

PHENOTYPIC AND GENOTYPIC ASSESSMENT OF VIRULENCE FACTORS IN BETA-HEMOLYTIC STREPTOCOCCI ISOLATED FROM KINDERGARTEN INFANTILE POPULATION, WITH OR WITHOUT CLINICAL SYMPTOMS, INCLUDING SCARLET FEVER

ION ANGHEL^{1*}, MARIANA CARMEN CHIFIRIUC^{2*}, MAGDA MITACHE³, LUMINITA MARUTESCU², ALINA GEORGIANA ANGHEL⁴, MARCELA POPA², DIANA PELINESCU², CORALIA BLEOTU⁵, VERONICA LAZAR²

¹University of Medicine Carol Davila Bucharest, Romania

²University of Bucharest, Faculty of Biology, Microbiology Immunology Department, Ale. Portocalelor 1-3, 60101 Bucharest, Romania

³Public Health Direction, Bucharest, Romania

⁴ENT Clinic, Coltea Hospital, Bucharest, Romania

⁵Institute of Virology, St. S. Nicolau, Bucharest, Romania

*corresponding authors : ionangheldoc@yahoo.com,

carmen_balotescu@yahoo.com

Abstract

To determine some biochemical and virulence hallmarks of beta-hemolytic streptococci isolates isolated from kindergarten infantile population, with or without clinical symptoms, including scarlet fever.

The isolated strains were identified by the help of conventional tests (sheep blood beta-haemolysis, bacitracin susceptibility, latex agglutination and API 20 Strep). Production of cell associated virulence factors (adhesins) and soluble enzymatic factors (haemolysins and other pore-forming toxins, proteases activity, DNA-ase and esculin hydrolysis) was assessed by inoculation of HeLa cells and respectively, specific enriched culture media containing different biochemical substrata, with bacterial inocula prepared from fresh cultures. The beta-haemolytic group A streptococci were investigated by PCR (polymerase chain reaction) for the streptococcal superantigenic toxin (*speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speJ*, *ssa*, *smeZ* and *speI*) gene profiles.

All tested strains exhibited the ability of adherence to HeLa cells (with adherence indexes ranging from 35 to 100% and a predominant diffuse-aggregative pattern). The soluble virulence factors implicated in the pathogenicity of the streptococcal strains were represented by beta-haemolysins, proteases and DNA-ses. The most frequent alleles detected in GAS (Group A streptococci) strains were the chromosomally located genes *speB* and *speF*, as well as *speG* and *ss*, while the other genes were randomly present among the analyzed strains. The *speA* allele was detected in one of the two GCS strains.

Conventional PCR techniques are important to identify streptococcal strains harboring SAg genes irrespectively of their gene expression status, these methods allowing the identification of SAg (superantigen) gene-less strains, that could be used to clarify the role of SAgs in *Streptococcus pyogenes* infection, which is still a matter of debate.

Rezumat

Obiectivul prezentului studiu a fost identificarea biochimică și determinarea unor markeri fenotipici și genotipici de virulență la tulpini de streptococi beta-hemolitici izolate de la populația infantilă din grădinițe, cu sau fără simptome clinice, inclusiv scarlatină.

Tulpinile izolate au fost identificate cu ajutorul testelor convenționale (beta-hemoliză, sensibilitate la bacitracină, latex aglutinare și API 20 Strep). Producerea de factori de virulență asociați peretelui celular (adezine) și factori solubili enzimatici (hemolizine și alte toxine formatoare de pori, proteaze, DN-aze și hidroliza esculinei) a fost evaluată după inocularea pe celule HeLa și, respectiv, pe medii specifice îmbogățite cu substrat biochimic incorporat. Streptococii beta-hemolitici de grup A au fost investigați prin PCR (*polymerase chain reaction*) pentru profilul genic al toxinelor superantigenice streptococice (*speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speJ*, *ssa*, *smeZ* și *speI*).

Toate tulpinile testate au prezentat capacitatea de aderare la celulele HeLa (cu indici de aderență variind de la 35 la 100% și un *pattern* predominant difuz –agregativ). Factorii de virulență solubili implicați în patogenitatea tulpinilor de streptococi au fost reprezentați de beta-hemolizine, proteaze și DNA-aze. Alelele cele mai frecvente codificatoare de superantigene au fost cele cromosomale *speB* și *speF*, precum și *speG* și *ssa*. Alela *speA* a fost detectată exclusiv la o tulpină de streptococ beta-hemolitic de grup C. Tehnicile convenționale bazate pe PCR sunt importante pentru identificarea profilului de gene codificatoare pentru superantigene streptococice, indiferent de expresia fenotipică a acestora, prin aceste metode putând fi evidențiate tulpini mutante pentru o anumită alela codificatoare a superantigenelor, utile pentru elucidarea rolului diferitelor superantigene prezente la streptococii de grup A în evoluția procesului infecțios.

Keywords: beta-haemolytic streptococci, virulence factors, streptococcal superantigenic toxins genes

Introduction

Group A streptococci (GAS, *Streptococcus pyogenes*) is one of the major human pathogens, giving rise to various suppurative complications of infection, e.g. acute throat and skin infections, as well as severe systemic disease such as cellulitis, puerperal sepsis, streptococcal toxic shock syndrome (STSS). In addition, GAS infections can give rise to nonsuppurative sequelae, such as acute rheumatic fever and acute glomerulonephritis [12]. Many extracellular virulence factors are also produced by GAS [5], including several superantigens (SAGs), such as streptococcal pyrogenic exotoxins. This Gram-positive bacterium produces a variety of exotoxins, known as streptococcal pyrogenic exotoxins (SPEs), which are believed to be involved in pathogenicity or virulence. These proteins are also known as superantigens (SAGs), due to their ability to stimulate large populations of T cells. In contrast to conventional Ags, SAGs are not processed inside APCs (antigen-presenting cells), but instead directly bind to the MHC (major histocompatibility complex) class II

protein outside the Ag binding groove. Simultaneously, they bind to all TCRs (T-cells receptors) bearing particular Vb (variable region of the *b* chain) regions. This trimolecular complex subsequently cross-links a large number of APCs and T cells resulting in the production of high systemic levels of the cytokines TNF- α and IL-1 β and of T cell mediators, such as IL-2 and IFN- α (interferon alpha) [6]. Presently, 11 SAgS from *Streptococcus pyogenes* are described: SPEA, SPEC, SPEG, SPEH, SPEI, SPEJ, SPEK, SPEL, SPEM, SSA, and SMEZ [3, 4, 9, 10, 13, 15-19, 23]. Five alleles have been reported for speA [4], including the alleles 1–3 and 5, which are very homologous with only one base pair difference, and allele 4 showing 26 base pair exchanges. Similarly, smeZ consists of more than 20 different alleles [18].

Most SAgS are encoded on phages or plasmids (Ekelund et al., 2005). However, Proft [15,19] described that speG and speJ are genomic because they found that these SAg genes were present in all *Streptococcus pyogenes* tested strains, while smeZ is a frequently detected SAg gene found in more than 90% of *Streptococcus pyogenes* strains [18]. The rarest SAg genes are speL (15%) and speM (5%) [15,19]. The aim of the study was to determine, using a complex phenotypic and genotypic approach, some biochemical and virulence hallmarks of beta-hemolytic streptococci isolates isolated from kindergarten infantile population, with or without clinical symptoms, including scarlet fever.

Materials and Methods

Bacterial strains

Samples were collected from epidemiological triage after vacations from kindergarten and school infantile population. Usually three screenings per year were performed, in autumn, winter and spring. In 2009, 2210 samples (15 being positive for GAS and 5 for nonA hemolytic streptococci) were collected containing isolated from healthy children, while 1300 (with 29 GAS positive cases) from scarlet fever contacts. We have selected 10 GAS and two GCS (group G streptococci) strains for further studies.

Biochemical and serological identification

The isolated GAS and GCS strains were identified by the help of conventional tests (beta-haemolysis production, bacitracin susceptibility, latex agglutination and API 20 Strep, following the manufacturer instructions).

Expression of virulence factors

Adherence to HeLa cells assay and biofilm development on inert substrata

Cell adherence assay was performed using Cravioto's adapted method [20,21]. The HeLa cell monolayers were washed 3 times with PBS (phosphate buffer saline) and 1 mL of fresh medium without antibiotics was added to each well. Suspension of enterobacterial strains from bacterial mid-logarithmic phase cultures grown in nutrient broth was adjusted to 10^8 cells/mL and 1 mL was used for the inoculation of each well. The inoculated plates were incubated for 2 hours at 37°C. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold methanol (3 min), stained with Giemsa solution (1:10) for 20 min. The plates were examined microscopically to evaluate the adherence index and patterns. The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria and 100 eukaryotic cells counted on the microscopic field.

Expression of soluble enzymatic factors

Bacterial strains were evaluated for the following virulence factors expression [2,14,22]: haemolysins, other pore forming toxins (lecithinase, lipase), proteases (caseinase, gelatinase), DNA-ase and mucinase. An enriched agar base medium was used for preparing the media for soluble virulence factors, in order to enable the growth of streptococci. Detection of haemolysin production was performed by spotting the fresh cultures on 5% sheep blood agar medium and incubation at 37°C for 24 h. The colorless area around the culture revealed the presence of haemolysis activity. Investigation of lipase production the strains were spotted on 1 % Tween 80 agar as a substrate and were followed by incubation at 37°C for 72 h. An opaque (precipitation) zone around the spot was registered as positive reaction; for lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37°C for 72 h. A clear zone around the spot indicated the lecithinase production; the DNA-ase production was studied on DNA supplemented agar with blue toluidin. A zone colored in pink around the culture was interpreted as positive reaction; The caseinase activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37°C for 72 h, a white precipitate surrounding the growth indicating casein proteolysis. The amylolytic activity was assessed on agar medium supplemented with starch for 72 h; the enzyme activity was detected by the presence of a clear area around the culture spot; the clear area became more evident when some Lugol drops

were poured upon. For production of gelatinase the strains were spotted in the solid medium and incubated with gelatin 72h at 37°C. The presence of a precipitation area around the culture growth indicated proteolysis of gelatin by an active gelatinase.

Molecular detection of SAg genes

For genomic DNA isolation, an overnight culture was prepared and DNA was extracted by using Wizard DNA Genomic Purification kit (Promega, U.S.), according to the manufacturer's recommendations. Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in an Applied Biosystems 2700 Thermal Cycler. Toxin gene detection was performed using two multiplex PCR. The following streptococcal superantigenic toxin genes were screened: *speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speJ*, *ssA*, *smeZ* and *speI*. The composition of PCR reactions and the amplification conditions were those described by Luca-Harari [12].

Results and Discussion

Identification of streptococcal beta-haemolytic strains

The GAS and GCS strains were confirmed by the agglutination tests along with biochemical assays.

Expression of virulence factors

Streptococcus pyogenes is one of the most frequent pathogens of humans. It is estimated that between 5-15% of normal individuals harbor the bacterium, usually in the respiratory tract, without signs of disease. When the bacteria are introduced or transmitted to vulnerable tissues, a variety of types of suppurative infections can occur. *Streptococcus pyogenes* owes its major success as a pathogen to its ability to colonize and rapidly multiply and spread in its host while evading phagocytosis and confusing the immune system.

The first step in the establishment of infection is the attachment to host cells. GAS express a large number of adhesion proteins, generally called adhesins, that can bind to host cells and extracellular matrix. Many of the adhesins are M or M-like proteins. GAS also express pili, which mediates attachment to tonsils and skin. Studies of colonization of skin revealed that the M-protein mediate the binding to membrane cofactor CD46 on keratinocytes, while protein F mediates the binding to Langerhans cells in epithelia. In our study, all tested strains exhibited the ability to adhere to HeLa cells, with adherence indexes ranging from 35 to 100% and a predominant diffuse-aggregative pattern (Fig. 1).

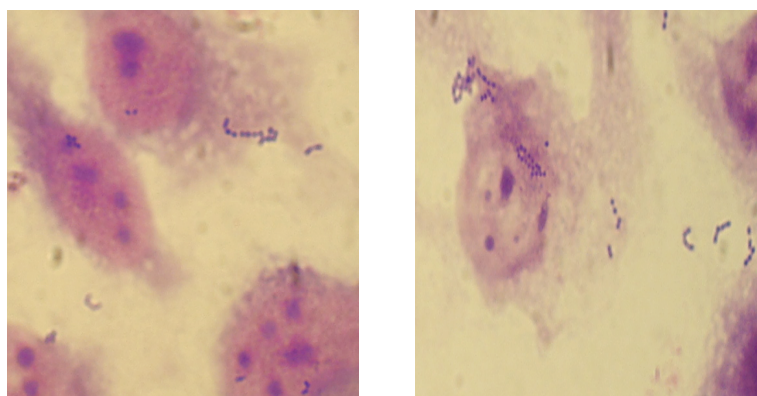


Figure 1

Giemsa stained wells, showing a diffuse adherence pattern (left) and an aggregative one (right)

The destructive nature of wound infections prompted the popular press to refer to *S. pyogenes* as "flesh-eating bacteria" and "skin-eating streptococci". The invasive infections are mediated by an impressive number of soluble virulence factors, represented both by extracellular growth products as well as by toxins. In our study, all tested strains expressed soluble virulence factors implicated in the pathogenicity represented by beta haemolysins, proteases and DNA-ase.

Streptolysins lyse eukaryotic cells, including red blood cells and phagocytes.

GAS strains produce several proteases that are important for virulence. IdeD and SpeB have been shown to degrade immunoglobulins by inhibiting opsonisation. Another recently described protease, streptococcal chemokine protease C, have been shown to degrade the chemokine IL-8 and consequently impair recruitment neutrophils into the infected tissue.

Initially four DNA-ases were described and named DNA-ase A-D, but lately two new DNA-ses have been characterized Streptococcal DNA-ase 1 (Sda 1) and streptococcal DNA-ase α (sd α). DNases have been suggested to lichefy pus, allowing bacteria to spread among tissues, to degrade DNA from lysed bacteria, and to degrade neutrophil extracellular traps. The newly described GAS DNA-ase Sda 1 is both necessary and sufficient for neutrophil extracellular trap degradation and neutrophil resistance.

Toxin gene detection

Superantigens are a family of microbial proteins with the ability to induce massive inflammatory responses. This is achieved by circumventing the normal rules for antigen processing and presentation. Superantigens interact without prior cellular processing with the MHC class II molecules on antigen presenting cells binding outside of the antigen-binding cleft. They also interact with V β -regions of the $\alpha\beta$ heterodimeric T-cell receptor and each superantigen activates T cells in a V β -specific manner, i.e. only T cells bearing certain V β will be activated. This activates the interacting cells to proliferate and to produce large amounts of cytokines. There are about 20-50 different V β regions recognized in humans and in mice, hence one superantigen can activate 2-20% of the resting T-cell population, as compared to approximately 0.1% for a conventional antigen. The activation phase may be followed by apoptosis of most of the expanded T-cell clone, leaving a few surviving anergized (non-responsive) cells [11].

GAS produces several potent superantigens, including SpeA-M, the streptococcal superantigen (SSA) and the streptococcal mitogenic exotoxin SmeZ. The number of superantigens that have been described in GAS points to an important role in the pathogenesis, and it has been suggested that a dysregulated immune response is beneficial for the bacteria. *speB* and *speF* are not SAg genes. *speB* is a streptococcal extracellular cysteine proteinase [25] and *speF* is identical to streptococcal DNA-ase B [8]. DNA-ase B was renamed to mitogenic factor in 1993 due to its mitogenic activity, but was shortly thereafter named Streptococcal pyrogenic exotoxin F (SpeF) due to its induction of pyrogenic cytokines. DNases possess deoxyribonuclease as well as ribonuclease and allow the bacteria to spread among tissues by increasing the secretions fluidity.

In this study, the most frequent alleles detected in GAS strains were the chromosomally located genes *speF*, *speG* and *ssa* (83.33% isolated streptococcal strains), followed by *speB* (50%), *speC* (3.33%) and *speH*, *speI*, *smeZ* (1.66%), (Fig. 2, 3 and 4).

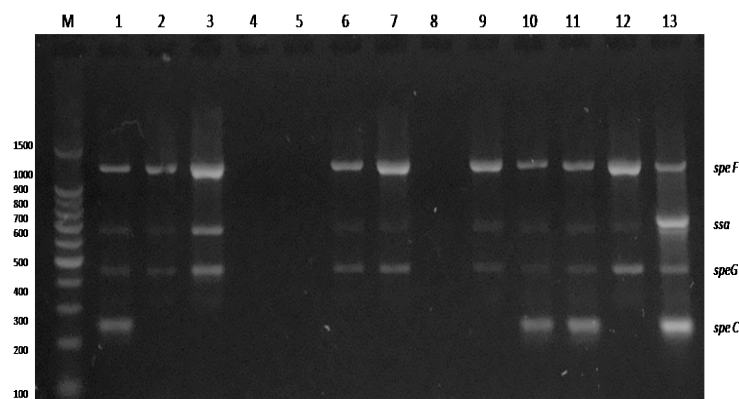


Figure 2
SAs allelic pattern evidenced by multiplex PCR followed by agarose electrophoresis

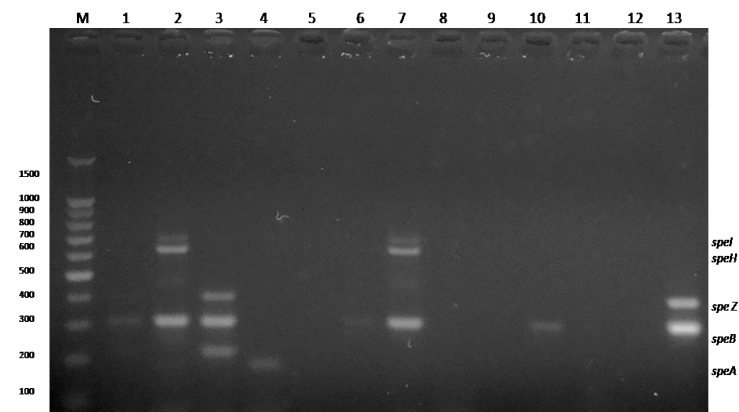


Figure 3
SAs allelic pattern evidenced by multiplex PCR followed by agarose electrophoresis

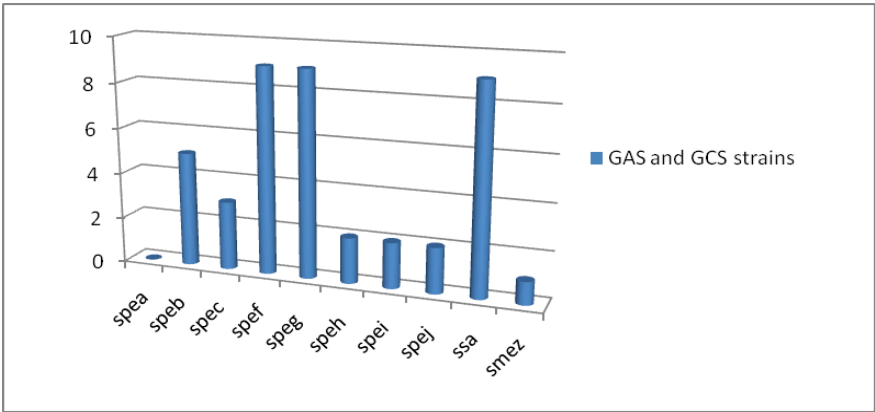


Figure 4
Distribution of different SAs alleles among the tested strains

The *spea* allele was detected in one of the two GCS strains. Two streptococcal strains, one GAS strain and one GCS strain didn't express any of the toxin genes investigated. Also the genetic analysis showed that the SAg gene *spej* and *spea* were not present in the case of the tested strains. In the case of GCS strains we did not find any SAg genes. Notwithstanding the obvious deleterious role of superantigens in toxic shock, the evolutionary advantage conferred by these toxins remains a subject of speculation.

Conclusions

Conventional PCR techniques are important to identify streptococcal strains harboring SAg genes irrespectively of their gene expression status, these methods allowing the identification of SAg gene-less strains, that could be used to clarify the role of SAgs in *Streptococcus pyogenes* infection, which is still a matter of debate.

Acknowledgements

This research was financed by the Human Resources Project no. 135/2010 Contract no. 76/2010).

References

1. Andries A., Cioaca D., Gutiu IA, Oprea I, Radulescu F, Georgescu A, Detection of CYP2D6*6 allele by real time polymerase chain reaction in Romanian population, *Farmacia*, 2010, 58(3), 353-361.
2. Banu O., Bleotu C., Chifiriuc M.C., Savu B., Stanciu G., Antal C., Alexandrescu M., Lazăr V., Virulence factors of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains involved in the etiology of cardiovascular infections, *Biointerface Res. in App. Chem.*, 2011, 1:72-77.
3. Beres, S. B., Sylva, G. L., Barbian, K. D., Lei, B., Hoff, J. S., Mammarella, N. D., Liu, M. Y., Smoot, J. C., Porcella, S. F. & other authors, Genome sequence of a serotype M3 strain of group A streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc Natl Acad Sci U S A* 99, 2002, 10078-10083.
4. Bessen, D. E., Izzo, M. W., Fiorentino, T. R., Caringal, R. M., Hollingshead, S. K. & Beall, B., Genetic linkage of exotoxin alleles and *emm* gene markers for tissue tropism in group A streptococci. *J Infect Dis* 1999, 179, 627-636.
5. Bisno AL, et al., Molecular basis of group A streptococcal virulence, *Lancet Infect. Dis.* 2003, 3(4):191-200.
6. Darenberg J., Luca-Harari B., Jasir A., Sandgren A., Pettersson H., et. al., Molecular and Clinical Characteristics of Invasive Group A Streptococcal Infection in Sweden. *Clinical Infectious Diseases*, 2007, 45:450-8
7. Ekelund, K., Darenberg, J., Norrby-Teglund, A., Hoffmann, S., Bang, D., Skinhoj, P., Konradsen, H.B., Variations in *emm* type among group A streptococcal isolates causing invasive or noninvasive infections in a nationwide study. *J Clin Microbiol* 2005, 43, 3101-3109.

8. Eriksson A., Erikson B., Holm, S.E., Norgren M., Streptococcal DNase B is immunologically identical to superantigen SpeF but involves separate domains. *Clin. Diagn. Lab. Immunol.* 1999, 6, 133-136.
9. Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N. & other authors, Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A* 98, 2001, 4658-4663
10. Igwe, E. I., Shewmaker, P. L., Facklam, R. R., Farley, M. M., van Beneden, C. & Beall, B., Identification of superantigen genes *speM*, *ssa*, and *smeZ* in invasive strains of β -hemolytic group C and G streptococci recovered from humans. *FEMS Microbiol Lett* 2003, 229, 259-264.
11. Lavoie, P.M. et al., Quantitative relationship between MHC class II superantigen complexes and the balance between T cell activation versus death. *J. Immunol.*, 2001, 166 (12): 7229-37.
12. Luca-Harari B, Straut M., Cretoiu S., Surdeanu M., Ungureanu V., van der Linden M. and Jasir A., Molecular characterization of invasive and noninvasive *Streptococcus pyogenes* isolates from Romania. *Journal of Medical Microbiology*, 2008, 57, 1354-1363.
13. McCormick, J. K., Yarwood, J. M. & Schlievert, P. M., Toxic shock syndrome and bacterial superantigens: an update. *Annu Rev Microbiol*, 2001, 55, 77-104.
14. Panus E., Chifiriuc M.C., Banu O., Mitache M., Bleotu C., Rosoiu N., Lazăr V., Comparative study of resistance and virulence markers in *Escherichia coli* strains isolated from hospital surfaces, clinical specimens and drinking/ marine waters, *Biointerface Res. in App. Chem.*, 2011, 1:24-30.
15. Proft, T., Superantigens and streptococcal toxic shock syndrome. *Emerg Infect Dis* 2003, 9, 1211-1218.
16. Proft, T., Arcus, V. L., Handley, V., Baker, E. N., Fraser, J. D., Immunological and biochemical characterization of streptococcal pyrogenic exotoxins I and J (SPE-I and SPE-J) from *Streptococcus pyogenes*. *J Immunol* 2001, 166, 6711-6719.
17. Proft, T., Moffatt, S. L., Berkahn, C. J., Fraser, J. D., Identification and characterization of novel superantigens from *Streptococcus pyogenes*. *J Exp Med* 1999, 189, 89-102.
18. Proft, T., Moffatt, S. L., Weller, K. D., Paterson, A., Martin, D., Fraser, J.D., The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. *J Exp Med* 2000, 191, 1765-1776.
19. Proft, T., Webb, P. D., Handley, V. & Fraser, J. D., Two novel superantigens found in both group A and group C *Streptococcus*. *Infect Immun*, 2003, 71, 1361-1369.
20. Saviuc C, Grumezescu A.M., Holban A., Bleotu C., Chifiriuc C., Balaure P., Lazar V., Phenotypical studies for raw and nanosystem embedded *Eugenia carryophyllata* buds essential oil effect on *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains, *Biointerface Res. in App. Chem.*, 2011, 1:111-118.
21. Saviuc C., Grumezescu A., Chifiriuc M.C., Bleotu C., Stanciu G., Hristu R., Mihaiescu D., Lazăr V., *In vitro methods* for the study of microbial biofilms, *Biointerface Res. in App. Chem.*, 2011, 1:31-35.
22. Saviuc C., Grumezescu A.M., Oprea E., Radulescu V., Dascalu L., Chifiriuc M.C., Bucur M., Banu O., Lazar V., Antifungal activity of some vegetal extracts on *Candida* biofilms developed on inert substratum, *Biointerface Res. in App. Chem.*, 2011, 1:15-23.
23. Smoot, L. M., McCormick, J. K., Smoot, J. C., Hoe, N. P., Strickland, I., Cole, R. L., Barbican, K. D., Earhart, C. A., Ohlendorf, D. H. et al. Characterization of two novel pyrogenic toxin superantigens made by an acute rheumatic fever clone of *Streptococcus pyogenes* associated with multiple disease outbreaks. *Infect Immun* 2002, 70, 7095-7104.

24. Sriskandan S, Faulkner L, Hopkins P. Streptococcus pyogenes: Insight into the function of the streptococcal superantigens. *Int J Biochem Cell Biol*, 2007, 39(1):12-9.
25. Tamura F., Nakagawa R., Akuta T., Okamoto S., Hamada S., Maeda H., Kawabata S., Akaike T., Proapoptotic effect of proteolytic activation of matrix metalloproteinases by Streptococcus pyogenes thiol proteinase (Streptococcus pyrogenic exotoxin B), *Infect. Immun*, 2004, 72, 4836-4847.

Manuscript received: July 22nd 2011