Frequency and phenotypic virulence features of *Streptococcus pyogenes* strains isolated from throat carriage in Romanian population

**Abstract**

*Streptococcus pyogenes* is a Gram-positive bacterial pathogen responsible for a wide variety of human diseases. The aim of this study was to investigate the rate of *Str. pyogenes* infections and to determine the virulence patterns in isolates obtained from patients diagnosed in the SYNEVO Laboratory in Bucharest, during December 2012 - April 2013. **Material and methods.** The strains were isolated from throat swabs, identification was performed by conventional tests and by using a Bruker Maldi-Tof analyzer. The bacterial adherence to the cellular substratum was assessed by using the adapted Cravioto’s method. The expression of soluble virulence factors was assessed by inoculating the *Str. pyogenes* strains on enriched culture media. **Results.** From the total of the 10691 individuals, 560 (5.14%), were found to be *Str. pyogenes* carriers. All tested strains adhered to the HeLa cells exhibiting different adherence patterns. At least one of the investigated soluble virulence factors with DN-ase being the most prevalent (77.1%) was produced by the streptococcal studied strains. **Conclusions.** The *Str. pyogenes* strains isolated from throat carriage in Romanian population exhibited a high adherence capacity to epithelial cells and expressed at least one of the investigated soluble virulence factors, suggesting that *Str. pyogenes* possess a high variety of pathogenic features that can be correlated with the high frequency, diverse location and severity of the produced infections.

**Keywords:** *Str. pyogenes*, virulence factors, infections, phenotypic, adherence, Maldi-Tof

**1. Introduction**

*Str. pyogenes* is a chain-forming Gram-positive bacterial pathogen responsible for a wide variety of human diseases (E. Lappin, AJ Ferguson [1]). It is the most common bacterial cause of acute pharyngitis, accounting for 15-30% of cases in children and 5-10% of cases in adults (K. Kasper [2]; N. Karaky & al. [3]). During the winter and spring in temperate climates, up to 20% of asymptomatic school-aged children may carry *Str. pyogenes* (D. Buiuc, M. Neguț [4]). Clinical manifestations range from superficial, common infections, such as pharyngitis (strep throat) (S. Tokajian [5]) and impetigo, to life-threatening invasive conditions, such as
necrotizing fasciitis (flesh-eating disease) and toxic shock syndrome, or sepsis (TL. Lamagni & al. [6]; M. Cunningham [7]). It has been estimated that over 18 million people worldwide currently suffer from serious invasive Str. pyogenes infections, with almost 2 million new cases each year (J.R. Carapetis & al. [8]; Y. Ma & al. [9]). Over half a million deaths annually can be attributed to severe Str. pyogenes infections, highlighting the need for a deeper understanding of Str. pyogenes infection (TN. Cao & al. [10]; DA. Watkins & al. [11]).

The Strep-EURO program was conducted in 11 countries, including for the first time Romania, on a number of 5522 Str. pyogenes infections diagnosed during 2003 and 2004. In the southern countries a lower incidence of streptococcal infections was observed with a rate of 0.3 infections per 100,000 population in comparison to the northern countries which had a 2.3 percentage per 100,000 population. The low incidence is partly attributed to the poor investigative methods used (TL. Lamagni & al. [6]). The morbidity related to severe Str. pyogenes infections in all 11 countries was highlighted in this study with one in five patients dying within 7 days of infection diagnosis (JL. Danger & al., [12]; J. Tsatsaronis & al. [13]). A study conducted in Galaty County between 1990-2000 on the incidence of Str. pyogenes infections, revealed that the yearly morbidity associated with scarlet fever was between 8.2 to 54.1 cases/100,000 inhabitants (A. Chirita & al. [18]; EJ. McDowell & al. [19]).

Post-infective sequelae including rheumatic heart disease are serious consequences of Str. pyogenes infections (RJ. Commons [14]; MJ. Walker & al. [15]). The pathogenic potential of Str. pyogenes is mediated by its ability to colonize and disseminate in the infected host, while avoiding the effectors of the immune system (Y. Terao [16]; A. Timmer [17]).

The objective of the present study was to establish the percentage of Str. pyogenes infections in the studied population represented by patients investigated in Synove laboratory and to investigate some of the phenotypic features implicated in the Str. pyogenes virulence.

2. Materials and Methods

**Bacterial strains**

Str. pyogenes strains have been isolated following the analysis of 10691 throat swabs during December 2012 - April 2013, from patients diagnosed in Bucharest SYNEVO Laboratory.

**Identification of Str. pyogenes strains**

The pharyngeal swabs were cultivated on Columbia Agar medium supplemented with 5% sheep blood. The Str. pyogenes strains were identified with the use of conventional tests: beta-haemolysis production, latex agglutination (using the Pastorex Strep latex agglutination kit produced by Biorad following the manufacturer instructions) and by the Bruker Maldi-Tof analyzer.

**Bruker Maldi-Tof identification**

Maldi-Tof uses mass spectrometry allowing the identification of microorganisms by the analysis of unique protein structures. The bacterial culture of 24h is mixed with a matrix solution provided by the manufacturer and applied to a coded plate. The sample is irradiated by a laser beam that induces desorption and ionization, after which the resulting molecules pass through a tube at which point, the separation of the molecules occurs depending on their individual mass. The main advantage of this method is that it measures the mass of the molecules at a very high speed making it a very specific and rapid method (J. Ragoussis & al. [20]).

**Adherence to HeLa cells**

The study of Str. pyogenes strains adherence to the mammalian cells was performed using the Cravioto’s adapted method (V. Lazar [21], A. Holban & al. [23], [24]). In this purpose 1 mL
of bacterial suspension with a turbidity of 0.5 McFarland was added over the cell monolayers (HeLa cell line), after the removal of the growth medium enriched with antibiotics and three steps of washing with medium without antibiotic. After incubation at 37°C for 2 h, during which the bacteria adhered to the cell substratum, the cell monolayers were washed four times with PBS (phosphate buffered saline), fixed with methanol for 5 minutes, followed by staining with Giemsa solution. After staining, the plates were washed with tap water, dried at room temperature and examined in optic microscopy with immersion oil objective.

The study of soluble virulence factors expression

The phenotypic expression of virulence factors was highlighted using 7 different substrata for the following enzymes: amylase, esculinase, caseinase, gelatinase, DNase, lipase and lecithinase.

Amylase production

The presence of amylase was detected using agar medium enriched with 1% starch. The strains were spotted in the medium. After incubation at 37°C for 72 h, a HCl or Lugol solution was poured on the medium in order to make the results more evident. In the case of the HCl solution the presence of a clear area around the culture spot was considered to be a positive result and in the case of the Lugol solution the presence of a yellow ring around the culture spot meant a positive result (V. Lazar & al. [22]; A. Holban & al. [23],[24]).

Esculin hydrolysis

Esculin is hydrolyzed to glucose + esculitol. In the presence of iron citrate (FeC₆H₅O₇) (Fe³⁺), the released esculotol in contact with beta-galactosidase generates a black precipitate of ferric esculentin. The strains were spotted with a wire loop in the medium enriched with 1% esculin and iron citrate and distributed in haemolysis tubes. After incubation at 37°C, for 72 h, the hydrolysis of esculin determined the formation of esculotol that combined with iron salts from the medium (1% ammonium ferric citrate) caused the darkening of the medium around the positive strain culture spot (V. Lazar & al. [22]; A. Holban & al. [23],[24]).

Caseinase production

The caseinase activity was determined by spotting the strains in the agar medium supplemented with 15% casein. After incubation at 37°C for 72 h, around the positive strains cultures spots a white precipitation area could be seen due to the formation of calcium para-caseinate (V. Lazar & al. [22]; A. Holban & al. [23], [24]).

Gelatinase production

The strains were seeded on agar enriched with gelatin and incubated for 48 h at 37°C. The gelatinase is a mixture of proteolytic proteins which are highlighted through this test. The presence of a precipitation zone around the growing area indicates the presence of gelatinase (V. Lazar & al. [22]; A. Holban & al. [23], [24]).

DN-ase production

The DN-ase degrades bacterial DNA releasing mono- or dinucleotides. The strains were spotted in the medium supplemented with DNA and incubated at 37°C for 72 h. After incubation, the plates were flooded with 1N HCl solution, which precipitated undegraded DNA causing the opacification of the medium, the only exceptions being the areas around the positive strains culture spots, which remained transparent due to the degradation of the DNA, with the release of mononucleotides (V. Lazar & al. [22]; A. Holban & al. [23], [24]).

Lipase production

The bacterial strains were spotted in the agar medium enriched with 1% Tween 80 and incubated for 72 hours at 37°C. The presence of a precipitation area around the strain, after the appearance of crystals formed from the fatty acids and Ca²⁺, was considered a positive reaction (V. Lazar & al. [22]; A. Holban & al. [23], [24]).
The production of lecithinase was studied on agar medium supplemented with yolk (2.5%) where the strains were spotted and incubated at 37°C for 72 hours. The presence of a precipitation zone around the strain was considered a positive reaction (production of lecithinase) (V. Lazar & al. [22]; A. Holban & al. [23], [24]).

3 Results
In the present study, from the 10691 samples analyzed during a period of 5 months, from patients investigated in the Synevo Laboratory from Bucharest between December 2012 and April 2013, 5.14% were found to be positive for *Str. pyogenes* growth. In our study, from the total of the 10691 individuals, 550 (5.14%), out of which 304 boys and 246 girls were found to be *Str. pyogenes* carriers. The highest frequency of *Str. pyogenes* carriage was registered both in boys and girls aged from 3 to 7 years. The distribution of streptococcal infections in the studied population on the basis of sex and age is shown in Fig. 1. It is to be noticed that in the age groups of 3-7 and 7-19 years, the carriage of *Str. pyogenes* predominated in male population, while after 19 years the frequency of *Str. pyogenes* infections was significantly higher in females (Fig. 1).

![Fig. 1. Distribution of *Str. pyogenes* infections on the basis of sex and age.](image)

The diverse pathogenicity of *Str. pyogenes*, varying from asymptomatic carriage to the most severe forms of invasive infection is the results of a complex interaction between the bacterial virulence factors and the human host (L.R. Marks & al., 2014 [25]; K. Sjöholm & al., 2014 [26]; P. Bidet and S. Bonacorsi, 2014 [27]). A number of 114 strains were analyzed for the expression of cell-associated and soluble virulence factors in order to establish the virulence profiles of *Str. pyogenes* infections.

In our study the capacity of *Str. pyogenes* strains to adhere to the cellular substratum was investigated in vitro using HeLa cells. Three types of adhesion patterns have been revealed, i.e. i) localized adhesion - microcolonies are formed on the surface of the host cell; ii) aggregative adhesion - cells adhere to the host cell surface and between them in large aggregates; iii) diffuse adhesion –the single bacterial cells adhere to the surface of the host cell. Most of the analyzed strains presented the aggregative adhesion pattern (79%), followed by those with diffuse (13%) and localized pattern (8%) (Fig. 2).
The expression of the following soluble virulence factors was evaluated: amylase, lecithinase, lipase, caseinase, gelatinase, esculin hydrolysis and DN-ase.

A great percentage of the isolates expressed concomitantly four soluble virulence markers. All strains expressed at least one of the tested virulence factors, the most prevalent being DN-ase (77.1%), caseinase (71.9%), esculin hydrolysis (62.2%) and amylase (55.2%) (Fig. 3).

4. Discussion

The spontaneous nature of the Str. pyogenes infections limits the possibility of prevention, the transmission occurring mainly in overcrowded communities. Approximately 15% of schoolchildren and 4 to 10% of adults may suffer an episode of Str. pyogenes pharyngitis each year in developed countries whereas incidence rates in developing countries are 5 to 10 times higher (A.K. Shrestha & al., 2013 [28]). An overall rate of Str. pyogenes infections in Europe was determined in the Strep-Euro program to be 2.79 cases / 100,000 individuals. The program identified a north-south incidence gradient regarding these infections which evolves for high to low. Following this gradient we Finland, Denmark, Sweden and United Kingdom have a combined incidence rate of 3.14 cases / 100,000 individuals, while in Central European countries, the rate is 1.48 infections / 100,000 individuals. In this study, Romania exhibited a similar incidence rate (0.36) more similar to those observed in Italy (0.40) and Cyprus (0.30) (TL. Lamagni & al. [6]). The incidence rate resulted from our study was of 0.48, which is higher than that reported by the Strep-Euro
program, as expected, taking into account that only severe infections accompanied by the clinical signs of STSS have been selected (R. Breiman & al. [29]).

The frequency of Str. pyogenes throat carriage in the studied Romanian population was 5.14% and predominated in the male population, in the age group of 3-7 years. Similar results were obtained in studies conducted in other countries as shown by the Strep-EURO surveillance program which included positive cases from 11 countries, where 53% of the infected individuals were males with most countries showing a slightly higher number of cases affecting the male population in comparison to women. Regarding the age of the individuals included in this European study, in the case of Romania 42% of the study population was represented by children (0-17 years). At the European level 14% of these infections affect children, with a higher proportion of cases reported in children but the most severe infections were reported in the elderly population. In our study, an incidence of 80.7% was determined in young patients (0-19 years). Our results regarding the age criteria which showed a higher incidence in the age group under 19, also similar with other studies (L. Olafsdottir & al. [30]).

A rich repertoire of virulence mechanisms are involved in the Str. pyogenes infectious process. The development of Str. pyogenes infections is influenced by age, underlying disease, such as diabetes, and acute or chronic skin lesions (M. Walker et al., 2014 [31]). It is thought there are three stages in the pathogenesis of Str. pyogenes pharyngitis: i) adherence to the host pharyngeal epithelial tissue, often followed by invasion into and persistence in host epithelial cells, ii) nutrient acquisition to enable proliferation in the host, and iii) evasion of the host immune response (R.J. Olsen & al., 2009 [32]; A. Henningham & al., 2012 [33]).

The ability of bacterial pathogens to adhere to the host cells is mediated by structures located on their surface, generically called adhesins. The adhesion phenomenon has been studied in a large number of pathogenic bacteria, because their adherence to host cells represents the initial stage and a mandatory precondition for the infectious process. Adherence involves the close proximity of two cell types, which allows complementary interaction at specific sites on their surface. The ability of the pathogen to adhere to the host cell surface is a first stage of the microbial colonization and invasion, resulting in the development of a variety of microbial structures that mediate adhesion to host tissues as a consequence to the co-evolution of host-parasite complexes (V. Lazar, 2003 [21]).  

All of the studied strains adhered to the HeLa cells, exhibiting mostly an aggregative adhesion pattern suggesting the capacity of Str. pyogenes to colonize specific sites, in particular the naso-pharyngeal pathway. After colonization the bacterial pathogen can spread to other tissues. Studies on this subject suggest that the most virulent aspect of Str. pyogenes strains is the adherence to epithelial host cells.

Invasive infections produced by Str. pyogenes are mediated by an impressive number of soluble virulence factors (C.Y. Okumura, V. Nizet, 2014 [34]). To the best of our knowledge, no other studies were conducted on strains isolated in Romania regarding the phenotypic expression of the soluble virulence factors expressed by throat Str. pyogenes.

All analyzed Str. pyogenes strains expressed at least one soluble virulence factor, with more than half them expressing most of the tested soluble virulence factors. The DN-ase was the most preponderant virulence factor expressed by the studied strains, its role being the reduction of local viscosity determining the evasion of the immune system and the hydrolysis of DNA molecules thus obtaining nucleotides that are ready to use in the replication process (M. Chifiriuc & al., 2013 [35]). The invasive capabilities of the isolates are also demonstrated by the proteolytic enzyme caseinase being the second most prevalent virulence factor, which
destroys the structural host proteins that are a part of the host tissues as well as its immune effectors.

**Conclusions**

In Romania, the rate of Str. pyogenes throat carriage is 0.48 similar or higher that those reported for other South-European countries. The Str. pyogenes strains circulating in Romania posses a high variety of pathogenic features represented by the high adherence capacity to epithelial cells and by soluble virulence factors that can be correlated with the high frequency, diverse location and severity of the produced infections. Active and continuing surveillance is required to provide an accurate assessment of the disease burden and to provide epidemiological data on the Str. pyogenes circulating in our country. The present study provides an experimental basis for further molecular and immunological analyses allowing a more detailed epidemiological characterization of Romanian strains and a better understanding of the pathogenicity of these bacteria.

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**REFERENCES**

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