ACTA MICROBIOLOGICA BULGARICA Volume 33 / 4 (2017)

Distribution of Super-Antigens and Toxins in Bulgarian Invasive and Non-Invasive Clinical Isolates *Streptococcus pyogenes*

Adile Muhtarova, Raina Gergova, Lena Setchanova, Ivan Mitov
Department of Medical Microbiology, Medical University, Sofia, Bulgaria

Abstract

Streptococcus pyogenes, or Group A streptococcus (GAS), is the major pathogen of bacterial tonsilo-pharyngitis tonsillopharyngitis and of skin and soft-tissue infections, which can also cause severe invasive disease or dangerous post-streptococcal autoimmune complications. The differences in the pathogenesis are due to the existing variety in the GAS genome, which often occurs as a result of the cumulative effect of a greater number of various virulence genes**.** The aim of this study was to determine the frequency and distribution of genes encoding super-antigens and toxins in Bulgarian GAS isolates according to their source and kind of infection. Clinical isolates (n=238) from throat samples, wounds, punctures from peri-tonsilar peritonsillar abscesses, middle ears and sinuses, blood cultures and cerebrospinal fluid, identified as GAS, were screened for the presence of 21 virulence genes, using multiplex polymerase chain reaction (PCR). All of the tested strains were shown to carry the typical of GAS genes: *slo, speB, sypCEP, sdaB*; more than 80%, *mac* and *smeZ*; followed by *speF, speG, speJ* and genes for DNA-ses *sdc,* sdaD, Spd3 (between 50% and 75%). An attempt was made to seek an association between the PCR-detected GAS virulence genes, the kind of infection and the source of isolates. A large genetic diversity was found in the studied strains. The genes *speA, speF*, s*peL* and *speM* or their combinations were detected more often in invasive isolates ($p < 0,05$) than in non-invasive ones.

Key words: *Streptococcus pyogenes*, virulence, PCR

Резюме

Streptococcus pyogenes или стрептококи от група А (GAS) са основният причинител на бактериалния тонзило-фарингит, инфекции на кожата и меките тъкани, които също могат да причинят тежко инвазивно заболяване или опасни пост-стрептококови автоимунни усложнения. Разликите в патогенезата се дължат на съществуващи различия в генома на GAS, който често се проявява в резултат на кумулативния ефект на по-голям брой различни гени на вирулентност. Целта на това изследване е да се определи честотата и разпределението на гени, кодиращи супер-антигени и токсини в българските изолати GAS, в зависимост от техния произход и вид инфекция. Клиничните изолати (n = 238) от проби от гърло, рани, пунктати от перитонзиларни абсцеси, от средно ухо и синуси, хемокултури и цереброспинална течност, идентифицирани като GAS, бяха скринирани за наличието на 21 гени на вирулентност, използвайки мултиплекс полимеразо-верижна реакция (PCR). Всички от изпитваните щамове са показали, че носят типичните за GAS гени *slo, speB, sypCEP, sdaB*; повече от 80%, *mac* и *smeZ*; последвани от *speF, speG, speJ* и гени за ДНК-зи *sdc, sdaD, Spd3* (между 50% и 75%). Беше направен опит да се търси връзка между откритите чрез PCR GAS гени на вирулентност, вида на инфекцията и източника на изолатите. В изследваните щамове е установено голямо генетично разнообразие. Гените *speA, speF, speL* и *speM* или техните комбинации се откриват по-често в инвазивни изолати ($p < 0,05$), отколкото при неинвазивни.

Introduction

Streptococcus pyogenes (Group A streptococcus - GAS) causes a broad range of diseases in humans including invasive and non-invasive infections like pharyngitis, impetigo, erysipelas, cellulitis, scarlet fever, necrotising fasciitis (NF), streptococcal toxic shock syndrome (STSS) In addition, self-limited streptococcal infections, which can start as local, benign ones, may often become the reason

for development of dangerous post-streptococcal suppurative (abscess, sepsis) or nonsuppurative autoimmune complications, such as acute rheumatic fever, rheumatic heart disease, reactive arthritis, Kawasaki disease, psoriasis and acute glomerulonephritis (Descheemaeker *et al*., 2000; Carapetis *et al*., 2005; Proft and Fraser, 2016). Some of these are with high mortality rates (15%): more than half a million deaths each year are due to streptococcal diseases worldwide, with no effective vaccine prevention against GAS infections currently (Carapetis *et al*., 2005; McMillan *et al*., 2012). Increases in invasive *S. pyogenes* disease have been reported from various European countries during the 1990s and into the 2000s (Lamagni *et al*., 2005; Lamagni *et al*., 2008). A large number of virulence factors are involved in the complex pathogenicity of this organism (Borek *et al.,* 2011; Cunningham, 2000; Golińska *et al*., 2016; Yang *et al*., 2013). These factors include M protein, streptococcal inhibitor of complement - *sic,* streptococcal pyrogenic exotoxins, which have properties of super-antigens (SAgs) - *speA, speC, speH, speI, speK, speL, speM, ssa*, haemolysins and several DNases: *spd3, sdc, sdaB, sdaD* (Hauser *et al*., 1991; Murakami *et al*., 2002; Hasegawa *et al*., 2010; Borek *et al*., 2012). Some of the GAS virulence factors are chromosomally encoded, however, a large fraction of virulence factors such as a majority of DNAses and SAgs are encoded by mobile genetic elements (Vlaminckx *et al*., 2003; McMillan *et al*., 2012). The differences in the pathogenesis are due to the existing variety in the GAS genome, which often occurs as a result of the cumulative effect of a greater number of various virulence genes (Proft and Fraser, 2016; Golińska *et al*., 2016).

The aim of this study was to determine the frequency and distribution of genes encoding super-antigens and exotoxins in Bulgarian GAS isolates according to their source and kind of infection.

Material and methods

Strains

A collection of clinical non-duplicate GAS strains (n=238) isolated in the period October 2013 – April 2017 were used. The first group consisted of non-invasive isolates from mucosal samples: pharyngeal (185) and vaginal (12) swabs, and from erysipelatous skin lesions (6). The second group consisted of invasive ones: punctures from peritonsillar abscesses (5), middle ears (10) and sinuses (8), wounds (10) blood culture (1), and one cerebrospinal fluid (1). The isolates were identified as previously described by Gergova *et al.* (2015). GAS strains were stored in skim milk at 70°C, and before experiments were subcultured three times on Columbia agar (BBL, Germany) supplemented with 5% sheep blood.

Extraction of DNA

DNA extraction was performed using a DNAsorb-AM nucleic acid extraction kit (AmpliSens), according to the manufacturer's guidelines. For the purpose of DNA extraction, GAS strains were cultured on Columbia blood agar (BBL, Germany) for 24 h at 35°C in an atmosphere with 5% CO_2. Then, the bacterial lysates were obtained from this growth of pure microbial culture.

Polymerase chain reaction (PCR) assay.

PCR was performed in a 25 µl reaction mix, using primers for the genes of DNA-ses: *spd3, sdc, sdaB, sdaD*; exotoxins and SAgs: *speA, speC, speH, speF, speI, speJ, speK*, *speL, speM, ssa*; protease inhibitors: *spe B, spyCEP, mac, sic* and gene for hemolysin - streptolysin O (*slo*) shown in Table 1. DNA was amplified using modifications of the protocols of Borek *et al.* (2011, 2012) and Gergova *et al.* (2015). The amplified genes were separated in a 2% agarose gel for 70-90 min at 120 V, stained with ethidium bromide (0.5 µg /mL) and detected by UV transillumination (wavelength 312 nm).

Statistical Analysis

The data were analyzed using the *Chi*-square test, Fisher's exact test for categorical variables. All analytical procedures were performed using SPSS for Windows, Version 16.0. (SPSS Inc., Chicago, USA). Differences were considered statistically significant at $P < 0.05$.

Results

Our results are shown in Table 2. All of the tested strains were shown to carry the typical of GAS genes *slo, speB, sypCEP, sdaB*. More than 80% of the strains had genes *mac* and *smeZ*; followed by *speF, speG, speJ* and genes for DNA-ses: *sdc,* sdaD, spd3 (between 50% and 75%). The predominant combinations in both groups were: *slo, speB, sypCEP, sdaB, mac*, *smeZ*, *speG, speJ* with or without *sdc,* sdaD, spd3. The genes *speA, speF*, s*peL* and *speM* or their combinations were detected more often in invasive isolates ($p < 0,05$) than in non-invasive ones. Representative amplicons formed via multiplex PCR using six mixes by protocols 1 to 6 are presented in Fig. 1.

Table 1. Oligonucleotide primers and protocols used for gene detection

Virulence genes	Primer sequence (5`→3`)	Annealing	Amplicon size (bp)
Protocol 1 and mix 1		60°C 30 sec	
sdc	AAGCTTAGAAACTCTCTCGCCA AGTTCCAGTAATAGCGTTTTTCCGT		600
sdaB	TATAGCGCATGCCGCCTTTT TGATGGCGCAAGCAAGTACC		440
sdaD	TTTACGCTGAATCGGGCACT GGCTCTGGTTTGCTTTCCCA		295
spd3	GCCGCTTCTTCAAACTCTTCG ATCGTCGTACTTGGCAAGGTT		784
Protocol 2 and mix 2		59°C 40 sec	
speL	CCTGAGCCGTGAAATTCCCA ACACCAGAATTGTCGTTTGGT		657
speK	CCTTGTGTGTGTATCGCTTGC TTGCTGTCCCCCATCAAACT		568
speC	GCCAATTTCGATTCTGCCGC TGCAGGGTAAATTTTTCAACGACA		405
smeZ	TTTCTCGTCCTGTGTTTGGA TTCCAATCAAATGGGACGGAGAACA		246
speM	ATCGCTCATCAAACTTTTCCT TTGTGTGTGTATCGCTTGC		496
Protocol 3 and mix 3		58,8°C 35 sec	
speH	TGAGATATAATTGTCGCTACTCACAT CCTGAGCGGTTACTTTCGGT		480
speG	TGGAAGTCAATTAGCTTATGCAG GCGAACAACCTCAGAGGGCAAA		384
speJ	TCCTTGTACTAGATGAGGTTGCAT GGTGGGGTTACACCATCAGT		286
Protocol 4 and mix 4		57°C 40 sec	
ssa	AAGAATACTCGTTGTAGCATGTGT AATATTGCTCCAGGTGCGGG		678
speI	TTCATAGACGGCGTTCAACAA TGAAATCTAGAGGAGCGGCCA		176
mac	TCTTGCCCTGTTGAAAGTGT CGAGGTGGTATTTTTGACGCC		389
Protocol 5 and mix 5		53°C 40 sec	
speB	AGACGGAAGAAGCCGTCAGA TCAAAGCAGGTGCACGAAGC		952
spyCEP	GATCCGGCCCATCAAAGCAT AGCTGCCACTGATGTTGGTG		786
slo	GCCAATGTTTCAACAGCTATT CGGAGCTGCACTAAAGGC		400
Protocol 6 and mix 6		55 °C 45 sec	
speA	AGGTAGACTTCAATTTGGCTTGTGT GGGTGACCCTGTTACTCACG		576
speF	TACTTGGAT CAAGACG GTAATTAATGGTGTAGCC		782
sic	TTACGTTGCTGATGGTGTATATGGT TTGATAGAGGGTTTTCAGCTGGC		150

Table 2. Distribution of super-antigens and toxins in Bulgarian invasive and non-invasive clinical isolates of *Streptococcus pyogenes*.

Virulence genes in GAS in Bulgarian strains (n= 238)	Non-invasive strains carrying gene % (n=203)	Invasive strains carrying gene % (n=35)	*P – value**
speB, spyCep, slo, sdaB	100	100	1.000
mac	86	82	0.5957
smez	81	82	1.000
speJ	65	71	0.5647
speG	50	51	1.000
sdc	65	68	0.8475
sdaD	55	57	0.8561
spd3	51	54	0.8556
speA	20	45	0.0022
speF	54	77	0.0152
speL	16	40	0.0049
speM	18	42	0.0031
speK	28	31	0.6886
speI	26	25	1.000
speC	30	28	1.000
speH	2	6	0.1567
ssa	28	31	0.6886
sic	36	37	1.000

******P< 0.05* is statistically significant

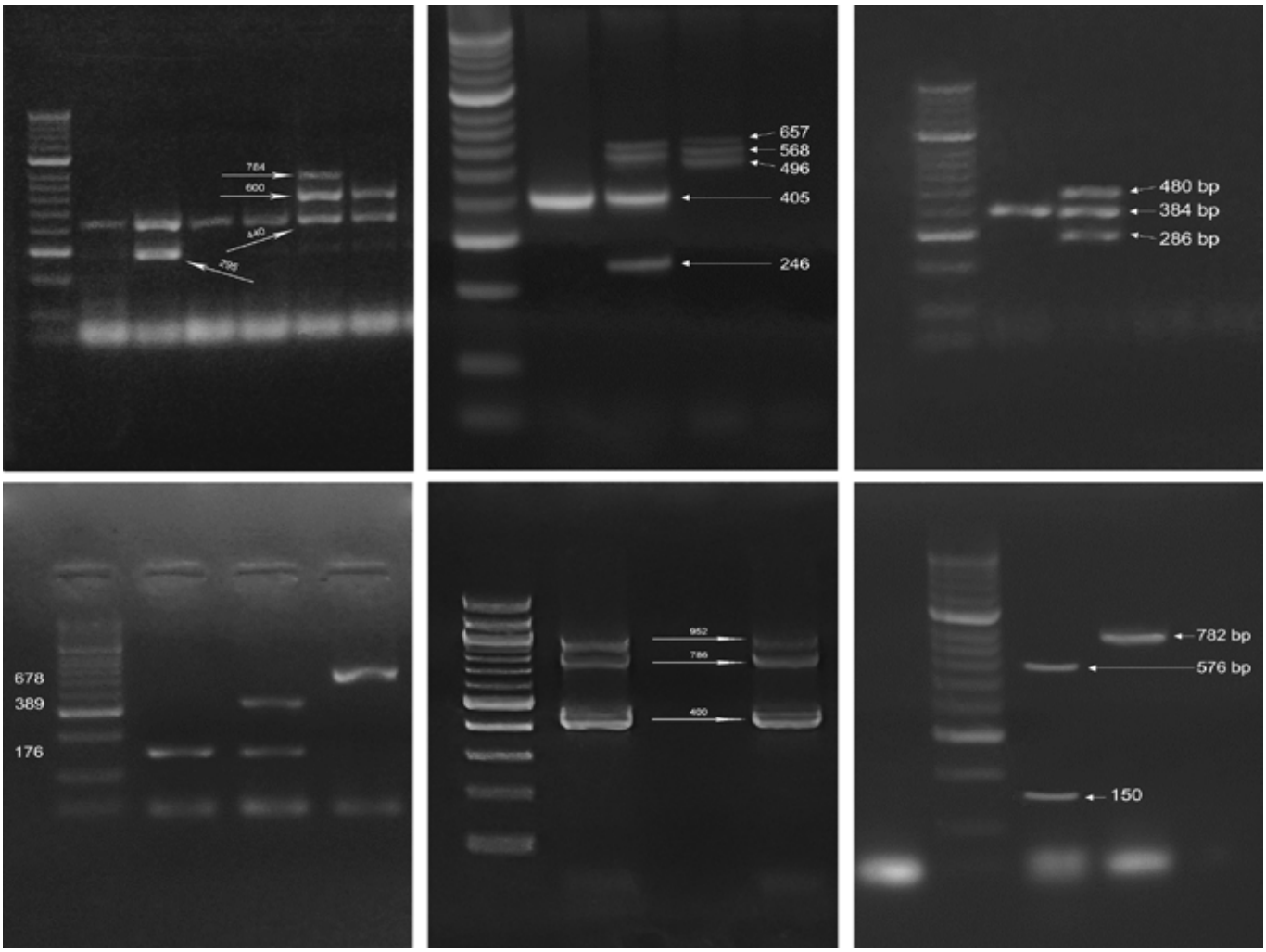

Fig. 1. Gel electrophoreses - amplicons of GAS genes encoded DNA-ses, SAgs and exotoxins.
(Mixes 1, 2, 3, 4, 5, 6)

Discussion

The results from this study revealed a trend towards a strong presentation of various genes encoding SAgs and toxins in Bulgarian GAS isolates (Table 2**)**. SAgs are a large family of heat-stable exotoxins and highly potent mitogens that share the ability to trigger excessive stimulation of human and other mammalian T lymphocytes. They are produced by a small number of bacterial species such as *S. pyogenes, S. dysgalactiae* (group C *Streptococcus*) and *S. equi* (group G streptococci), *Staphylococcus aureus* and some viruses (Proft and Fraser, 2016). These virulence factors play a major role in the development and pathogenesis of invasive infections (Hauser *et al*., 1991). In all tested Bulgarian GAS isolates from the last five years, *slo, speB, sypCEP* genes were detected*,* and also frequently the SAg gene *smeZ,* against which no neutralizing antibodies could be detected in the acute serum, but were found in convalescent serum (Yang *et al*., 2013). They are chromosomal genes and the first three may be used to identify GAS (Gergova *et al*., 2017) or as positive controls for PCR amplification (in this study). Both s*peB* and spyCEP are vaccine candidates. The s*peB* gene is involved in the post-translational regulation of the synthesis of virulence factors. *SpeB* encodes information for the synthesis of GAS proteins associated with mucosal colonization and biofilm formation in invasive phenotypes (Dmitriev *et al*., 2010). The *spyCEP* protease inhibits neutrophils by specifically cleaving IL-8 and other chemokines, one of the multifunctional host defense peptides and promotes resistance to neutrophil killing. This repression of phagocytic protection contributes to the higher virulence and plays a key role in the regulation of the proteolytic activity and pathogenesis of invasive soft tissue GAS infection by aiding systemic bacterial spread (Zinkernagel *et al*., 2008). In addition, all of the tested isolates had at least one (*sdaB*), in approximately 70% - two DNA-se genes and more than 50% - three or four ones. The production of multiple DNA-ses with various substrates is a survival advantage of these GAS strains in the host, which contributes to disease progression. The multiple DNA-ses genes, including chromosomally determined and prophage encoded variants, contribute to DNA-se activity and thus play an important role at various phases of the infection (Hasegawa *et al*., 2010). The invasion potential of the GAS strains tested in this study and in our previous work is higher than in many other countries (Gergova *et al*., 2015). Changes in the epidemiology of *S. pyogenes* have drawn the attention of researchers towards various virulence factors of these bacteria: pyrogenic exotoxins and streptococcal SAgs (Lamagni *et al*., 2005; 2008). GAS with the help of many SAgs and extracellular DNA-ses have the capacity to breach epithelial barriers and cause a variety of invasive diseases (Hasegawa *et al*., 2010; Proft and Fraser, 2016).

The studied GAS strains contained more than twelve genes, often close to nineteen, when streptococci were isolated from a patient with invasive infection ($p < 0.05$), than in the cases with non-invasive ones - usually with eight to ten of the examined 21 virulence elements. New data have indicated that the pathogenic properties of GAS strains are often linked to the production of a greater number of virulence factors and their cumulative effect may be the predictor of its of GAS strains invasiveness (Vlaminckx *et al*., 2003; Sumby *et al*., 2005; Golińska *et al*., 2016). The genes for important Sags, such as *speA, speF, speL, speM* (Table 2), were very frequently detected ($p < 0.05$) in Bulgarian isolates from punctures, aspirates, wounds, blood and cerebrospinal fluid, probably regarding the more invasive potential of the strains. The *speA* gene was found in a majority of *S. pyogenes* isolates from the USA, associated with invasive disease and STSS, but only in a minority of isolates from non-invasive diseases, similar to our results (Proft and Fraser, 2016). Some other virulence elements, such as *ssa,* were detected in Bulgarian GAS strains with different frequency according to the findings of other authors and did not confirm its significance at invasive streptococcal disease (Descheemaeker *et al*., 2000; Hasegawa *et al*., 2010). Results from our previous study on a smaller number of strains showed the other important SAg *speF* in 34.92% only (Gergova *et al*., 2015). After optimization of PCR and examination of new strains, we detected speF in about 70%, again predominantly in invasive isolates. The *speL* and *speM* genes were detected in a small number of tested isolates, 15% - 16% of non-invasive, and 28% - 29% of invasive ones, but were found together, suggesting their stable genetic linkage (Proft and Fraser, 2016).

Some other studies suggest a link between *emm* genotypes and combinations of SAgs. In a longer period the *spe* genotype can change profiles in different M type isolates. These results suggest that the distribution of *emm* genotypes is related to super-antigens, and are the first to show the profiles of *speG* and *speH* (Murakami *et al*., 2002) However, most SAg have not been characterized precisely,

and more information is needed to clarify the association between the clinical features and pathogenic roles of SAgs.

Conclusion

All of the examined strains presented the typical for GAS genes *slo sdaB*, *speB* and *sypCEP*; more than 80% genes *mac* and *smeZ*; followed by *sdc*, *spe F, speG*. and *speJ*. A large genetic diversity was found in the tested GAS strains, with a greater number of virulence determinants detected when their source was an invasive infection than from a non-invasive one ($p < 0.05$). The significant difference among invasive and non-invasive isolates was shown with the combination of SAgs: *speA, speF, speG, speL, speM,* which was the most commonly detected in link to invasive isolates.

Our results illustrated that the cumulative effect of a large number of genes, encoded SAgs, toxins and DNA-ses in Bulgarian GAS strains may be predictors of their possible invasiveness. This could hinder the treatment of the diseases due to GAS and must be related to the selection and the duration of their therapy.

Acknowledgments

This study was supported by a Grant from the Medical University of Sofia (Council of Medical Science, Project No 8263/2016, Grant No D-56/2017)

References

Borek, A., J. Wilemska, R. Izdebski, W. Hryniewicz, I. Sitkiewicz (2011). A new rapid and cost-e ective method for detection of phages, ICEs and virulence factors encoded by *Streptococcus pyogenes*. *Pol. J. Microbiol*. **60**: 187-201.

Borek, A., K. Obszańska, W. Hryniewicz, I. Sitkiewicz (2012). Detection of *Streptococcus pyogenes* virulence factors by multiplex PCR. *Virulence* **3**: 529-533.

Carapetis, J. R., A. C. Steer, E. K. Mulholland, M. Weber (2005). The global burden of group A streptococcal diseases. *Lancet Infect. Dis*. **5**: 685-694.

Cunningham, M. (2000). Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **13**: 470-511.

Descheemaeker, P., F. Van Loock, M. Hauchecorne, P. Vandamme, H. Goossens (2000). Molecular characterisation of group A streptococci from invasive and non-invasive disease episodes in Belgium during 1993–1994. *J. Med. Microbiol.* **49**: 467-471.

Gergova, R., A. Muhtarova, G. Petrova, I. Mitov (2018). Comparison of cultural, immunological and new PCR techniques for detection of *Streptococcus pyogenes*. *Cr. Acad. Bulg. Sci*. in press.

Gergova, R., A. Muhtarova, I. Mitov (2015). Virulence potential of Bulgarian *Streptococcus pyogenes* clinical isolates. *Cr. Acad. Bulg. Sci*. **68**: 1061-1070.

Golińska, E., M. van der Linden, G. Więcek, D. Mikołajczyk, A. Machul, A. Samet, M. Strus (2016). Virulence factors of *Streptococcus pyogenes* strains from women in peri-labor with invasive infections. *Eur. J. Clin. Microbiol. Infect. Dis*. **35**: 747-754.

Hasegawa, T., M. Minami, A. Okamoto, I. Tatsuno, M. Isaka, M. Ohta (2010). Characterization of a virulence-associated and cell-wall-located DNase of *Streptococcus pyogenes*. *Microbiology* **156**: 184-190.

Hauser, A., D. Stevens, E. Kaplan, P. Schlievert (1991). Molecular analysis of pyrogenic exotoxins from *Streptococcus pyogenes* isolates associated with toxic shock-like syndrome. *J. Clin. Microbiol*. **29**: 1562-1567.

Lamagni, T., A. Efstratiou, J. Vuopio-Varkila, A. Jasir, C. Schalen (2005). The epidemiology of severe *Streptococcus pyogenes* associated disease in Europe. Euro surveillance: bulletin Europeen sur les maladies transmissibles. *European communicable disease bulletin.* **10**: 179-184.

Lamagni, T., J. Darenberg, B. Luca-Harari, T. Siljander, A. Efstratiou, B. Henriques-Normark, M. Koliou (2008). Epidemiology of severe *Streptococcus pyogenes* disease in Europe. *J. Clin. Microbiol.* **46:** 2359-2367.

McMillan, D., M. Sanderson-Smith, P. Smeesters, K. Sriprakash (2012). Molecular markers for the study of streptococcal epidemiology. In: Chhatwal, G., (ed), Host-Pathogen Interactions in Streptococcal Diseases. *Current Topics in Microbiology and Immunology*. Springer, Berlin, pp. 29-48.

Murakami, J., S. Kawabata, Y. Terao, K. Kikuchi, K. Totsuka., A. Tamaru, S. Hamada (2002). Distribution of *emm* genotypes and super-antigen genes of *Streptococcus pyogenes* isolated in Japan, 1994–9. *Epidemiol. Infect.* 128: 397-404.

Proft, T., J. Fraser (2016). Streptococcal Superantigens: Biological properties and potential role in disease, In: Ferretti, J., D. Stevens, V. Fischetti, (eds). *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations [Internet]. Oklahoma City, pp. 1- 43.

Sumby, P., K. Barbian, D. Gardner, A. Whitney, D. Welty, R. Long, F. DeLeo (2005). Extracellular deoxyribonuclease made by group A *Streptococcus* assists pathogenesis by enhancing evasion of the innate immune response. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 1679-1684.

Vlaminckx, B., E. Mascini, J. Schellekens, L. Schouls, A. Paauw, A. Fluit, F. Schmitz (2003). Site-specific manifestations of invasive group A streptococcal disease: type distribution and corresponding patterns of virulence determinants. *J. Clin. Microbiol*. **41**: 4941-4949.

Yang, P., X. Peng, D. Zhang, S. Wu, Y. Liu, S. Cui, J. Li (2013). Characteristics of group A *Streptococcus* strains circulating during scarlet fever epidemic, Beijing, China, 2011. *Emerg. Infect. Dis.* **19**: 909.

Zinkernagel, A. S., A. M. Timmer, M. A. Pence (2008). The IL-8 Protease SpyCEP/ScpC of Group A Streptococcus promotes resistance to neutrophil killing. *Cell Host Microbe* **4:** 170–178.