ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2019;5(4):1-7

Concurrent Molecular Study and Expression Analysis of *Streptococcus pyogenes* Superantigens under the Effect of Bacteriocin Nisin by Real-Time PCR Method

ARTICLE INFO

Article Type
Original Research

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How to cite this article
Sheikhabbasi A.H, Amini K, Tabatabaee Bafroee A.S. Concurrent Molecular Study and Expression Analysis of *Streptococcus pyogenes* Superantigens under the Effect of Bacteriocin Nisin by Real-Time PCR Method. Infection Epidemiology and Microbiology. 2019;5(4):1-7

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Article History
Received: August 22 ,2019
Accepted: December 2 ,2019
Published: January 8 ,2020

ABSTRACT

Aim: Group A *Streptococcus* (*GAS*) is the causative agent of several invasive and non-invasive diseases. Several virulence factors contribute to the pathogenesis of GAS, such as M protein, hemolysins, and extracellular enzymes. Due to the improper use of antibiotics, the resistance of these microorganisms to antibiotics is increasing. Bacteriocins as an alternative to antibiotics are of great importance. In this study, the effect of antimicrobial Bacteriocin nisin was investigated on the expression of *sme*Z gene.

Materials & Methods: Samples were taken from the site of infection on the skin surface of the patients at the dermatology clinics of Tehran public hospitals. The specimen was immediately transferred to the primary culture medium or basal medium. Chromosomal DNA extraction was performed using the standard method for the extraction of *Streptococcus pyogenes* genomes. Multiplex PCR was performed to identify the presence of *sme*Z, *spe*I, and *spe*H genes in the isolates. The expression of *sme*Z gene was evaluated using the real-time PCR technique.

Findings: The frequencies of *sme*Z, *spe*I, and *spe*H genes in 12 *S. pyogenes* isolates were 25, 8.3, and 8.3%, respectively. The fold change rate for *sme*Z gene was -1.209, indicating that this gene was decreased 1.209 folds in the treated group compared to the untreated group.

Conclusion: Bacteriocin not only reduces the number of pathogens but may also affect the metabolism of the bacteria by producing toxins. The use of new antimicrobial agents in place of previous drugs for psoriasis patients could be considered as a way to treat the disease more effectively in the future.

Keywords: *Streptococcus pyogenes*, Superantigens, Bacteriocin, Nisin, Real-time PCR

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[DOR: 20.1001.1.25884107.2019.5.4.2.9]

Introduction

Streptococcus is an essential genus of Gram-positive bacteria. These bacteria are spherical (cocci or cocoon), and splitting them into one axis leads to the formation of a chain or pair. *GAS* is a human pathogen causing more than 600 million infections with over 500,000 deaths worldwide annually [1]. It causes mild skin and mucosal infections, but could also cause aggressive and dangerous diseases such as Streptococcal Toxic Shock Syndrome (STSS) or necrotizing fasciitis. The severity of *GAS* infections depends on numerous bacterial and host factors [2].

*spe*H, *sme*Z, and *spe*I are superantigen-encoding genes produced by *Streptococci*. *Streptococcus*-secreted superantigens and toxins by binding to the beta chain of T cell receptors could induce the expression of T cell receptors required to settle this cell in skin cells. Superantigens released by *GAS* in the tonsils could stimulate T cells within the lymph nodes [3]. As a result, T cells could settle in the skin, where they are most active, and trigger an autoimmune reaction. *spe* gene is a plasmid-encoded gene that could be transmitted from one bacterium to another one and spread among *Streptococci* [4].

*sme*Z is a very small superantigen which is only detectable by biological methods that could reliably detect T cell molecules. Virulence factors are not evenly distributed among *S. pyogenes* strains. Some of them are chromosomal, and some are plasmid-encoded. Their presence or absence could be used as a diagnostic method and simple tool in clinical diagnosis. So far, determining how *S. pyogenes* species/strains relate to each other has been a costly and time-consuming task. Although sequence-based methods are relatively easy and easily comparable, the cost of sequencing reactions for routine use could be very high. Bacteriocins are synthetic and non-toxic ribosomal peptides with antimicrobial activity, produced by lactic acid bacteria. They are resistant to acid and heat and are easily digestible [5]. Bacterial resistance to antibiotics is an essential issue in the treatment of infectious diseases. Bacteriocins produced by bacteria appear to be suitable alternatives to conventional antibiotics in the treatment of human infections, which have attracted much attention in recent years.

The bacteriocin term comprises a large group of extracellular ribosomal antimicrobial proteins or peptides that have been introduced as antibacterial agents. Bruno et al. (1992) suggested that bacteriocins should have two characteristic properties [3]; firstly, they should have a protein nature, and secondly, they should have non-lethal effect on the cells producing them.

Nisin is the most common digestible bacteriocin that has been used as a preservative and antimicrobial agent for microbiological immunity for many years [6]. Nisin is highly regarded because of its inhibitory effect on the growth of many Gram-positive bacteria and some Gram-negative bacteria causing damage to the outer membrane.

Objectives: The purpose of this research was the molecular study and expression analysis of *S. pyogenes* superantigens affected by bacteriocin nisin via the real-time PCR method in lab conditions.

Materials and Methods (in vitro)

Sampling: The statistical population was selected from all psoriasis patients referred to the dermatology clinics of Tehran public hospitals. Sixty patients were randomly selected from among the psoriasis patients who completed a written consent form and had not received antibiotics during the last two weeks prior to sampling. Samples were taken from the site of infection on the skin surface of the patients.

Identification tests: The samples were immediately transferred to the primary culture medium or basal culture medium containing 10% sheep blood using a sterile swab. The

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culture medium was incubated for 48 hrs at 37-38 °C in the presence of 8-10% carbon dioxide until a clear halo was discernible around the colony, indicating beta hemolysis.

In the next step, grown colonies were gram stained. After staining, colonies were assayed. Then catalase test was performed on pure colonies [7].

DNA extraction: Genomic DNA extraction of Gram-positive bacteria (*S. pyogenes*) was done using G-spin ™ Genomic DNA Extraction Mini Kit.

Preparation of primers: Three pairs of primers were chosen based on the previous studies and purchased from Bioneer Co [8-9]. The PCR reaction was performed in a final volume of 25 μL in 35 cycles, including an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 55°C for 40 sec, extension at 72oC for 70 sec, anf final extension at 72°C for 7 min. PCR products were then loaded on 1% agarose gel and evaluated by electrophoresis.

Preparation of Bacteriocin Nisin Stock Solution: About 2048 mg of nisin was weighed on a digital scale and suspended in 1 cc of DMSO, and for proper dispersion, ultrasonic apparatus was used for 30 min [11].

Micro Broth dilution method: Using the standard Broth microdilution procedure proposed by Clinical and Laboratory Standards Institute (CLSI, 2015), the minimum growth inhibitory concentration (MIC) was obtained for each bacterium in contact with nisin suspension [12].

About 100 μL of microbial suspension at 0.5 McFarland concentration was added to all wells. At First, well concentration was 2048 μg/mL, and the minimum growth inhibitory concentration (MIC) was obtained using Micro broth. Two wells were used as positive (culture medium and microbial suspension) and negative (medium and nisin) controls. The microplates were then incubated in the incubator for a maximum of 24 hrs at 37 °C [13].

Expression analysis of *sme*Z gene by real-time PCR technique: Before performing the polymerase chain reaction, 1.0 mg/mL of bacteriocin was poured into 20 mL volume of DMSO solvent, and half McFarland of *S. pyogenes* positive microbial suspension was added to it. After 15 hrs of incubation (late log phase) is the best time for RNA extraction. After 15 hrs, RNA extraction was performed as follows [1].

RNA extraction: Qiagen Company RNeasy microcytes were used for RNA extraction. The

Table 1) Primers used in this research by Multiplex PCR Method

Target Gene	´5→3´Sequences of Primers	Product Size (bp)	Reference
spe H	F_TTGGATCCAATTCTTATAATACAACC R_CCACTTCCTGAGCGGTTACTTTCGG	175	[10]
sme Z	F-TGGGATCCTTAGAAGTAGAAGTAGATAATA R-AAGATTTTAGGAGTCAATTTC	148	[10]
spe I	F: AAGGAAAAATAAATGAAGGTCCGCCAT R: TCGCTTAAAGTAATACCTCCATATGAATTCTTT	90	[10]

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bacterial suspension (bacteria containing smeZ gene with their determined MIC in the presence of bacteriocin nisin) was used in the logarithmic phase of growth. DNase Qiagen kit was used to remove genomic DNA [13].

Quantitative and qualitative evaluation of RNA: Extracted RNA was quantitatively and qualitatively evaluated to ensure the accuracy of extraction and sample assay. Quantitative evaluation of RNA extraction was performed using the 280/260 nm optical absorption method. In this method, 2 μL of extracted RNA was placed in a spectrophotometer, and absorption of wavelengths of 260 and 280 nm, the ratio between these two wavelengths, and the ratio of 260/230 were investigated [14].

CDNA Synthesis: CDNA synthesis was performed using Roche Company AMV reverse enzyme at a concentration of 25 μL/unit. The extracted RNA was then incubated at 65 °C for 3 min. Then reverse transcription (RT) was performed at 42 °C for 60 min with 2 μL Random Primer, 0.8 μL of AMV reverse transcriptase, 2 μL of 10 mM dNTP, 1 μL Rnase inhibitor, and 2 μL of 10x AMV buffer. The AMV was then incubated at 99 °C for 5 min and inactivated [14].

The polymerase chain reaction was performed using the Company Kit (Genet bio CAT. NO: Q9210) from South Korea in a final volume of 20 μL containing 10 μL of Prime Qmaster mix (2x) with syber green, 5 μL of Depc water, one μL of forward and revers primers each, one μL of Rox dye, and 2 μL of cDNA. Fragments proliferation was completed in ABI-STEP-ONE PLUS with an initial denaturation at 95 °C for 1 min, followed by 35 cycles of 95°C for 30 sec, 59°C for 40 sec, and 72°C for 60 sec. The domestic gene was used as the internal control of the test. Then to calculate gene expression and plot the corresponding graphs, specific software (micPCR) was used, and target gene expression was calculated. Expression analysis was performed by relative measurement of mRNA expression compared to the standard strain [15].

Findings

Sampling: From a total of 60 specimens studied, 12 (20%) cases were identified as *S. pyogenes*. Group A *Streptococci* were isolated and identified after performing the identification tests.

Gram-positive cocci of *S. pyogenes* were observed singly and in chains. Catalase test was negative for *S. pyogenes,* and the results of hemolysis test determined a clear halo around the bacterial colony, indicating beta hemolysis.

Multiplex-PCR: As shown in Figure 1, out of 12 *S. pyogenes* isolates, one isolate (8.3%) had *spe*H gene, three isolates (25%) had *sme*Z gene, and one isolate (8.3%) had

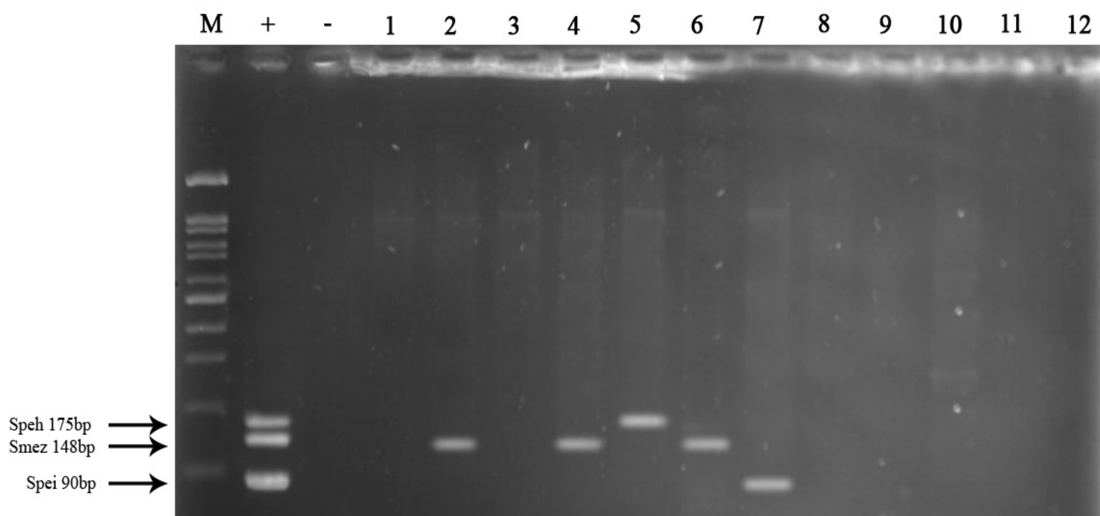

Figure 1) PCR test result on some positive isolates from Sample 1 to 12. From left to right: 100bp DNA Ladder, + Positive Control, - Negative Control

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*spe*I gene.

Real-time PCR: The results of real-time PCR showed that the fold change for smeZ gene was -1.209, indicating that it was decreased 1.209 folds in the treated group compared to the untreated group. There was a statistically significant difference in the expression of smeZ gene between the treated and untreated groups (p value < .05).

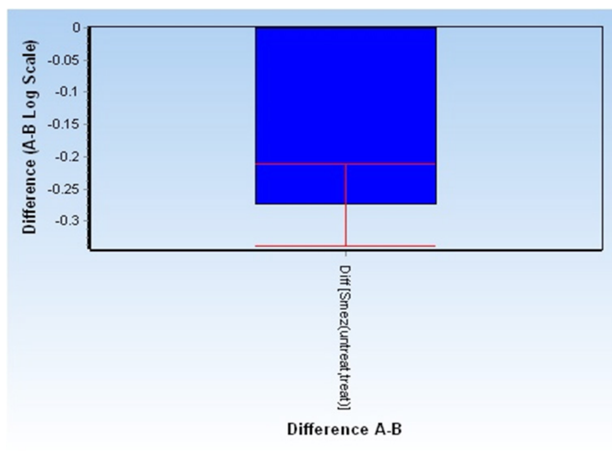

Figure 2) Fold Change Variation

Discussion

GAS is a Gram positive, beta-hemolytic coccus occurring in chains, which is responsible for a wide range of diseases in humans. These diseases include strep throat (acute pharyngitis) and skin and soft tissue infections such as impetigo and cellulitis. In a study by Siasi et al. (2017), 60 individuals with psoriasis were isolated by biochemical tests. After DNA extraction from 48 isolated bacteria, polymerase chain reaction was performed to investigate the presence of exotoxin genes C, B, and A. The results of PCR amplification of these bacteria indicated the presence of *spe*A gene in 28 samples (58.3%), *spe*C gene in 16 samples (33.3%), and *spe*B gene in all samples (100%). The frequency of samples with more than one gene was studied during the study, and the results showed that 28 (58.3%) samples contains *spe*A and *spe*B, 16 (33.3%) samples contained *sep*B and *sep*C, and 10 (28.8%) samples contained all three *sepA*, *sep*B, and *sep*C genes. The results showed a significant relationship between the presence of the three *sep*A, *sep*B, and *sep*C genes and psoriasis. Among the infectious, physiological, and genetic factors that are responsible for psoriasis, *S. pyogenes* is one of the critical causative agents of psoriasis, confirmed by this and other studies results [16].

In a study by Bor bor Azimi et al. (2016), the profile of streptococcal *spe*A, *spe*B and *spe*C exotoxin genes was evaluated in 72 *GAS* strains collected from blood, urine, wound, and nasopharynxes of carriers (32 isolates) and patients (40 isolates) with clinical symptoms. Gene profiles were determined by double-stranded polymerase chain reaction (PCR-duplex) to identify two *spe*A and *spe*C genes and by single PCR to identify *spe*B gene. The results showed that *spe*A, *spe*B, and *spe*C pathogenic genes were present in 69.4, 69.4, and 66.7% of the strains, respectively. In the strains isolated from patients, the frequency of *spe*A, *spe*B, and *spe*C exotoxin genes was 67.5, 62.5, and 67.5%, respectively. Although 71.9, 78.10, and 65.60% of the strains isolated from the carriers had *spe*A, *spe*B, and *spe*C genes, there was not a significant difference between the carrier and patient isolates regarding the frequency of exotoxin genes [14].

Mir Hosseini et al. (2007) investigated the inhibitory effect of *Lactococcus lactis* using broth culturing and 24-hour culture supernatant; in their study, the effect of bacteriocin produced on the growth curve of *Listeria monocytogenes* and *Bacillus cereus* was also evaluated. The results showed that bacteriocin produced by *L. lactis* had a more inhibitory effect on *L. monocytogenes* and *B. cereus*. Due to the observed inhibitory effect, the use of bacteriocin directly in food or starter culture in liquid medium is

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recommended to reduce food poisoning caused by undesirable bacterial activities [17]. Taheri et al. (2013) investigated the role of microbial agents in the development or exacerbation of psoriasis. The primary purpose of their study was to provide a general overview of the research conducted on the role of microbial agents in psoriasis. Articles published during 1961-2011 in MEDLINE/PubMed and internal information resource databases were reviewed to determine the role of microbial agents in psoriasis. The results of these reviewed studies were carefully analyzed. According to the results of these studies, it was suggested that microbial agents, especially *S. pyogenes*, could alter the psoriasis disease process by altering the specific characteristics of patients with psoriasis, and that superantigens appear to be very important in the pathogenesis of *S. pyogenes*. The effect of microbial bacteriocin nisin was also monitored on the expression of resistant *S. pyogenes* super-families carrying *sme*Z gene [18].

Conclusion

S. pyogenes is one of the causative agents of psoriasis. The role of *semZ* exotoxin gene as a superantigen could be prominent in this disease. Due to the improper use of antibiotics, the resistance of microorganisms to antibiotics is increasing. Today, the biggest challenge for researchers is to eliminate antibiotic resistant agents and virulence factors with other antimicrobial agents. The aim of this study was to investigate the effect of bacteriocin nisin on the expression of *smeZ* gene. The fold change rate for *smeZ* gene was -1.209, indicating that this gene was 1.209 folds lower in the treated group than in the untreated group. Bacteriocin not only reduces the number of living cells in the pathogens but may also affect the metabolism of the bacteria by producing toxins. Further research in this area, especially on the expression of this gene in psoriasis patients, could open the way to treat the disease more effectively in the future.

Acknowledgements: The authors would like to extend their sincere appreciation to all the staff in Department of Microbiology, Science and Research Branch, Islamic Azad University, Tehran, Iran

Ethical Permissions: Not applicable.

Conflicts of Interests: The authors declare that they have no conflict of interest.

Authors Contribution: AHS: Statistical analyst, Original researcher, Introduction author, Methodologist, Discussion author; KA: Original researcher, Methodologist, Discussion author, ATB: Methodologist, Discussion author.

Fundings: We have no funding to declare.

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