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ORIGINAL ARTICLE

The virulence factors of group A streptococcus strains isolated from invasive and non-invasive infections in Polish and German centres, 2009–2011

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Abstract *Streptococcus pyogenes* (GAS) is one of the major human pathogenic bacteria that cause a wide range of diseases. Currently, increased incidence of streptococcal invasive infections is observed worldwide. In this study, we focused on the prevalence of genes encoding superantigens and type M proteins in the population of GAS strains from invasive versus non-invasive infections. We tested 253 GAS strains: 48 strains from patients with invasive infections (18 from wound/deep skin localization, 30 from women in labour) and 205 strains from non-invasive forms (147 from common infections of the upper respiratory, 49 from the vagina of females with genital tract infections and 9 from non-invasive wound and superficial skin infections). Significant differences were found in the occurrence of genes: *speG, speI, speJ* and *smeZ*, which were more common in GAS isolated from invasive than from non-invasive strains; *speJ* and *smeZ* occurred more frequently in strains from invasive perinatal infections versus strains from women without symptoms of invasive infection; *speH* and *speI* in strains from invasive skin/wound infection versus strains isolated from non-invasive wound and superficial skin infections. Emm types 1 and 12 predominated in the group of strains isolated from superficial infections and type 28 in those from puerperal fever. Occurrence of genes encoding virulence factors is common in genomic DNA of most of *S. pyogenes*, regardless whether these streptococcal infections are invasive or non-invasive. On the other hand, it appears that strains with *speG, speI, speJ* and smeZ genes may have a particular potential for virulence.

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Introduction

Group A *Streptococcus* (GAS) (*Streptococcus pyogenes*) infections vary from superficial infection of the pharynx (strep throat, pharyngitis) to serious skin and soft tissue infections that can lead to lethal invasive disease, despite antibiotic treatment [1]. Currently, increased incidence of invasive infections such as puerperal fever, streptococcal toxic shock syndrome and sepsis caused by *S. pyogenes* is observed worldwide [2–4]. Protein M is considered the main virulence factor, which limits phagocytosis, disturbs the function of complement and is responsible for adhesion [5]. Furthermore, the *emm* gene, which encodes this protein, forms the basis for epidemiological typing of GAS, to correlate serotype with pathogenicity [6]. Although GAS possess many virulence factors that engage a wide variety of host defences, the streptococcal superantigens play a pivotal role in triggering potent inflammatory responses. GAS strains that cause invasive infections usually produce one or more superantigens: SpeA, Spe C, Spe G, Spe H, Spe I, Spe J, Spe K, Spe L, SpeM, SmeZ, and Ssa [6–8]. Epidemiological studies, which provide the distributions of the types of streptococci prevalent in communities, are of basic importance for the identification and control of streptococcal infections. The US Centres for Disease Control and Prevention (CDC) reported an average

of 3.5 cases of invasive GAS infections per 100,000 population in the United States in 2000–2004 [9]. During nearly the same period of time, a European epidemiological survey of GAS infection was reported. The ten most predominant M/emm types were M/emm type 1 (M/emm1), M/emm28, M/emm3, M/emm89, M/emm87, M/emm12, M/emm4, M/emm83, M/emm81, and M/emm5, in descending order, but the M/emm type distribution varied broadly between participating countries. Unfortunately, no data on GAS strains isolated in Poland were included in this survey [10]. It should be stressed that in the last 10 years there has been an increase in invasive GAS infections with associated mortality, and these data are especially alarming from an epidemiological point of view; however, the factors underlying the worldwide resurgence of this pathogen remain unknown [11, 12].

Aim

The primary objective of this study was to compare virulence factors of *S. pyogenes* strains isolated both from invasive and non-invasive infections, in Polish and German centres, in the years 2009–2011. Additionally, we focused on the prevalence genes encoding superantigens and M protein type in the population of invasive GAS strains.

Materials and methods

A total of 253 GAS strains were tested in that 48 originated from patients with clinical signs of invasive infection and 205 from non-invasive cases. In the group with invasive infections, 18 *S. pyogenes* strains were isolated from wound and deep skin infections, half of which required surgical intervention due to development of necrotising fasciitis. Additionally, 30 invasive *S. pyogenes* strains originated from women in labour with clinical symptoms of puerperal fever, sepsis and four cases of streptococcal toxic shock syndrome. The control group consisted of 147 *S. pyogenes* strains isolated from common infections of the upper respiratory tract in children and in adults associated with *S.pyogenes*, 49 *S. pyogenes* strains isolated from the vagina of females manifesting clinical symptoms of genital tract infection, and nine GAS originated from non-invasive wound and superficial skin infections (Table 1). All *S. pyogenes* strains were collected in the years 2009–2011 in outpatient and inpatient clinical centres in southern and northern Poland and in different healthcare institutions in Germany. The strains were initially characterized in the local microbiological laboratories and then sent to the Department of Bacteriology, Microbial Ecology and Parasitology, Chair of Microbiology, Jagiellonian University Medical College, Kracow, Poland for further testing. The strains originating from Germany were characterized and collected in the German National Reference Center for Streptococci in Aachen. Speciation of the strains was performed using phenotypic methods (API, bioMérieux, France) and latex agglutination test for serological grouping of β-haemolytic streptococci (Oxoid, UK). In case of inconclusive results, polymerase chain reaction (PCR) was performed with species-specific primers, i.e. spy1258F and spy1258R, constructed for transcriptional regulator gene spy1258. Amplification was performed according to the methodology described by Liu et al. [13].

Table 1 Origins of the collected *S. pyogenes* strains

Number of strains source type	Invasive infections	Non-invasive infections
Wound/skin infections	18	9
Genital tract	30	49
Upper respiratory tract	0	147
Total	48	205

Polymerase chain reaction-based gene detection of streptococcal exotoxins

Presence or absence of the genes coding for different streptococcal pyrogenic exotoxins (SpeA, SpeC, and SpeG through SpeM), streptococcal superantigen (SSA), and streptococcal mitogenic exotoxin Z (SmeZ) were evaluated using PCR (for *speI, speJ, speK, speL, speM, smeZ,*) and multiplex PCR (for *speA, speB, speC, speF, speG, speH, and ssa*). Primers used in the reactions are shown in Table 2. Amplification was performed according to methodologies previously described [13, 15–17], and the obtained results are presented in Fig. 1 and Table 3.

Detection of *emm* gene serotype by PCR

The presence of the *emm* gene encoding protein M was verified in all strains from the study group. The PCR method, as described by Podbielski et al. [14] designed for the N-terminal region of the *emm* gene, was used. The PCR reaction products were sequenced using an ABI Prism® 310 Genetic Analyser (Applied Biosystems, Weiterstadt, Germany). The sequences obtained were compared with all available reference sequences on the United States (US) Center for Disease Control and Prevention (CDC) website (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp).

The *emm* gene was considered if the degree of homology between the sequences reached 95%. Studies related to detection and sequencing of the *emm* gene were performed at the Department of Medical Microbiology, National Reference Center for Streptococci and Institute of Medical Statistics, RWTH Aachen University, Aachen, Germany.

Table 2 List of primers used in the reactions

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)	References
spy1258	AAAGACCGCCTTAACCACCT	TGGCAAGGTAAACTTCTAAAGCA	407	Liu D et al. [13]
emm	TATT (C/G) GCTTAGAAAATTAA	GCAAGTTCTTCAGCTTGTTT	–	Podbielski A. et al. [14]
speA	CTT AAG AAC CAA GAG ATG GC	ATA GGC TTT GGA TAC CAT C	200	Luca-Harari B. et al. [15]
speB	TTC TAG GAT ACT CTA CCA GC	ATT TGA GCA GTT GCA GTA GC	300	Luca-Harari B. et al. [15]
speC	CAT CTA TGG AGG AAT TAC GC	TGT GCC AAT TTC GAT TCT GC	246	Luca-Harari B. et al. [15]
speF	GCG AAA TTA GAA AAG AGG AC	GCT GAG CAA AAG TGT GTG	1193	Luca-Harari B. et al. [15]
speG	TAT AAT ATT ACC CCA TGC GA	AAG GCT CCC CGA TG	447	Luca-Harari B. et al. [15]
speH	AAG CAA ATT CTT ATA ATA CAA CC	TTA GCT GAT TGA CAC ATC TAC A	630	Luca-Harari B. et al. [15]
speI	ATGAGTAGTGTGGGAGTTATTAA	TTATTTATTAAATTTAACTAAG	678	Rivera A et al. [16]
speJ	GATAGTGAAAATATTAAAGACG	GCTCCTATCTTATTTAGTCC	639	Rivera A et al. [16]
speK	GTGTGTCTAATGCCACCGTCT	GGAACATATATGCTCCTAGAT	564	Banks DJ et al. [17]
speL	TTAGGATGGTTTCTGCGGAAGAGAC	TTCCTCTTTCTCGCCTGAGCCGTG	596	Rivera A et al. [16]
speM	GCTCTATACACTACTGAGAGTGTC	CATATCAATCGTTTCATTATCTG	612	Rivera A et al. [16]
smeZ	TAGAAGTAGATAATAATTCC	TTAGGAGTCAATTTCTATAT	629	Rivera A et al. [16]
ssa	AGT AGT CAG CCT GAC CCT AC	TTT GGT AAG GTG AAC CTC TAT	691	Luca-Harari B et al. [15]

Statistical analysis

Statistical analyses were performed to demonstrate significant differences in the distribution of the genes encoding superantigens and M protein types in the population of invasive vs. non-invasive GAS strains. The Kołmogorow-Smirnov test was used. Calculations were made in the R environment (version 3.1.0).

Results

The prevalence of the genes encoding superantigens (pyrogenic exotoxins, superantigen, mitogenic exotoxin) in the *S. pyogenes* population was high and they were commonly found in the GAS genomes, regardless of the type of infection. Based on statistical analysis, we found significant differences in the occurrence of only four genes: *speG, speI, speJ* and *smeZ* in GAS isolated from total invasive versus total non-invasive infections (Fig. 1).

A more detailed analysis of these results (Table 3) showed that in case of 18 *S. pyogenes* strains isolated from patients with invasive wound, skin and soft tissue infections, percentage distribution of genes was as follows: *speA*–none, *speB*–77.8%, *speC*–44.4%, *speF*–100%, *speG*–83.3%, *speH*–50%, *speI*–61.1%, *speJ*–5.6%, *speK*–16.7%, *speL*–5.6%, *speM*–5.6%, *smeZ*–77.8% and *ssa*–16.7%, with two genes speH and speI significantly more often present in the genome of GAS strains isolated from invasive skin/wound infection compared with strains isolated from non-invasive wound and superficial skin infections.In case of 30 women who developed invasive perinatal streptococcal infections, isolated *S. pyogenes* had the following profile of genes encoding superantigens: *speA*–26.7%, *speC*–56.7%, *speG*–93.3%, *speH*–10%, *speI*–10%, *speJ*–60%, *speK*–36.7%, *speL*–6.7%, *speM*–33.3%, *smeZ*–83.3% and *ssa*–6.7%. Two genes, *speJ* and *smeZ*, were significantly more often present in the genome of *S. pyogenes* strains isolated in

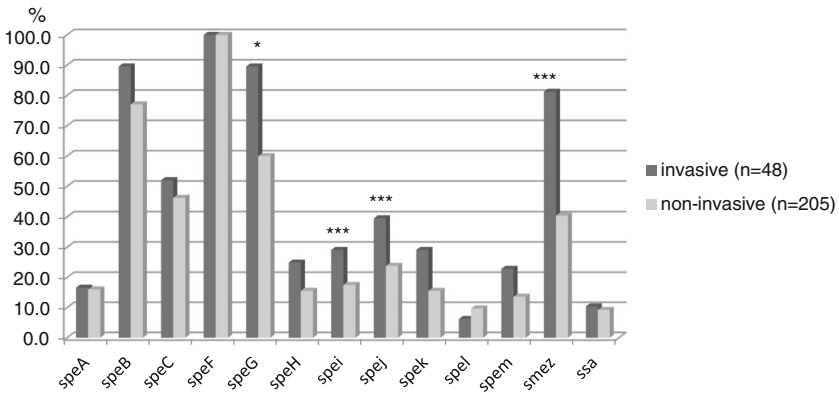

Fig. 1 Proportional occurrence of virulence genes in Streptococcus pyogenes strains (*$p < 0.05$, ***$p < 0.001$)

Table 3 Occurrence of various virulence genes in GAS strains isolated from different sources

Genes	Wound/skin infections (n = 27)			Genital tract (n = 79)			Upper respiratory tract (n = 147)
	Invasive (n = 18)	Noninvasive (n = 9)	p-value	Invasive (puerperal sepsis/ fever) (n = 30)	Noninvasive (n = 49)	p-value	
speA	0	2	>0.05	8	5	>0.05	26
speB	14	7	>0.05	29	17	>0.05	134
speC	8	2	>0.05	17	9	>0.05	84
speF	18	9	>0.05	30	49	>0.05	147
speG	15	8	>0.05	28	13	>0.05	102
speH	9	1	**0.0113**	3	0	>0.05	31
speI	11	2	**0.0189**	3	0	>0.05	34
speJ	1	3	>0.05	18	2	**0.0356**	44
speK	3	1	>0.05	11	5	>0.05	26
speL	1	0	>0.05	2	4	>0.05	16
speM	1	0	>0.05	10	4	>0.05	24
smeZ	14	5	>0.05	25	5	**0.0262**	73
ssa	3	1	>0.05	2	4	>0.05	14

Bold values indicate statistical significance

this patient group compared with *S. pyogenes* strains isolated from the genital tract of women without symptoms of invasive infection. Two genes coding for enzymes related to virulence were also detected in a vast majority of the strains*: speB*–96.7% and *speF*–100%.

In the 147 *S. pyogenes* strains from the control group, derived from patients with non-invasive infections of the upper respiratory tract, the profile of genes encoding superantigens was as follows: *speA*–17.7%, *speC*–57.1%, *speG*–69.4%, *speH*–21.1%, *speI*–23.1%, *speJ*–29.9%, *speK*–17.7%, *speL*–10.9%, *speM*–16.3%, *smeZ*–49.7% and *ssa*–9.5%. Two genes coding for enzymes related to virulence were also detected*: speB*–91.2% and *speF*–100%.The *speF* (100%) and *speB* (91.2%) genes were commonly present in the genome of *S. pyogenes* colonizing the throat, in contrast to *ssa* (9.5%), which occurred least frequently in the genomic DNA.

Distribution of the emm types among all tested GAS strains belonged to 23 different types (Table 4). Only in case of emm type 28, statistically significant differences were observed, namely, the type 28 M protein was much more frequently isolated in the population of GAS causing invasive streptococcal infections of women in childbirth, as compared to non-invasive GAS originating from genital tract infections in women. Among 147 *S. pyogenes* strains isolated from non-invasive upper respiratory tract infections (control group), emm type 1 (31 /147), emm type 12 (20/147), emm type 2 (18/147) and emm type 28 (16/147) predominated over the other types. *S. pyogenes* isolated from invasive wound infections belonged to ten different emm types; the most frequent were types 44 (3/18) and 89 (3/18), while those from puerperal sepsis were mostly of types 28 and 1, but five other types (12, 77, 89, 2, 75) were also noted. The strains from invasive infections belonged more frequently to types 28 and 1, while those isolated from non-invasive GAS infections to types 2, 1 and 28.

Discussion

A special role in pathomechanisms of the invasive infections caused by *S. pyogenes* is played by the superantigens: a group of proteins that cause excessive activation of T lymphocytes. Rapid stimulation of the immune system leads to both the secretion of proinflammatory cytokines such as IL-1, TNF alpha, IL-2, as well as sudden symptoms of acute inflammation manifested by high fever, altered respiration, heart failure, multiple organ dysfunction ending in shock and death of the patient [1, 5]. At present, in the tested populations of *S. pyogenes* strains, there are several genes that encode superantigens including, among others, *speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa, smez*, and enzymes *spe*B and *spe*F. At the turn of the 1980s and the 1990s, it was proven that in case of *speB* and *speF* genes, there are more genes encoding proteins of cysteine protease and streptococcal DNase, that were not included in the group of superantigens [18, 19]. The above conclusion was confirmed in our results, as indeed *speB* and *speF* were the most common genes in genomic DNA of group A streptococci and both types of infections—invasive and non-invasive. We can, therefore, say that the distribution of genes encoding the superantigens is a feature common in most of the genomic

Table 4 Emm types of GAS strains isolated from different sources

emm-type	Wound/skin infections (n = 27)			Genital tract (n = 79)			Upper respiratory tract (n = 147)
	Invasive (n = 18)	Non-invasive (n = 9)	p-value	Invasive (puerperal sepsis/fever) (n = 30)	Non-invasive (n = 49)	p-value	
1	0	2	>0.05	7	7	>0.05	31
2	2	0	>0.05	2	10	>0.05	18
3	0	0	>0.05	0	3	>0.05	1
4	0	0	>0.05	0	2	>0.05	12
6	0	0	>0.05	0	0	>0.05	13
11	0	0	>0.05	0	0	>0.05	3
12	1	0	>0.05	3	1	>0.05	20
27G	2	0	>0.05	0	0	>0.05	0
28	0	0	>0.05	11	7	0.0284	16
32	1	0	>0.05	0	0	>0.05	0
44	3	0	>0.05	0	0	>0.05	0
58	2	0	>0.05	0	0	>0.05	0
66	0	3	>0.05	0	0	>0.05	1
73	1	0	>0.05	0	0	>0.05	0
75	0	0	>0.05	1	0	>0.05	0
77	0	1	>0.05	3	4	>0.05	13
78	0	0	>0.05	0	0	>0.05	1
81	2	0	>0.05	0	0	>0.05	0
89	3	1	>0.05	3	4	>0.05	14
108	0	1	>0.05	0	0	>0.05	0
122	1	1	>0.05	0	0	>0.05	0
159	0	0	>0.05	0	0	>0.05	1
123	0	0	>0.05	0	0	>0.05	1
Not analysable	0	0	>0.05	0	9	>0.05	2

DNA of *S. pyogenes*, regardless of whether these streptococcal infections caused invasive or non-invasive inflammation of the skin or respiratory system. Interestingly, non-invasive *S. pyogenes* strains isolated from women of childbearing potential during routine diagnosis of inflammatory conditions of the genital tract, also have most of the genes encoding superantigens. According to our results of such genes, including *speG, speI, speJ* and *smeZ*, they occurred significantly more often in the genomic DNA of streptococci isolated from invasive GAS infections. Based on the publication of Unnikrisnan et al. [20] we suppose that the gene *smeZ* encoding streptococcal mitogenic exotoxin Z (SmeZ) plays a special role in stimulating the secretion of pro-inflammatory cytokines. These authors indicated that human mouse HLA-DQ transgenic cells stimulated with supernatant from the *S. pyogenes* without *smeZ* gene (−) led to a complete inability to elicit cytokine production (TNF-alpha, lymphotoxin-alpha, IFN-gamma, IL-1 and -8) from cells. According to other authors, *S. pyogenes* strains capable of producing mitogenic exotoxin Z, have particular potential for virulence but also the ability to colonize and proliferate in the human body [20–22]. The gene *smeZ* demonstrates extensive allelic variation (*smeZ1, smeZ2*), thereby producing polymorphic protein that displays antigenic variation, especially detected during invasive infection [23].

It seems that the SmeZ protein is often released into the extracellular space by streptococci responsible for the invasive forms of infection. This information may be important from an epidemiological point of view.

Of course, the presence of a pool of genes in genomic DNA of *S. pyogenes* does not necessarily lead to the production and release of proteins/superantigens. There are probably many mechanisms that regulate the process of protein synthesis, and these are both strain-dependent factors, as well as factors related to the individual characteristics of the patient, which may include diabetes, immunosuppression, alcoholism, or extensive surgery with the discontinuity of the skin, including caesarean section. All these risk factors were accumulated in a group of homeless people and injectable drug abusers who were involved in an outbreak of invasive GAS infection in England and Wales [24]. However, there has been limited investigation into how combinations of transcriptional

regulators control gene expression during infection despite the clear importance of regulatory networks to microbial pathogenesis. The data generated by Shelbourne et al. [25] demonstrate that the global metabolic gene regulator CcpA and the virulence factor regulator CovR act together to control expression of diverse GAS genes. The streptococcal pyrogenic exotoxins (*SpeI, SpeJ and SpeH*) bind to the beta-chain of CD4 T cells and MHC class II molecules on B cells, monocytes and dendritic cells, resulting in the overstimulation of the inflammatory response and subsequent systemic toxicity, tissue necrosis, organ failure and shock. Despite the increasing knowledge on GAS virulence factors and their role in disease pathogenesis [8, 23, 26], there is no clear view on their involvement in the pathomechanisms of different forms of invasive infections. We have recently reported [8] on the main virulence factors of GAS strains of *emm*28 serotype (less commonly *emm*1, *emm*12, *emm*75, and *emm*89) responsible for invasive perinatal infections which possessed *speF, speG*, and *speB* genes as well as genes responsible for mechanisms allowing for the binding and metabolism of iron ions. In the European study [10], a high rate of occurrence of *speA* was found among isolates of *emm1* and *emm3*, types that were often involved in severe infections, and also for the less frequent type *emm43*. In the same study, GAS strains from puerperal sepsis harboured mostly *speG, speF* and *speC* genes, while our strain of this origin had predominantly *smeZ* and *speJ* genes. Similarly, cellulitis derived strains in the European study had mostly *speG* and *speF* genes, while our strains from wound infections clinically presenting as cellulitis possessed more frequently *speI* and *speH* genes. These discrepancies confirm differences among GAS strains isolated in different regions and countries related to local epidemiology, which, unfortunately, pose many problems in the ongoing attempts at creating a universal vaccine against GAS disease [27]. Distribution of the M types among GAS strains tested in this study was compared with epidemiological data summarized by Walker et al. [5]. Types 1 and 12 predominated in the group of strains isolated from superficial infections and type 28 in those from puerperal fever. On the other hand, types 2, 77 and 89, which were present more often than others in our GAS, were not mentioned in this review. It is known that epidemiological studies have shown a remarkable difference in the distribution of *emm* types in geographically and socioeconomically distinct regions of the world [3, 4]. In general, frequency of the types found by us seems to be similar to that reported for other European countries [10], with some exceptions, such as types 2 or 77 more frequently present in our GAS from non-invasive infections. We cannot offer an explanation for this discrepancy. Our strains were isolated from infections in three centres distributed in different regions of Poland and many centres in Germany. However, it should be emphasized here that *emm* types found in different outbreaks vary considerably and, for example, GAS strains isolated from very recent outbreaks described in Canada and England, were different and uncommon, namely, *emm59* and *emm66*, respectively [24, 28]. Szczypa et al. [29] found an association between severe invasive GAS diseases and M type emm1 GAS isolates bearing the speA2 gene in their study on virulence factors of 41 GAS strains isolated in Poland between 1997 and 2005 from various invasive infections. Our data obtained several years later and reported in this study showed no difference between GAS isolated from invasive infections versus those from non-invasive infections in occurrence of *speA* gene and *emm1* type. In fact, the *speA* was quite rarely present. Also, invasive GAS strains from streptococcal toxic shock syndrome post-caesarean section outbreak characterized by us [30], belonged to emm type 28 and had *speC* gene.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Since this study was based on bacterial strains isolated from human cases for routine diagnostic purposes and collected over several years, for this type of study formal consent is not required.

Informed consent Informed consent was obtained from all individual participants included in the study.

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