because it ferment dietary sugar (sucrose) to lactic acid , which is dissolving tooth structures and lead to cavity (2).

Virulence factors of *S. mutans* help to protect the bacterium against possible defenses and maintain its ecological niche in the oral cavity , acid tolerance , protease production and production of glucosyltransferases and intracellular polysaccharides (3) .Beyond initial adherence, it appears that a variety of genes are required for the adaptation of *S. mutans* and other oral streptococci in biofilms.These include genes associated with intercellular communication systems and environmental sensing systems, regulators of carbohydrate metabolism, and adhesion-promoting genes

(4). Among the genes were *brpA* (1YtR), *comDE* and *vicR* encoding regulatory proteins (5), A number of studies have indicated that expression of the genes responsible for biofilm formation depends on environmental conditions (6), and is also genetically regulated (7). It was therefore of interest to identify transcriptional changes for several genes that accompany the formation of an in vitro biofilm by the most important pathogens in the development of dental caries, such as S.*mutans.* To assess the expression of genes known to be involved in biofilm formation by *S.mutans*, for comparison with the expression of genes in planktonic cells, real time PCR was used.

Previous studies have shown that *BrpA* play a major role in acid and oxidative stress tolerance and biofilm formation by *S.mutans*. Mutant strains lacking *BrpA* also display increased autolysis and decreased viability, suggesting a role for *BrpA* in cell envelope integrity(8).

Aim of the study:

In the present study we attempt to quantify the intensity of biofilm formation by *S. mutans* clinical strains by measuring biofilm volume and further analyze how expression differ between various isolated *S.mutans* strains that showed either high or low levels of ability to form biofilm, and we compared the results with those regarding biofilm formation ability by using reverse transcriptase Polymerase Chain Reaction (PCR) method.

Methods

Specimens collection: A total of 100 swabs were taken by inserting into the middle of cavity in case of carious tooth, while swabs were taken from dental plaque in case of periodontitis and gingivitis over the tooth surfaces. Swabs for culture were placed in tubes containing transport media to maintain the bacterial swab vital until being taken to the laboratory. Each specimen was immediately inoculated on blood agar plates, nutrients agar and chocolate agar. All plates were incubated aerobically and anaerobically by candle jar at 37 C^{o} for 24-48 hrs.

Isolation and identification: Each transport media swab was inoculated into universal tube containing 5ml of nutrient broth and incubated at 37 C^{o} for 18-24 hrs, a loop full of broth was streaked on surface of nutrient agar, blood agar, MacConkey agar which then incubated at the same conditions. Colonies had been primarily identified depending on morphological colonies characteristics were recorded on the media used in this study for primary identification of microorganisms and microscopic properties. Gram's stained slide was done for each individual isolate of microorganism recovered from the different agar media to study the microscopic properties of genera (9) and the biochemical tests done by using VITEK 2 system.

Quantitative Reverse Transcriptase Real-Time PCR:

Quantitative Reverse Transcriptase Real-Time PCR technique was performed for measurement of absolute quantification (mRNA copy numbers) and relative quantification (gene expression analysis) for Biofilm regulation protein A gene in treatment and control *S. mutans* isolates. The Biofilm *S. mutans* plate design for Real-time PCR is included:

1- **Treatment (T)** *S.mutans* isolates which grown in trypticase soya broth with 1% glucose and curcumine(50%) then incubated at 37 ^{0}C for 18 hours.
2- **Control (C)** *S.mutans* isolates which grown in trypticase soya broth with 1% glucose only then incubated at 37 ^{0}C for 18 hours.

This technique was done according to the method described by (10) as follow:

- **Total RNA extraction:** Total RNA were extracted from *S.mutans* experimental isolates of treatment and control groups by using (TRIzol® reagent kit) and done according to the company instructions of Bioneer /South Korea**.**

- **Assessing of RNA yield and quality:** The extracted total RNA was assessed and measured by Nanodrop spectrophotometer**.**
- **DNase I Treatment:** The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using DNase I enzyme kit and the reaction was done according to method described by promega company, USA instructions as follow**:**

Mix	Volume
Total RNA 100ng/µl	10µl
DNase I enzyme	1µl
10X buffer	4µl
DEPC water	5µl
Total	20µl

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl 25mM EDTA was added and incubated at 65C° for 10 minutes for inactivation of DNase action.

- **cDNA synthesis:** DNase-I treatment total RNA samples were used in cDNA synthesis step by using AccuPower® RocktScript RT PreMix kit that provided by Bioneer company, South Korea and the reaction was done according to company instructions as follow

RT master mix	**Volume**
Total RNA 100ng/ul	10µl
Random Hexamer primer	1µl
DEPC water	9µl
Total	20µl

This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then dissolved completely by vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Step	Temperature	Time
cDNA synthesis (RT step)	50 °C	1 hour

Heat inactivation	95 °C	5 minutes

- **Quantitative Real-Time PCR (qPCR):** qPCR results was performed for measurement of absolute quantification (mRNA copy numbers) and relative quantification (gene expression analysis) for Biofilm regulation protein A gene and housekeeping gene *16S rRNA* in treatment and control of *S.mutans* isolates. This assay was performed by using AccuPower™ Green Star Real-Time PCR kit that depended on SYBER Green dye detection of gene amplification in Real-Time PCR system as follow:

Absolute quantification: RT-qPCR absolute quantification analysis was included in the measurement of mRNA copy numbers of target gene (*brpA*) and compared with housekeeping gene (*16S rRNA*) in test and control by using Dilution of genomic DNA standard curve for ($1x10^8$ $1x10^6$, $1x10^4$, $1x10^2$ copy number) as follow; Genomic DNA standard curve for *S. mutans* standard strain was prepared by estimation the concentration of extracted DNA by Nanodrop spectrophotometer ng/µL and whole genomic sequence of *S.mutans*, then used DNA copy number calculation formula online. The ($1x10^8$ copy number) that obtained by DNA copy number formula was diluted for 10 fold as ($1x10^8$, $1x10^7$, $1x10^6$, $1x10^5$ $1x10^4$,$1x10^3$, $1x10^2$, $1x10^1$) and only ($1x10^8$, $1x10^6$, $1x10^4$, $1x10^2$) genomic DNA dilution was used for preparation of standard curve for amplification of *brpA*(Table 1) and *16S rRNA* genes (Table 2):

Table 1: qPCR master mix for *brpA* standard curve

qPCR master mix		Volume
Genomic DNA dilution template ($1x10^8$, $1x10^6$, $1x10^4$ $1x10^2$)		5µl
Primers (*brpA*) (10pmol)	Forward primer	1 µl
	Reverse Primer	1 µl
DEPC water		13 µl
Total		20 µl

Table 2: qPCR master mix for *16S rRNA* standard curve

qPCR master mix		Volume
Genomic DNA dilution template ($1x10^8$, $1x10^6$, $1x10^4$ $1x10^2$)		5µl
Primers (*16S rRNA*) (10pmol)	Forward primer	1 µl
	Reverse Primer	1 µl
DEPC water		13 µl
Total		20 µl

After that, qPCR master mix components mentioned above loaded on Accopwer Green star qPCR premix standard plate tubes that contain the SYBER Green dye and other PCR amplification components, then the plate mixed by Exispin vortex centrifuge for 3 minutes, than placed in Exicycler Real-Time PCR system, for thermocycling.

Results and Discussion:

The *S. mutans* was comprised the high ratio (40.47%) in comparison with the other *streptococcus* species (*S. sangius* 11.90%, *S. mitis* 10.70%, *S.intermedius* 10.70%, *S.oralis* 9.50%, *S.salivarius* 5.95%, *S.pneumoniae* 4.76%, *S.pyogenes* 3.57%, *S.sorbinus* 2.38%). No isolate of *S.mutans* were isolated from the healthy control. This explains that *S.mutans* represent the main causative agent of dental diseases, especially dental caries and to a lesser degree the periodontal diseases. All *S. mutans* colonies showed α-hemolysis (green coloration on blood agar plates) which was not evident after 24 hrs. of incubation and required further incubation. Gram staining were adopted and showed Gram positive cocci arranged in chains. Biochemical and physiological characteristics of all *S. mutans* isolates showed negative reactions with catalase, oxidase tests and positive for sugars(glucose. lactose, sorbitol. manitol, and raffinose).

Bacterial identification was specified by using VITEK 2 compact system. Based on the results of VITEK 2, *S. mutans* was the most frequent species of streptococcus.

Biofilm formation can be considered as a developmental process, which is characterized through the identification of structural and regulatory genes required at the various steps of the process (11). To gain an insight into molecular estimation differences in gene expression of biofilm regulation protein A gene in test and control of *S. mutans* isolates, the researcher used quantitative Reverse Transcription Real-Time PCR (RT-qPCR) was performed .This assay also used by Shemesh(12) whom used real-time RT-PCR in differential gene expression profiling of *S. mutans* cultured under biofilm and planktonic conditions.RT-qPCR absolute quantification analysis included the measurement of mRNA copy numbers of target gene (*brpA*) and compared with housekeeping gene (*16S rRNA*) in test and control groups by using Dilution of genomic DNA standard curve for ($1x10^8$ $1x10^6$, $1x10^4$, $1x10^2$ copy number). The results of DNA standard curve were shown in Real-Time PCR amplification plot (Fig. 1) and Real-Time PCR standard curve (Fig. 2).

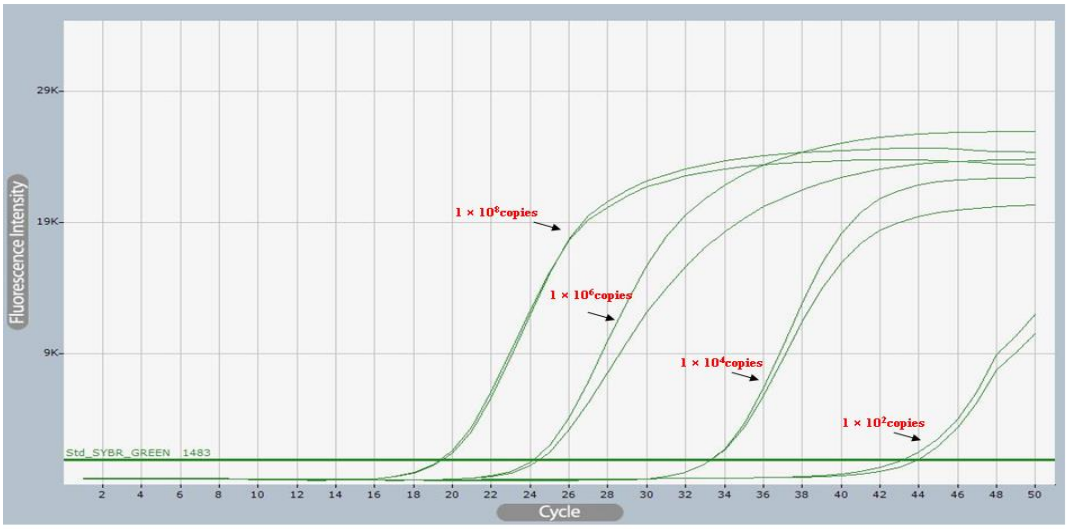


Figure (1): Real-Time PCR amplification plot that shown four standard dilutions DNA used with target (*brpA*) and housekeeping gene (*16s rRNA*).

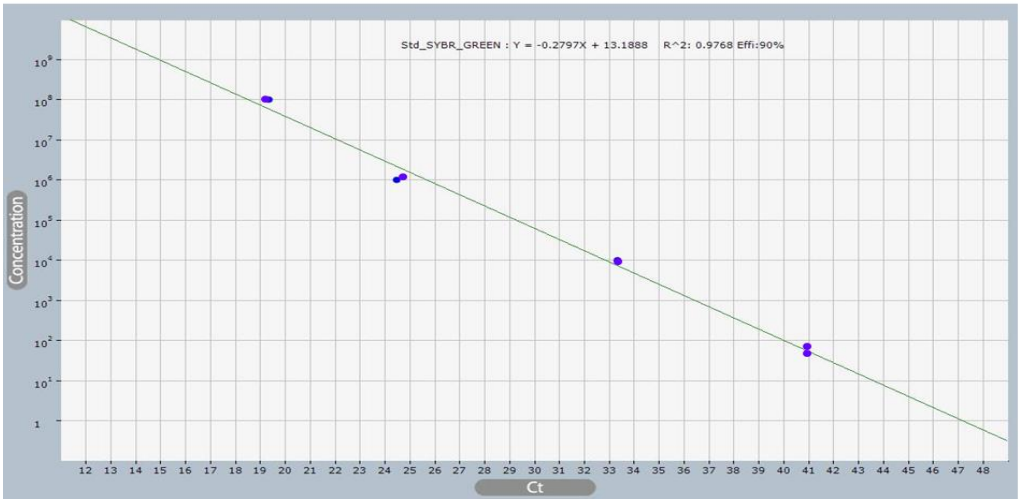


Figure (2): Real-Time PCR standard curve that shown dilutions DNA that used in absolute quantification of target (*brpA*) and housekeeping gene (*16s rRNA*).

The results of housekeeping gene (16S rRNA) are shown in Real-Time PCR amplification plot (Fig. 3) and Real-Time PCR standard curve (Fig. 4).

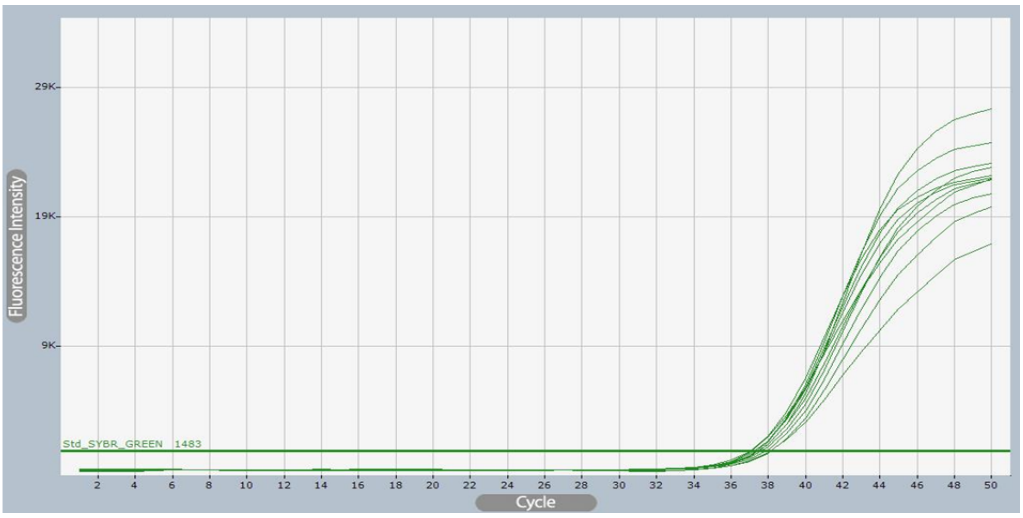


Figure (3): Real-Time PCR amplification plot of housekeeping gene (*16S rRNA*) in treatment and control *S. mutans* isolates.

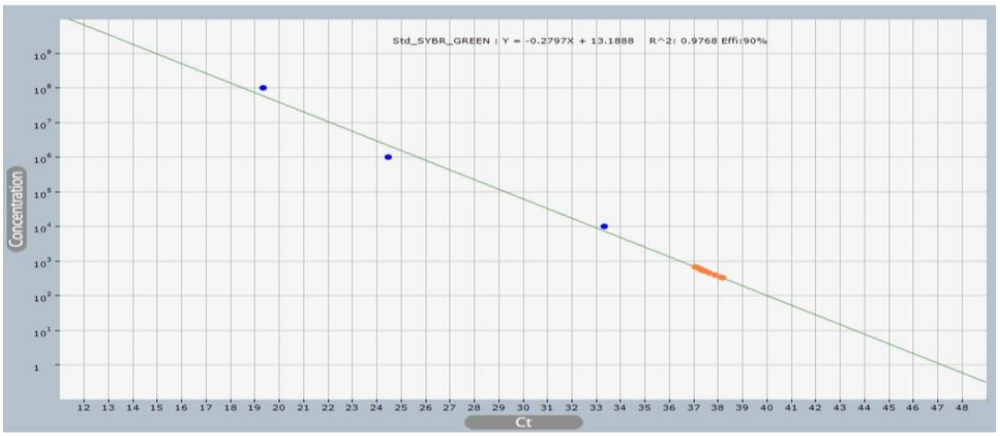


Figure (4): Real-Time PCR standard curve of housekeeping gene (*16S rRNA*) in test and control of *S.mutans* isolates.

The results of target gene (*brpA*) are shown in Real-Time PCR amplification plot (Fig. 5) and Real-Time PCR standard curve (Fig. 6).

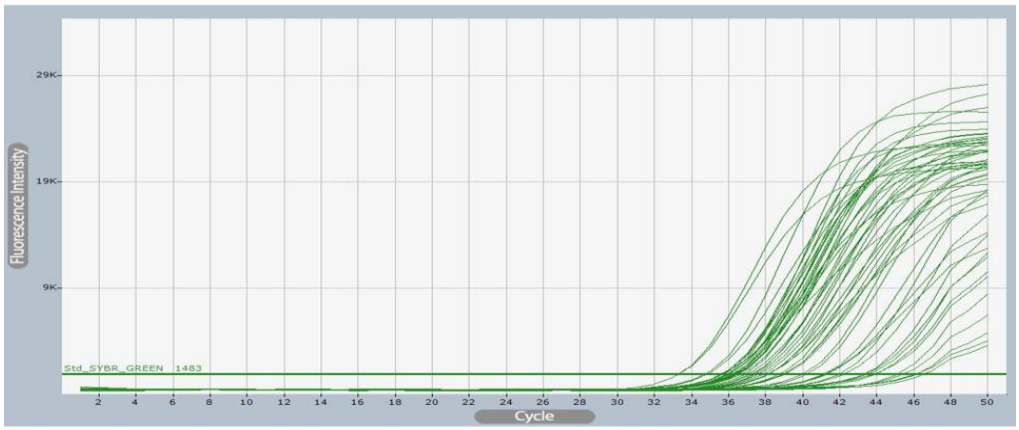


Figure (5): Real-Time PCR amplification plot of target gene (*brpA)* in test and control S.*mutans* isolates.

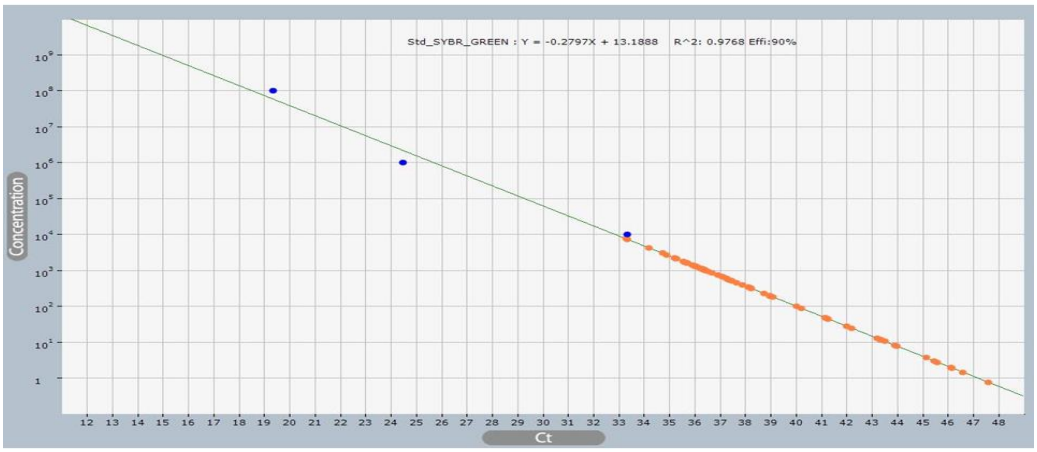


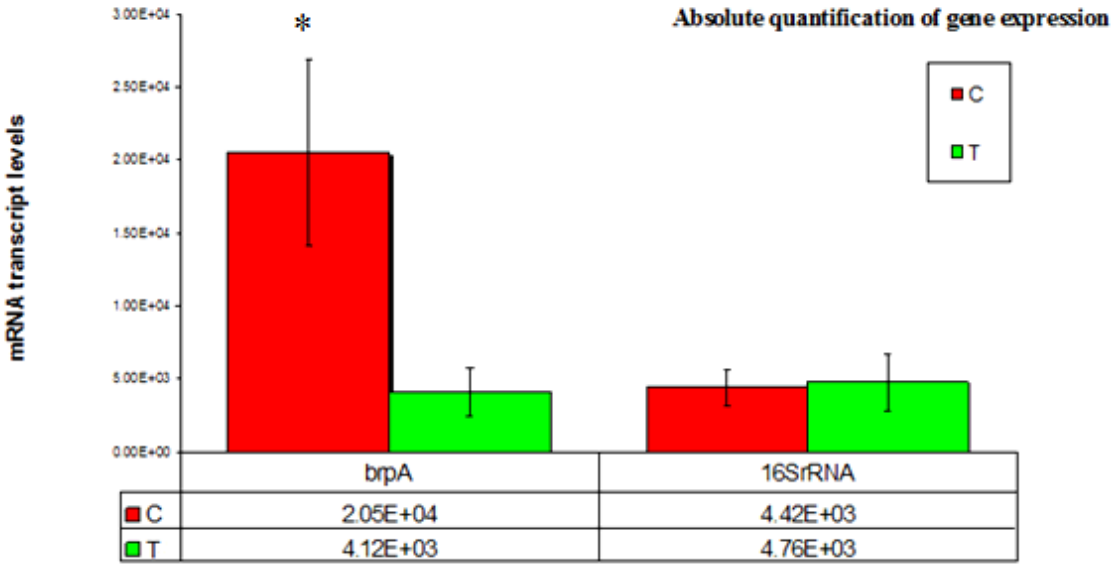
Figure (6): Real-Time PCR standard curve of target gene (*brpA*) in test and control of *S. mutans* isolates.

Absolute quantification of mRNA transcript levels in *brp A* gene and house keeping gene (*16S rRNA*) were obtained by compared threshold cycle numbers of these genes with threshold cycles of genomic DNA standard curve in Real Time PCR system.

This procedure is consistent with Bustin (13) who indicated that when appropriate standard curves are used, absolute copy numbers of mRNA can easily be calculated.

The absolute quantification results of Biofilm regulation protein A gene showed a clear decrease in mRNA copy numbers in test isolates (4.12E+03± 1705.22074) compared with control isolates (2.05E+04± 1035.28311) . The statistical analysis of absolute quantification of *brpA* gene found significant differences ($P \leq 0.05$) in test isolates compared with control. Whereas, the absolute quantification results of housekeeping gene (*16S rRNA*) revealed no differences in mRNA copy numbers in test isolates (4.76E+03±1012.04414) compared with control groups (4.42E+03±260.34166). The statistical analysis of absolute quantification of 16SrRNA gene was found no significant differences ($P \geq 0.05$) in test isolates compared with control (Fig. 6).

The absolute quantification results of housekeeping gene (*16S rRNA*) showed no significant differences ($P \geq 0.05$) in mRNA copy numbers in test isolates. These results interpreted that house keeping gene (*16S rRNA*) is suitable as reference gene for all *S. mutans* isolates that used in the present study, and these findings consistent with Paule & White (14) whom evaluated rRNAs, which constitute 85–90% of total cellular RNA, are useful internal controls, has expression stability when exposed to different stress conditions.



C: control isolates T: test isolates

Figure (6): Absolute quantification gene expression of target gene (*brpA*) and house keeping gene (*16S rRNA*) in *S. mutans* isolates.

Quantification of mRNA transcription can be either relative or absolute, relative quantification determines the changes in transcription of a gene and is often adequate. During the RT-PCR assay, the target Ct is compared directly with the calibrator Ct and is recorded as containing either more or less mRNA. In contrast, absolute quantification of transcription allows the precise determination of copy number per cell, total RNA concentration, or unit mass of tissue. It requires the construction of an absolute standard curve for each individual amplicon to ensure accurate reverse transcription and PCR amplification profiles (10). Therefore, absolute quantification was used in this study.

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