

Real-Time RT PCR Evaluation of the Xylitol Effect on the Expression of *Streptococcus pneumoniae* *cpsB*, *cpsD* and *psaA* Genes

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ABSTRACT

Background and Aim: The major pneumococcal pathogenesis factor is the capsular polysaccharide. The production of polysaccharide is regulated by *cpsB* and *cpsD* genes. Thus, every agent that induces or inhibition of expression of these genes probably increased pathogenesis of bacteria, intracellular survival and vis versa. The aim of this study was to assay the effect of Xylitol on *Streptococcus pneumoniae* *cpsB*, *cpsD*, *psaA* and *aroE* genes expression by Real Time RT-PCR.

Materials and Methods: During 2019 in Baqiyatallah university laboratories, 20 clinical isolates and a reference strain *Streptococcus pneumoniae* (ATCC 6305) were studied. To assay the effect of Xylitol and fructose, different concentrations of these sugars were exposed to BHI Broth media. Thereafter, the bacterial suspension was added to 5% Xylitol, 5% Xylitol + 5% fructose and control medium for 3 hours. RNA was extracted and cDNA synthesis was carried out. Then, the *cpsB*, *cpsD* and *psaA* genes expression levels were measured.

Results: The mean of *cpsB* gene expression ratio in presence of the % 5 Xylitol treated media compared with those in the control media and % 5 Xylitol + 5% fructose were 0.022 and 0.027 respectively.

The means of the *cpsD* and *psaA* genes expression ratio in the % 5 Xylitol treated media compared with those in the control media and % 5 Xylitol + 5% fructose were 1.659 and 0.056, and 0.633 and 3.294 respectively.

Conclusion: Our finding revealed that, the 5% Xylitol inhibits the *cpsB* gene expression properly. Therefore, it is possible to use of Xylitol for reductive on *cpsB* gene expression and superficial pneumococcal infections control.

Keywords: Capsular genes, RT Real-Time PCR, *Streptococcus pneumoniae*, Xylitol

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is an important human pathogen and a leading cause of morbidity and mortality worldwide (1). Invasive and noninvasive infectious diseases caused by *S. pneumoniae* strains such as meningitis, sepsis, bacteremia, pneumonia, sinusitis, acute otitis media in young children, elderly, and patients with immunodeficiency are increased (2,3). In previous studies, the frequency of pneumococcal meningitis in elderly patients was reported more than 35% (4-6).

The main virulence factor of the *S. pneumoniae* is capsular polysaccharide which protects the bacteria against phagocytosis and plays an important role in colonization the bacterium on the upper airway (7). Thus, expression of polysaccharide capsule genes is essential for survival in against of the superficial immune, and enhances the virulence and pathogenicity.

Currently, the strategy for the prevention of life-threatening pneumococcal disease, is based on the immunogenicity of polysaccharide capsule and the production of capsule base vaccines and existing vaccines (PCV7, PCV10, PCV13 and PPSV23) which made from capsule antigens (8). However, the causes such as the spread of serogroups, and emergence of new serotypes and serotype diversity (9) have reduced the efficacy of vaccines. Therefore, the use of vaccine in all cases could not be a good solution for preventing pneumococcal disease and its morbidity and mortality. In addition, the development of antibiotic resistance has increased the therapeutic problems (10). This bacterium has been resistant to many common antibiotics, therefore, since the production of polysaccharide capsules are introduced as the most important pathogenicity and virulence factor (11, 12). Several study on capsular synthesis mechanisms and also inhibit expression the involve genes were more considered (11).

Based on available data, in most serotypes, the capsule locus is located in the same region between the genes *dexB* and *aliA* locuses. The first four genes of this locus are conserved and named as: *cpsA*, *cpsB*, *cpsC* and *cpsD*, and thus, to determine the level of expression of the capsular genes locus are very important, (13, 14). It is well known that the *cpsB* and *cpsD* genes, plays major role in regulating the capsular synthesis and production (15)

It is reported that; Xylitol is a five-carbon sugar alcohol (16, 17) and have affected the bacterial growth and blocked the adhesion property of *S. pneumoniae* (18,19). Research results have shown that Xylitol can prevent acute otitis media infections in children (20). Some research results revealed that *psaA* gene produces a 37-kDa protein called Pneumococcal surface adhesin A (21), which acts as a superficial protein

for binding of *S. pneumoniae* to mucosal cells (22, 23). As a result, it is reported that the *psaA* gene is also involved in the disease. Since, all pathogenic *S. pneumoniae* have been shown to have this gene (24). Thus, the purpose of this study was assayed the effect of different concentration of Xylitol on the expression of the *cpsD* and *psaA* genes. If the genes were inhibited, probably Xylitol can be used as complementary treatment with no any side effects against the *S. pneumoniae* superficial infection hopefully.

Materials and Methods

Bacterial strain

This investigation was carried out during the year 2019 in Baqiyatallah university complex laboratories. A reference strain of *S. pneumoniae* (ATCC 6305, purchase from Baharafshan Laboratory, Tehran, Iran), and twenty *S. pneumoniae* clinical isolates which previously were identified and their serotypes were reported (25, 26) was used. In separately manner, the lyophilized bacteria vials in aseptic conditions were dissolved in 1 ml BHI- broth media (Merck Co, Cat No.110493). Then 50 μ L of the suspension was transferred into Blood agar (Merck Co, Cat No.110886) which supplemented by 5% sheep blood and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. After overnight incubation, bacterial colonies were harvested and transferred into 3 ml of BHI broth media containing 0.2% glucose and supplemented with 10%(vol/vol) FBS (Fetal bovine serum) and incubated at 37°C in a 5%CO₂ atmosphere until the OD₆₅₀ had been reached equal 0.5 McFarland approximately for 5 hours. For each strain the bacterial suspension as the same of standard 0.5 McFarland (10⁷ CFU/mL) were considered. In order to confirm-matic assay for all strain of the *S. pneumoniae*, a standard diagnostic procedure was carried out based on previously report (27, 28).

However, for each reaction the 300 μ L of bacterial suspension was added to 3 ml of test media that were prepared by adding various sugar alcohol or sugar concentrations (Table-1). The test tubes were incubated at 37°C in 5% CO₂ atmosphere for about 3 hours. In order to obtain proper concentration cells and the desired RNA level, the contents of each test medium was centrifuged in 4°C for 3 min at 12000g that supernatant containing the BHI broth was discarded and the resulting sediment was used to RNA extraction.

Sugars stock solution preparation and sterilization

In this research, the 40% stock solution of each sugar was prepared separately. Tested sugars were sterilized by filtration (0.22 μ m pore-sized filter; Millipore.). Afterward certain amount was added to relat-

ed test tubes. The Xylitol concentration used in this study were chosen on the basis of a previous study (19).

RNA Extraction

The kit of the TRIzol® Reagent solution (Cat No.-15596-026; Ambion; USA) was used in order to extract

RNA. It should be noted that in the whole process of extraction of RNA, it is necessary to note that due to the abundance of the RNase enzyme in the environment and the sensitivity of RNA, moreover to use filter micropipette tips and RNase free micro tubes, and the whole process is done alongside the ice.

Table 1. Media and ingredients used for bacterial growth and RNA extraction. The volume and different concentrations of Xylitol, Fructose and Glucose were prepared from the stock solution.

Media	BHI broth	FBS	Sugar	Total volume
BHI broth + 5% Xylitol	2425 µL	200 µL	375 µL	3000 µL
BHI broth + 5% fructose + 5% Xylitol	2050 µL	200 µL	375 µL Xylitol and 375µL fructose	3000 µL
BHI broth Glucose -free (Control)	2800 µL	200 µL	-	3000 µL

Based on the kit procedure, the RNA was extracted and stored at minus 70 ° degree centigrade. The probable DNA contamination, the extracted total RNA was subjected to treat by Dnase1 enzyme (Cat No. PR891627; GENEALL; South Korea). As follows: 1 µL of DNase I, 10x enzyme was added to 1 µg extracted RNA in a RNase free micro tubes. 1 µL of buffer 10 x was added and the final volume with DEPC reached 10 µL and incubated for 30 minutes at 37°C. The reaction was completed by adding 1 µL of 50 mM EDTA solution and incubation at 65°C. in order to analysis of the quality and quantity of the extracted RNA, the absorbance of 1.9 to 2 at 280/260 ratio revealed the purity of extracted total RNA by using Nano drop device (ND-1000; Thermo). In order to determine the quantification extracted specimens, the concentration was calculated according the absorbance at 260 nm.

To evaluate the quality of extracted RNAs, samples were run in agarose gel 1% (100 volts). The extracted RNA has shown a good quality, it will have two bands in gel, including 16srRNA and 23srRNA band. After that, all RNAs samples were rapidly converted to cDNA.

cDNA Synthesis

For cDNA synthesise, the PrimeScript™ RT reagent Kit (Cat No. RR037A; TAKARA; Japan) was used. The reaction mixture was prepared according to the instruction of the kit (Table-2). Briefly, this mixture was incubated 15 min at 37°C for rverse transcription and then for inactivation of reverse transcriptase was incubated 5 secoun at 95°C. To prevent contamination and experimental error until before the Real-Time PCR, wasn't use from specific primers for each of the genes and there were three total cDNA types.

Table 2. The Reagents and gradients utilized to synthesis cDNA.

Reagent	Amount	concentration
5X Prime Script Buffer	2 µL	1X
Prime Script RT Enzyme Mix I	0.5 µL	-
Oligo dT Primer	0.5 µL	50 µM
Random 6 mers	0.5 µL	100 µM
Extracted from control media		454.4 µL/ng
Total RNA	Extracted from media containing 5% Xylitol	3.5 µL
	Extracted from media containing 5% fructose + 5% Xylitol	395.8 µL/ng
	RNase Free DH ₂ O	3 µL
	Total volume	10 µL

RT Real Time PCR Performance

For this purpose, specific primers were designed with GenScript software for amplify target genes

(*cpsB*, *cpsD* and *psaA*), and to be synthesized were ordered (Macrogen; South Korea) (Table-3).

Briefly, 5 µL of synthesized cDNA was used in Real-Time PCR by applying the Real Q Pluse 2X Master Mix

Green low ROX™ with SYBR® Green (Cat No. A323499; Ampliqon; Denmark) and the Exicycler™ 96 Real-Time Quantitative Thermal Block (Cat No. A-2060; BIONEER;

Republic of Korea). The therm cycler programs and protocols and gradients reagent were used according the kit instruction.

Table 3. Characteristics of Designed primers for set up the RT Real Time PCR

Primer	Sequence 5' – 3'	GC%	Tm(°C)	Amplicon size	Concentration	
<i>cpsB</i> (<i>wzh</i>)	Forward	TAGATGACGGTCCCAAGTCA	50%	58.4°C	202bp	5 Pmol/μL
	Reverse	GCGCCATAAGCAATGACTAA	45%	56.4°C		5 Pmol/μL
<i>cpsD</i> (<i>wze</i>)	Forward	CCAAACCCTACAGCCTTGTT	50%	58.4°C	159bp	5 Pmol/μL
	Reverse	TGTTACCAAGATGAGCGCAT	45%	56.4°C		5 Pmol/μL
<i>psaA</i>	Forward	GTAGCATGTGCTAGCGGAAA	50%	58.4°C	254bp	5 Pmol/μL
	Reverse	TTTGTAACCAAGCATTGCC	40%	54.3°C		5 Pmol/μL
<i>aroE</i>	Forward	TTTGGCATCAATCTGTCCAT	40%	54.3°C	286bp	5 Pmol/μL
	Reverse	AAACGGAACGAACAAAGACC	45%	56.4°C		5 Pmol/μL

However, The Real-Time PCR was carried out in accordance with the instructions of the utilized kit. Real Time PCR was performed two times and every sample was run as triplicates in each time. Finally, the average C_T was totally obtained from the 6 collected C_Ts for each sample.

Results

As it was shown in [figure 1](#), Real-time PCR assay was performed to evaluate the expression of the *cpsB*,

cpsD and *psaA* genes on total cDNAs related to media containing 5% Xylitol and glucose -free (Control) media. According to obtained results of and by using REST 2009 software, the automatic statistical analysis by REST 2009 software revealed that, the expression of *cpsB*, *cpsD* and *psaA* genes was affected (see part A and B of [Figure 1](#)), but only the expression of *cpsB* gene in expose with 5% Xylitol was reduced significantly in compared to the glucose free media as Control ($P=0.005$). However, there was no significant reduction in the expression of the *cpsD* and *psaA* genes.

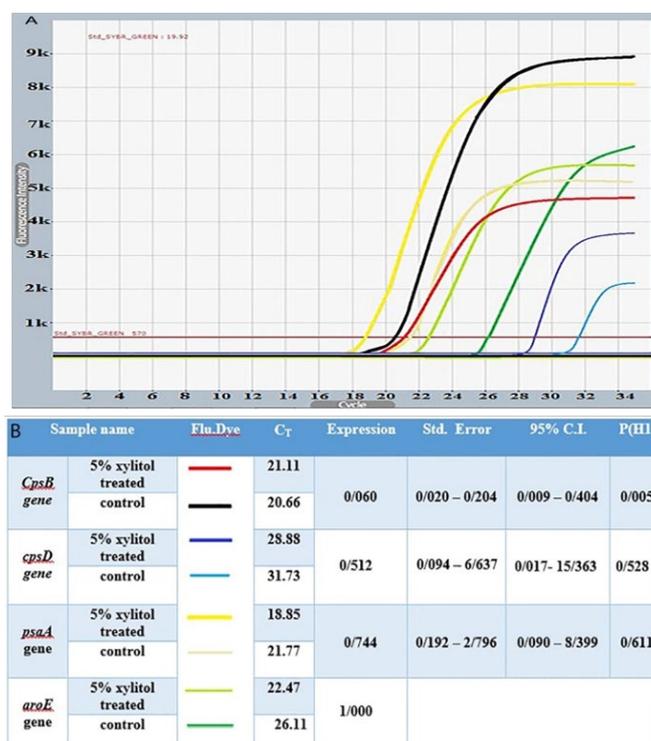


Figure 1. The Cyber green RT Real Time PCR amplification has showed. Part A), the pattern of implications cycle of *cpsB*, *cpsD*, and *psaA* genes in different media in this study. Part B), the results of REST 2009 software statistical analysis has shown.

In the second set up to simultaneously evaluate the effects of Xylitol and fructose; Real-time PCR assay was carried out on total cDNAs related to medias containing 5% Xylitol and 5% fructose + 5% Xylitol. It's just here, Only the expression of *cpsB* gene was

reduced and the expression of the *cpsD* and *psaA* genes did not change (Figure-2). This finding also confirmed the results of previous studies that the presence of fructose in the media prevents the inhibition of Xylitol.



Figure 2. Part A) The resulting pattern of replication of *cpsB*, *cpsD*, and *psaA* genes in media containing 5% Xylitol compared to media containing 5% fructose + 5% Xylitol. Part B) The REST 2009 software results statistical analysis has shown.

The analysis of melting curve also proved during the Real-Time PCR, only one specific product was amplified and there is no non-specific product and primer-dimer. More than one peaks show non-specific amplification. Melting curve results of *cpsB* gene showed that the T_m of the all product of this gene was equal to 82°C and also in the case of *cpsD* gene, in the analysis of melting curve only one peaks at 82°C was observed.

Discussion

Emerging a wide range of deadly infections by *S. pneumonia* (29) as well as the development of antibiotic resistance by this bacterium (30). Diagnosis, treatment and prevention of relative infections are global problem (31). In addition, a vaccine that can cover all pathogen strains is not yet available. Therefore, other ways to control pneumococcal infections have been considered in recent years.

One of the current hypothesis that could possibly prevent the infection from pathogenic strains is focused on the inhibition of some virulence genes.

Since the *S. pneumonia* several virulence genes that one of the most important of these genes is the

polysaccharide capsule regulator genes (as the most important virulence factor) and the *psaA* gene, which plays a major role in bacterial binding, can be mentioned.

Therefore, *cpsB*, *cpsD* and *psaA* genes were investigated in this study and as control, the *aroE* gene, also known as the House Keeping gene, was examined.

A published study results have shown that the fructose could prevent the inhibitory antibacterial action of Xylitol; this may be due to Xylitol entering bacteria through fructose receptors while in the absent of fructose, its inhibitory effect does not occur (19). As the same, our previous study results revealed that the presence of 5% fructose inhibit the anti- pneumococcal activity even after 24 hours (32).

Furthermore, our previous study had shown the growth pattern of bacteria based on the recorded ODs by MTT method, and concluded that the best time and concentration of the user sugars. Present study was conducted out to assay the effects of the expression of selected genes, and 3 hours after exposed of bacterial suspension to 5% Xylitol, 5% fructose + 5% Xylitol as a point out were considered. Thus, pertaining to the present study, it should be noted that in less

than 3 hours, there is not enough time to evaluate the inhibition of the expression of genes and in more than 3 hours, the growth rate of bacterial cells will vary in different media. Therefore, the time point of 3 hours, which was equivalent to MIC concentration of the 5% Xylitol, was chosen to assess the inhibition of the expression of the genes based on the previous study finding (32).

However, in this study based on our previous experience, we used 3 hours as a time point out for investigating the assay of inhibitory effects of utilized sugars against the selected genes expression. While, the Kuorola's research were implemented on standard strain ATCC 49619, the expression of the only *cpsB* gene was monitored 2 hours after exposure to 5% Xylitol and The *16SrRNA* gene was also selected as the reference gene (20). In addition, the Kuorola's research were focused on one target gene expression, but in this study, we assayed four target genes. However, pertaining to the one target gene the results were the same.

According to the results of the present study, the relative expression of *cpsB* gene in exposure to 5% Xylitol was significantly reduced compared with 5% glucose and control media. The outstanding of the current research was that, the results of the real-time PCR were analyzed by using REST 2009 software in order to determine the expression of *cpsB*, *cpsD* and *psaA* genes of bacteria strains and also reference ATCC 6305 strain in 5% Xylitol, 5% fructose + 5% Xylitol and control medias. Also the *aroE* gene was used as the reference gene. According to the REST 2009 software analysis (see attached Tables in Figures 1 and 2), although the expression of target genes was associated with a decrease, but the decrease in *cpsB* gene expression was significant renege in compared to control ($P=0.005$).

By comparing these results with the results of Kuorola studies, it was observed that in the same experimental conditions, including the same volume of BHI- Broth, FBS and similar concentration of Xylitol, the expression level of *cpsB* gene in this study was reduced significantly; the differences may be due to differences in standard bacterial strains, reference gene type, exposure duration, different quality of RNA extraction kits, cDNA synthesis, and Real-Time PCR protocols.

Conclusion

In recent years, the health benefits of using Xylitol have received much attention; not only act as an oral hygiene effects but also it stimulates the immune system. In addition, a research results have shown that the short-term consumption of Xylitol may decrease the release of pro-inflammatory cytokines and the counts of *Streptococcus mutans*. Although, a plenty of research has been reported on the benefits of Xylitol, its antibacterial mechanism of action is unknown. Thus, in the present study, three genes encoding the capsular- making proteins were investigated. The main finding was that, after 3 hours' exposure to 5% Xylitol, the relative expression of *cpsB* gene was reduced about 97.8 % compared with control media ($P=0.005$). This is an outstanding result, because of it may be very important and suggest the possibility of clinical use of this sugar.

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Conflict of Interest

The authors declared no conflict of interest.

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