Volume 6, Issue 6, 2016, 1607 - 1611 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

Original Research Article Open Access Journal

Received: 25.09.2016 / Revised: 28.10.2016 / Accepted: 30.10.2016 / Published on-line: 05.11.2016

Study of virulence gene profiles in β-hemolytic *Streptococcus* sp. strains

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ABSTRACT

Infections caused by *Streptococcus pyogenes* have significant medical importance because of the high number of complications and post-infectious sequelaes. The aim of this study was to analyze the gene profile of streptococcal superantigenic toxins found in *Streptococcus pyogenes* and β-hemolytic group C and G *Streptococcus* strains isolated from throat and nasal exudates. The analysis of the virulence *patterns* was conducted using 102 β-hemolytic streptococcal strains. The bacterial DNA was extracted and 3 Multiplex PCR assays were carried out. The results showed that the gene most commonly expressed was the *speF* gene (70.5% of cases) and the *smeZ* and *speI* genes had the lowest expression level (2% and respectively 3%). In conclusion, the β-hemolytic streptococcal strains analyzed possessed a rich repertoire of virulence genes showing the high pathogenic potential of streptococcal strains.

Keywords: *Streptococcus pyogenes, virulence genes, PCR*.

1. INTRODUCTION

Infections caused by *Streptococcus (Str.) pyogenes* have significant medical importance because of the high number of complications caused (sinusitis, otitis media, mastoiditis, tonsillar phlegmon, Ludwig angina) and post-infectious sequelaes (acute cardioarticular rheumatism, acute glomerulonephritis) [1, 2].

The pathogenic potential of streptococci is mediated by many virulence factors and is based on their ability to colonize and disseminate to the host tissues, avoiding the immune system [3-5].

Str. pyogenes produces a large variety of exotoxins known as streptococcal pyrogenic exotoxins which have an important role in pathogenicity and virulence [6, 7]. These proteins are known as superantigens because of their ability to stimulate large populations of T cells [8, 9]. In contrast to conventional antigens, superantigens bind directly to the components of the MHC (major histocompatibility complex) [10, 11].

It is believed that superantigens interfere with the activation of the immune response by inhibiting the production of specific antibodies through the disruption of specific responses to infections [12]. The virulence factors produced by *Str. pyogenes* cause the exacerbation of the inflammatory responses [13, 14].

The release of pro-inflammatory cytokines leads to reduced vascular tone, acidosis and organ failure [15, 16]. Superantigens and the streptococcal mitogenic exotoxin Z (smeZ) interact with class II MHC molecules and T cell receptors [17, 18]. *In vitro*, these interactions lead to the excess production of cytokines [19, 20]. Genes that encode for most superantigens can be found on phages or plasmids [21, 22]. However, it was noted that *speJ* and *speG* are located at the genome level and it has been discovered that these genes were present in all *Str. pyogenes* strains tested [23 -25]. The *speB* gene is located on the chromosome, while the genes encoding for exotoxins SpeA and SpeC are located on phages and are easily disseminated to other *Str. pyogenes* strains [26, 27]. The less common genes that encode for superantigens are *speL* (15%) and *speM* (5%) [28, 29].

The aim of this study was to analyze the gene profile codifying for streptococcal superantigenic toxins: *speA*, *speB*, *speF*, *speG*, *speH*, *speI*, *speJ*, *ssa*, and *smeZ* found in *Str. pyogenes* and β-hemolytic group C and G *Streptococcus* strains isolated from throat and nasal exudates between December 2012 and April 2013 in the Synevo Central Reference Laboratory from Bucharest.

2. EXPERIMENTAL SECTION

2.1. Isolation of the strains. The β-hemolytic *Streptococcus* strains were isolated from throat and nasal exudates belonging to a total of 10691 patients suspected of pharyngitis or scarlet fever, collected during December 2012 - April 2013 in the Synevo Central Reference Laboratory, Bucharest.

2.2. Identification of the strains. The throat and nasal exudates were seeded on Columbia agar medium supplemented with 5% erythrocytes. The β-hemolytic *Streptococcus* strains were identified using conventional test (appearance on Columbia nutrient agar with 5% sheep blood), latex agglutination and Maldi-Tof analysis. The study was conducted using 89 strains of *Str. pyogenes* and 13 β-hemolytic group C and G *Streptococcus* strains.

2.3. DNA extraction and PCR. For bacterial DNA extraction an

alkaline method was used. The chromosomal DNA was used as template for all PCR reactions performed.

The steps for alkaline DNA extraction were as follows: In a solution of 0.5 M NaOH 20 ml + 0.25% SDS between 1-5 *Streptococcus* colonies were resuspended. The next step is incubation in the thermocycler for 15 min. at 95^0 C followed by addition of 180 ml TE solution (Table 1). Afterwards, centrifugation for 3 minutes at 13,000 rpm and recovery of the supernatant. The final step is storage at -80°C freezer until use.

Table 1. Composition of TE.

Substance	Concentration	Quantity
TRIS	1 M pH 8	5 ml
EDTA	0.5M pH 8	1 ml
Distilled water	-	494 ml

The genes detection was performed by using three multiplex PCR assays (Table 2).

The presence or absence of the following 10 genes was determined: *speA*, *speB*, *speC*, *speG, speF, speH*, *speI*, *speJ*, *ssa* and *smeZ*.

A scheme that included 3 separate PCR reactions was preferred due to the high similarity between amplicons dimensions like *speJ* and *speH*, 629 and 630 bp respectively, or *speI* with *ssa*, 678 and 691 bp respectively (Table 2).

Table 2. Amplicons used in the detection of streptococcal virulence genes.

Gene	PCR reactions	Amplicon dimensions
speF	Multiplex I	1193 pb
ssa		691 pb
speG	Multiplex II	447 pb
speC		246 pb
speJ		629 pb
speA	Multiplex III	200 pb
speB		300 pb
speI		678 pb
speH		630 pb
smeZ		391 pb

The PCR reactions were performed in Thermal Cycler 2700 analyzer, according to the protocols described in literature.

The products obtained were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. A specific molecular weight marker (100 bp DNA Bench Top Ladder) was used.

3. RESULTS SECTION

Str. pyogenes strains express a large variety of superantigens, ranging from speA to speM, streptococcal superantigen (ssa) and streptococcal mitogenic exotoxin smeZ. These superantigens play an important role in the pathogenesis of streptococcal infections. Superantigens stimulate T cell activation without antigenic specificity due to their ability to bind directly to the MHC class II molecules [30, 31]. Activated T-cells cause a massive release of pro-inflammatory cytokines (TNF-α, IL-6 and IFN-γ). The inflammatory response becomes overactive and mobilizes cellular and humoral defense mechanisms: endothelial cells, epithelial lymphocytes, macrophages and releases pro-inflammatory mediators (IL-1β, IL-6, IL-8 and TNF) [32].

Simultaneously, the defense mechanisms related to the complement activation cascade are activated stimulating the production of C3a, C4a and C5a mediators of the inflammatory process [33]. The supernatigens speB and speF are not specific to *Str. pyogenes* strains, speB is a cysteine proteinase and the extracellular streptococcal superantigen speF is identical to the B streptococcal DNase. The DNase mitogenic factor was renamed in 1993 due to its mitogenic activity, but named shortly after streptococcal pyrogenic exotoxin F (speF) due to its pyrogenic capacity for cytokine stimulations. DN-ase possesses deoxiribonuclease as well as ribonuclease and allows the bacteria to disseminate through tissues by increasing secretion fluidity. Certain strains of *Str. pyogenes* produce the TSST-1 superantigen [34, 35]. This superantigen binds directly to the MHC molecules II activating a large number of T lymphocytes.

In the present study, the gene most commonly expressed was the *speF* gene, in 70.5% of cases (Fig. 4). The expression of this gene can be seen in Fig. 1.

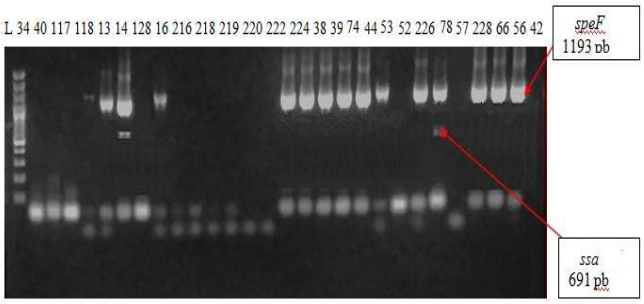

Figure 1. Multiplex PCR assay for simultaneous detection of *speF* and *ssa* genes.Line 1 - DNA ladder 100bp (Promega). Streptococcal strains 34, 13, 14, 16, 38, 39, 74, 44, 53, 52, 78, 57, 66, 56, 42 expressed the *speF* gene, and only strains 14 and 57 possessed the *ssa* gene.

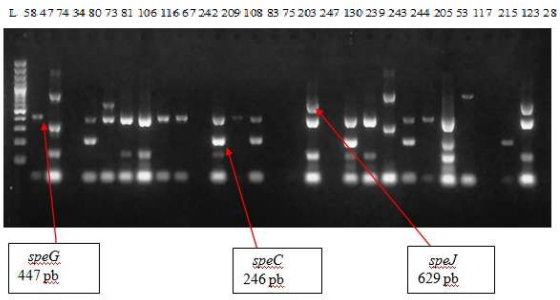

Figure 2. Multiplex PCR assay for simultaneous detection of *speG, speC* and *speJ* genes. Line 1 - DNA ladder 100bp (Promega). Strains 58, 34, 80, 73, 81, 106, 116, 242, 209, 108, 203, 130, 239, 53, 28 expressed the *speG* gene, strains 34, 242, 108, 130, 53, 123 presented the *speC* gene, and strains 203, 28 expressed the *speJ* gene.

The results are consistent with other studies from Romania, Anghel et al. (2012) [36] conducted a study on 12 β hemolytic

Streptococcus strains (10 group A and 2 group C β hemolytic strains) isolated from kindergarten and school children, following an epidemiological study. In that study, the *speF* gene was present in 83.33% of cases together with *speG* and *ssa* genes.

The expression of the *speC*, *speG* and *speJ* genes can be seen in Fig. 2.

The genes with the lowest expression level among the 102 tested strains from this study were those codifying for the superantigens smeZ and speI, with a rate of 2 and respectively 3% (Fig. 3, 4).

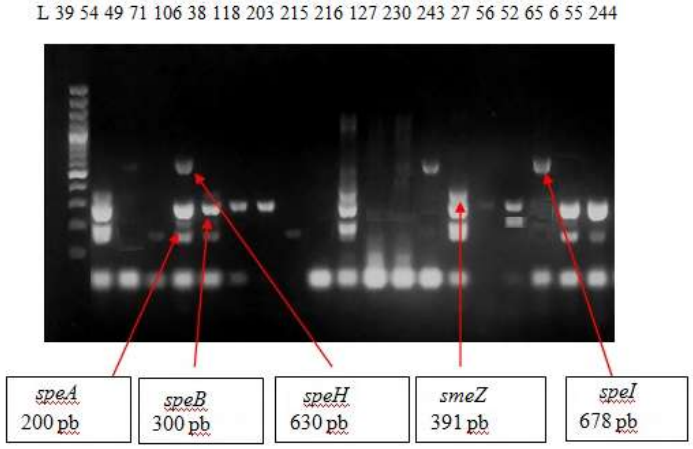

Figure 3. Multiplex PCR assay for simultaneous detection of *speA, speB, speH, smeZ and speI* genes. Line 1 - DNA ladder 100bp (Promega). Strains 39, 54, 106, 127, 56, 65, 55, 244 expressed the *speA* gene, strains 54, 106, 118, 203, 2, 56, 65, 55, 244 presented the *speB* gene, strains 106 and 27 expressed the *speH* genes, and the *smeZ* gene was present in the case of the 56 strain.

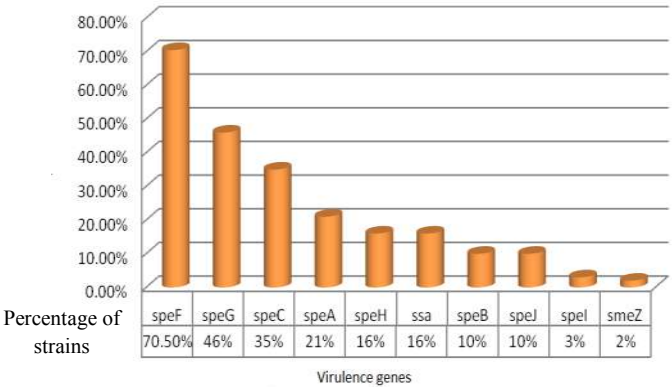

Figure 4. Distribution of genes encoding for superantigens.

Our findings are consistent with those obtained by Anghel et al. (2012) [36], who observed a expression rate of only 1.66% for both genes, but they are in contrast with the results obtained in a previous study conducted in the Cantacuzino Institute in 2008. In that study, conducted by Luca-Harari et al. (2009) [37], 92% of the streptococcal strains tested expressed *smeZ* gene. The difference between the results obtained by Luca-Harari et al. (2009) [37] and those found in this study could be explained by the fact that the 135 streptococcal strains studied in the Cantacuzino Institute came from invasive infections (33 strains) as well as from non-invasive infections. The non-invasive strains were isolated from different sites (throat swabs, skin infections, ear secretions).

The 102 β-hemolytic *Streptococcus* strains tested in this study were isolated from nasal and pharyngeal exudates obtained from outpatients with or without typical symptoms of pharyngitis. By comparison, we indirectly concluded that the streptococcal strains involved in invasive infections, but also those isolated from skin infections, ear secretions are more virulent than those isolated from throat and nasal exudates.

Of the 89 *Str. pyogenes* strains 4.5% did not possess any of the superantigen genes and 18% expressed only one of the genes, by comparison with the group C and G β-hemolytic streptococci where the lack of gene expression was observed in 38% of the strains, while 20% expressed only one of the genes, proving that a much lower virulence expression was seen in the case of the group C and G *Streptococcus* strains compared with the *Str. pyogenes* strains.

Of all strains 27.5% have presented three genes and 11.7% have expressed four genes. 5 virulence genes were expressed by two strains, one of them possessing the: *speG, speB, speF, speH, speJ* genes and the second strain expressing the *speC, speA, speB, speF, ssa* genes. Both strains were isolated from children.

Two strains expressed six virulence genes simultaneously, one isolated from a 9 years old child which possessed the *speC, speG, speA, speB, speF*, and *ssa* genes. The second strain was isolated from an adult person (30 years old) and the genes expressed were: *speC, speG, speA, speB, speF*, and *smeZ*.

In comparison, in the study conducted by Luca-Harari et al. (2009) [37], one strain presented 7 virulence genes simultaneously (*speC, speG, speA, speB, speF, speH, ssa*).

4. CONCLUSIONS

Streptococcus pyogenes analyzed strains possessed a rich repertoire of virulence genes, two strains expressing 6 genes while other two genes presented five of the 10 analyzed genes. In contrast 9% of the strains tested expressed none of the 10 genes. The analyzed group C and G *Streptococcus* strains showed a lower expression of virulence genes compared with the *Str. pyogenes* strains.

The highest gene expression was seen in the case of the *speF* gene, followed by *speG* (70.5% and 46%), while the least expressed genes were *speI* and *smeZ* (3% and 2% respectively).

In conclusion, the β-hemolytic streptococcal strains analyzed possessed a rich repertoire of virulence genes showing the high pathogenic potential of streptococcal strains.

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6. ACKNOWLEDGEMENTS

This paper was possible with the contribution of the Synevo Romania medical laboratory.

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