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Group A Streptococci from Invasive-Disease Episodes in Poland Are Remarkably Divergent at the Molecular Level[▽]

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Forty-one clinical isolates of group A streptococcus (GAS) were recovered in Poland from patients with severe invasive infections and were analyzed by phenotypic and genotypic techniques. All isolates were characterized by determining their susceptibilities to antimicrobial agents and by determining their types by pulsed-field gel electrophoresis, multilocus sequence typing, *emm* typing, and the detection of five streptococcal pyrogenic exotoxin genes (*speA*, *speB*, *speC*, *speF*, *ssa*). The isolates studied were fully susceptible to penicillin G, levofloxacin, quinupristin-dalfopristin, and linezolid. Resistance to tetracycline, chloramphenicol, and erythromycin was detected in 46.3, 12.1, and 9.8% of the isolates, respectively. A total of 23 different *emm* sequence types were identified, of which *emm1* and *emm12* (19.5% each) were the most common, followed by *emm81*, *emm44*/*61*, and *emm85*. All the *emm1* isolates had the *speA2* allele. Twenty-three unrelated sequence types (STs) were identified, with the most frequent STs, ST28 and ST36, corresponding to *emm1* and *emm12*, respectively. Six newly found STs (STs 375, 376, 377, 378, 379, and 385) corresponded to *emm* types 74, 102, 77, 76, 84 and 63, respectively. The *emm1* type and the presence of *speA2* gene were associated with the severity of GAS infections. This work presents the first molecular study on Polish invasive GAS isolates.

The past two decades have witnessed a worldwide resurgence in invasive group A streptococcus (GAS) disease, which includes various clinical syndromes, such as bacteremia, septic arthritis, pneumonia, peritonitis, puerperal sepsis, necrotizing fasciitis (NF), meningitis, and streptococcal toxic shock syndrome (STSS). These rapidly progressing infections are associated with high morbidity and mortality rates, even in patients receiving appropriate antimicrobial therapy (4, 7, 25). The extracellular pyrogenic exotoxins (SpeA, SpeB, SpeC), mitogenic factor (SpeF), and streptococcal superantigen (SSA), together with the surface-located M protein, play a major role in the pathogenesis of invasive GAS infections. The M protein, encoded by the *emm* gene, provides the basis for the identification of different GAS M types as a tool for epidemiological analyses (4, 11). Recently, classical serologic M typing in many laboratories has been replaced by molecular typing based on the sequencing of the 5′ region of the *emm* gene, and over 160 different *emm* genotypes are currently recognized (data available at ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/). The majority of GAS outbreaks studied worldwide so far have been caused predominantly by strains of *emm* types 1, 3, 12, and 28 (4, 7, 16). Pulsed-field gel electrophoresis (PFGE) of macrorestricted bacterial DNA and multilocus sequence typing (MLST) represent other important tools for discrimination among GAS strains (10, 11, 17).

The invasive GAS cases that occur in Poland pose a serious hazard to public health. GAS isolates from patients with invasive forms of disease were sent by local laboratories to the National Institute of Public Health for further species confirmation and identification of toxin genes. The present study constitutes the first one to describe the properties of the invasive Polish GAS isolates in terms of their susceptibilities to antimicrobial agents, *emm* types, sequence types (STs), and PFGE profiles and to analyze their virulence gene distribution.

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MATERIALS AND METHODS

Bacterial strains and patients characteristics. Forty-one GAS isolates from patients with severe symptoms of invasive GAS disease episodes were collected at 17 different hospitals distributed in different parts of Poland and sent to the National Institute of Public Health between 1997 and 2005. The criteria used to define severe GAS infection were in accordance with those described by The Working Group on Severe Streptococcal Infection (31). These isolates were recovered from blood ($n = 25$), pus ($n = 5$), wounds ($n = 6$), peritoneal fluid ($n = 2$), synovial fluid ($n = 2$), and pleural fluid ($n = 1$). Isolates were reidentified by standard procedures with a commercially available agglutination test kit (Streptex; Murex Biotech Ltd., United Kingdom) and the pyrrolidonyl-arylamidase test (PYR 50 test kit; Remel Inc., Lenexa, KS).

Patients' data were collected on a specially prepared questionnaire that included information on demographic characteristics (age, sex), underlying conditions, clinical manifestation, and the outcome of illness. The case-fatality ratio (CFR) was calculated on the basis of the number of cases with known outcomes.

Antimicrobial susceptibility testing. MICs were determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) by the standard microdilution method (21). *Streptococcus pneumoniae* strain ATCC 49619 was included for quality control purposes. For all isolates, susceptibility to the following antimicrobial agents was tested: penicillin G, erythromycin, tetracycline, and chloramphenicol (Sigma-Aldrich, Steinheim, Germany); clindamycin and linezolid (Pharmacia Upjohn, Inc., Kalamazoo, MI); spiramycin (Rhone-Poulenc Rorer, Collegeville, PA); and telithromycin, quinupristin-dalfopristin, and levofloxacin (Aventis Pharma, Romainville, France). The MIC breakpoints were interpreted according to the CLSI criteria (2). Breakpoints for spiramycin were those proposed by the French Society for Microbiology (3). All erythromycin-resistant isolates were assigned to one of the following macrolide,

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TABLE 1. Predisposing factors among patients with invasive GAS infections

Predisposing factor	No. (%) of patients
Predisposing factor(s) (one or more)	28 (68.2)
Alcoholism[a]	9 (32.1)
Surgical procedures[b]	8 (28.5)
Viral infections[c]	4 (14.2)
Pneumonia	3 (10.7)
Cancer	2 (7.1)
Chronic renal insufficiency	2 (7.1)
Cirrhosis of liver	2 (7.1)
Scabies	1 (3.5)
Epilepsy	1 (3.5)
Postpartum status	1 (3.5)
Hypothermia	1 (3.5)

[a] Six patients had more than one predisposing factor.
[b] Including appendectomy, postfrostbite transtibial amputation, thyroidectomy, cholecystectomy, and prosthetic joint replacement.
[c] Including varicella-zoster virus, human immunodeficiency virus, rotavirus, and Epstein-Barr virus.

lincosamide, and streptogramin B (MLS_B) phenotypes by the double-disk test described previously (27): inducible MLS_B ($iMLS_B$), constitutive MLS_B ($cMLS_B$), and efflux-mediated resistance (M phenotype).

PFGE analysis, MLST, *emm* typing, and detection of streptococcal pyrogenic exotoxin genes. Chromosomal DNA was isolated from bacterial cultures, digested with the SmaI restriction enzyme (Fermentas, Vilnius, Lithuania), and electrophoresed in 1% pulsed-field certified agarose (Bio-Rad Laboratories, Hercules, CA) in a CHEF-DR III system (Bio-Rad) as described by Stanley et al. (24). PFGE patterns were compared with the use of Molecular Analyst software, version 1.12 (Bio-Rad), by using the unweighted pair group method with arithmetic means clustering method with the Dice coefficient and a position tolerance of 1.5%. In the resulting dendrogram, isolates with a genetic relatedness of >80% were considered to represent the same PFGE type, with the subtypes designated A1, A2, etc. Total bacterial DNA, which was subsequently used as a PCR template, was isolated by using a Genomic DNA Prep Plus kit (A&A Biotechnology, Gdańsk, Poland). For MLST, the internal fragments of seven housekeeping genes were amplified and sequenced with primers by following the protocol described by Enright et al. (10). To be considered related, isolates had to share at least five alleles of the seven loci. The Internet-accessible database (www.mlst.net) was used to assign the allele numbers and the STs to particular allelic profiles. The *emm* types for all isolates were determined by sequencing according to the recommendations of the Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, and by using the *emm* sequence database (www.cdc.gov/nciod/biotech/strep). PCR was performed to detect the presence of the *speA*, *speB*, *speC*, *speF*, and *ssa* genes with primer pairs specific for each gene following previously described protocols (1, 28, 30). The alleles of the *speA* gene were identified by sequencing of the PCR product (20).

RESULTS

Patient characteristics and clinical features. Our study analyzed 41 isolates obtained from patients with invasive GAS disease. The clinical manifestations observed in these patients included sepsis (16 patients), STSS (10 patients), erysipelas (6 patients), septic arthritis (4 patients), peritonitis (2 patients), and necrotizing fasciitis (3 patients). The majority of patients (60.4%) were male. In one case the age was not reported; the remaining 40 patients ranged in age from 1 to 88 years (median age, 46 years), although the majority of cases (67.5%) occurred in patients aged 26 to 60 years (median age, 45 years). The five pediatric patients (median age, 3 years; range, 1 to 14 years) accounted for 12.2% of all isolates, and the remaining eight patients (19.5%) were older than 65 years (median age, 74 years). Among the patients with STSS, seven were adults (median age, 43 years; range, 26 to 72 years) and three were children (range, 1 to 4 years). In the non-STSS group of patients, the median age was 46 years (range, 3 to 88 years). Predisposing factors were identified for 28 (68.2%) patients (Table 1), of whom 10 patients (35.7%) had more than one predisposing factor. The CFR was assessed for 37 (90.2%) patients, as the outcome was unknown in 4 cases. The overall CFR was 35.1%; however, it reached 50% for the 10 patients who developed STSS. Among patients without STSS, the CFR was 29.6%.

TABLE 2. Susceptibilities of 41 invasive GAS isolates to 10 antimicrobial agents

Antibiotic	MIC (μg/ml)			% Resistant
	50%	90%	Range	
Penicillin G	0.008	0.015	0.008–0.015	0
Erythromycin	0.015	0.12	0.015–>128	9.8
Spiramycin	0.25	0.5	0.03–128	4.9
Clindamycin	0.06	0.12	0.03–128	4.9
Telithromycin	0.015	0.03	0.015–16	4.9
Q/D[a]	0.5	0.5	0.12–1	0
Tetracycline	0.25	32	0.12–128	46.3
Chloramphenicol	2	2	2–32	12.1
Levofloxacin	0.5	0.5	0.25–1	0
Linezolid	1	1	0.25–1	0

[a] Q/D, quinupristin-dalfopristin.

Susceptibilities to antimicrobial agents. All isolates were fully susceptible to penicillin G, levofloxacin, quinupristin-dalfopristin, and linezolid. The highest proportion of resistance was found for tetracycline (46.3%). Resistance to chloramphenicol and erythromycin was seen in 12.1% and 9.8% of the isolates, respectively (Table 2). According to the double-disk test, two of four erythromycin-resistant isolates exhibited the $iMLS_B$ phenotype and the other two exhibited the $cMLS_B$ phenotype. All four erythromycin-resistant isolates were also resistant to chloramphenicol and tetracycline. Clindamycin and spiramycin retained good activity against all erythromycin-susceptible isolates. Susceptibility to telithromycin was found in all but the two isolates which manifested the $cMLS_B$ phenotype.

PFGE analysis, *emm* typing, and MLST. A total of 23 different *emm* sequence types were identified, of which *emm1* and *emm12* (19.5% each) were the most common, followed by *emm81* (7.3%), *emm44/61*, and *emm85* (4.9% each). Together, these five *emm* types accounted for 56% of the isolates studied. Among the 10 isolates from patients with STSS, 5 and 2 isolates were *emm1* and *emm85*, respectively; the remaining 3 isolates were *emm12*, *emm81*, and *emm94*, respectively. The CFR among patients infected with *emm1* GAS isolates was 50% and was four times higher than that among the group of patients with *emm12* isolates. The four erythromycin-resistant isolates included two *emm12* isolates with the $cMLS_B$ phenotype and two *emm44/61* isolates with the $iMLS_B$ phenotype. MLST analysis revealed the presence of 23 unrelated STs (Table 3), which correlated very well with the *emm* types. The most frequent STs, ST28 and ST36 (eight isolates each), corresponded to *emm1* and *emm12*, respectively. Six newly found STs (STs 375, 376, 377, 378, 379, and 385) corresponded to *emm* types 74, 102, 77, 76, 84, and 63, respectively. Twenty-

TABLE 3. *emm* types, PFGE types, resistance profiles, MLST characteristics, toxin genes profiles, and clinical manifestations among 41 Polish invasive GAS isolates

emm type	PFGE type	Resistance profile[a]	ST[b]	MLST allelic profile[c]	No. of invasive isolates in DB[d]	Presence of toxin genes					Clinical manifestation (no. of isolates, no. of deaths)
						speA[e]	*speB*	*speC*	*speF*	*ssa*	
1	B1		28	4-3-4-4-4-2-4	33	2	+	−	+	−	STSS (5, 2), peritonitis (2, 1), sepsis (1, 1)
4	S1		39	5-11-8-5-15-2-1	14	−	+	+	+	+	Sepsis (1)
5	Y1		99	33-30-7-5-5-26-3	6	−	+	+	+	−	Sepsis (1)
8	N1	T	59	13-2-8-19-1-3-4		−	+	+	+	−	Erysipelas (1)
11	T1	T	22	3-4-6-7-1-5-4	2	−	+	+	+	−	Sepsis (1)
12	A1		36	5-2-2-6-6-2-2	6	−	+	−	+	−	Septic arthritis (1), erysipelas (1), STSS (2, 1)
12	A1 A2		36	5-2-2-6-6-2-2		−	+	+	+	−	Erysipelas (1), septic arthritis (1)
12	Q1 Q2	E S C Te T Ch	36	5-2-2-6-6-2-2		−	+	+	+	−	Septic arthritis (1), sepsis (1)
28	R1	T	244	11-6-14-5-9-44-19		−	+	−	+	−	Sepsis (1, 1)
44/61	E1	E T Ch	367	4-2-3-11-17-3-61		−	+	−	+	−	Erysipelas (1), sepsis (1)
49	X1	T Ch	190	4-6-28-7-21-7-1	1	1	+	+	+	+	Sepsis (1)
60	G1	T	53	11-6-22-7-9-2-17		−	+	+	+	−	Sepsis (1)
63	U1		**385**	78-53-52-5-81-68-4[c]		−	+	−	+	−	Sepsis (1)
64	C1	T	164	2-2-8-3-5-2-29		−	+	−	+	−	Sepsis (1)
73	W1		331	43-2-2-47-1-3-4		2	+	−	+	−	Sepsis (1, 1)
74	J1	T	**375**	92-2-2-2-31-3-2		−	+	−	+	−	Erysipelas (1)
76	L1	T	**378**	11-6-3-6-6-27-46		−	+	−	+	−	Sepsis (1)
77	I1		**377**	4-2-2-11-34-3-2		−	+	−	+	−	Sepsis (1, 1)
81	H1	T	341	91-2-65-7-1-3-60	2	−	+	+	+	−	STSS (1, 1), NF (2, 1)
84	F1	T	**379**	12-21-17-5-5-3-7		−	+	−	+	−	Sepsis (1)
85	D1 O1	T	336	87-9-8-7-5-57-54	1	−	+	+	+	−	STSS (1, 1), sepsis (1, 1)
94	M1		89	24-2-3-5-1-3-1	2	−	+	+	+	−	STSS (1)
95	K1	T	14	2-6-8-3-9-3-1	3	−	+	−	+	−	Erysipelas (1)
102	P1	T	**376**	4-2-2-5-70-2-1		−	+	+	+	−	NF (1, 1)
117	Z1		134	54-24-14-4-9-2-2	1	−	+	−	+	−	Septic arthritis (1)

[a] C, clindamycin; Ch, chloramphenicol; E, erythromycin; S, spiramycin; T, tetracycline; Te, telithromycin.
[b] Boldface type, new STs.
[c] Underlined, new allele; the MLST allelic profile is in the order *gki-gtr-murI-mutS-recP-xpt-yqiL*.
[d] Number of isolates reported to the MLST database (DB) as recovered from invasive disease-sterile site.
[e] Numbers indicate the *speA* allele.

seven different PFGE patterns were identified, and these constituted 25 types (Fig. 1). The two predominant types, types A (type A1, five isolates; type A2, one isolate) and B (eight isolates), comprised 36.6% of the isolates studied and were characteristic for isolates of *emm12*/ST36 and *emm1*/ST28, respectively. Most isolates with the same *emm* type and ST generally shared related chromosomal PFGE patterns. The exceptions to this were *emm12*/ST36 and *emm85*/ST336, which included more than a single PFGE type (types A1-A2 and Q1-Q2 and types D1 and O1, respectively; Table 3). The two predominant *emm1*/ST28 and *emm12*/ST36 types were isolates from patients in seven and six hospitals, respectively, recovered between 1998 and 2004. An epidemiological link between strains with the same PFGE/*emm*/ST type was observed for only two *emm*1/ST28 isolates, which were derived from two patients who were hospitalized in the same institution and who both developed STSS as a result of postoperative wound infections.

Distribution of virulence genes. The results of PCR amplification of streptococcal pyrogenic exotoxin genes showed that all the isolates possessed the chromosomal *speB* and *speF* genes (Table 3). The bacteriophage-encoded *speA* and *speC* genes were present in 24.4% (10/41) and 41.5% (17/41) of the isolates, respectively. The *ssa* gene was detected in only two isolates (4.9%), which were of *emm4* and *emm49*. All eight of the *emm1* isolates possessed the *speA2* gene, which was also present in a single isolate of *emm73*/ST331. Generally, five exotoxin gene profiles could be distinguished in the isolates studied, with three predominant profiles, *speA* negative (*speA*−), *speB* positive (*speB*+), *speC*−, *speF*+, and *ssa*− (15 isolates); *speA*−, *speB*+, *speC*+, *speF*+, and *ssa*− (15 isolates); and *speA2*+, *speB*+, *speC*−, *speF*+, and *ssa*− (9 isolates). The presence of all four *spe* genes (with the profile *speA1*+ *speB*+, *speC*+, *speF*+, and *ssa*+) was observed in a single isolate of *emm49*/ST190. Among the STSS isolates, three different exotoxin profiles were found, with the predominance of the profile *speA2*+, *speB*+, *speC*−, *speF*+, *ssa*− (five isolates). None of the STSS isolates possessed the *ssa* gene. All eight *emm1* isolates showed the presence of a single exotoxin gene profile (*speA2*+ *speB*+, *speC*−, *speF*+, and *ssa*−), whereas among the eight *emm12* isolates, two toxin gene profiles were found.

DISCUSSION

Recently, cases of severe invasive GAS infections have been reported in all parts of the world (7, 16). These infections occurred both in previously healthy individuals and in patients with weakened immune systems caused by other medical conditions (4). While some studies reported that the risk of invasive GAS disease associated with alcoholism was low (12),

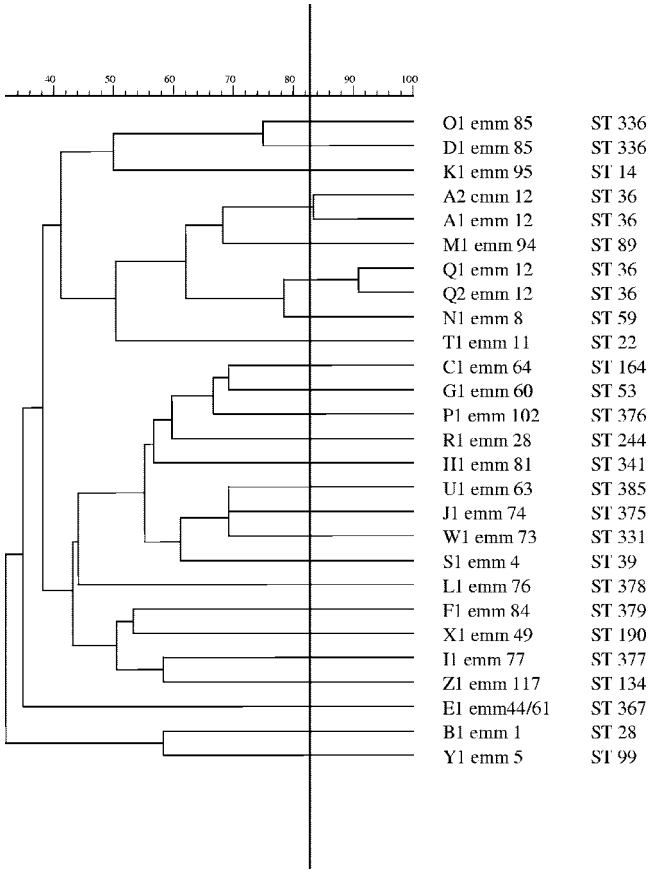

FIG. 1. PFGE-based dendrogram of invasive *Streptococcus pyogenes* isolates and their *emm* types and STs.

others indicated that it is a possible predisposing factor (5, 32), similar to the observation in this study. However, it must be underlined that the majority of alcoholic patients in our study had two or more other risk factors. Surgical procedures constituted another known factor associated with the development of invasive GAS infections found in the current study. Several studies reported the occurrence of invasive GAS disease among elderly individuals with underlying medical problems, while others found the highest rates of these diseases among young children (5, 22, 26). In the present study more than a half of the patients with invasive disease were adults with a mean age of 43.5 years, which is in accordance with reports from the United States (14, 32) and Taiwan (15). The CFR of 35% for the total sample was much higher than the CFRs described by others in Canada (5, 29), the United States (22, 32), Sweden (26), Denmark (9), and Israel (19), where it ranged from 12% to 24%. As only strains from the sickest patients are referred to our laboratory, this may explain the higher CFR reported here. Similar to other studies, we found that the risk of death due to invasive GAS infection is greatest when STSS develops (5, 8, 9, 15). Moreover, we observed a high risk of death among patients with NF. We tested a broad panel of antimicrobial drugs, including those not recommended for the treatment of streptococcal infections, for the purpose of epidemiological investigations. In this study, all isolates were susceptible to penicillin, the drug of choice for the treatment of GAS infections, and exhibited very low MICs. However, penicillin was shown to be ineffective in several clinical studies as well as in some experimental models of GAS invasive infections in which toxins were involved in the pathogenesis of that clinical condition and when a particularly large number of organisms was present (4). Therefore, the use of clindamycin in combination with penicillin for the treatment of necrotizing fasciitis or STSS was suggested (7, 25) since clindamycin inhibits protein (toxin) synthesis. As the prevalence of erythromycin- and clindamycin-resistant isolates from severe GAS infections reported in many countries varies (4, 7, 13, 22), it is obvious that clindamycin should not be used alone until an isolate is shown to be sensitive to this agent. Recently, a significant increase in the MLS_B phenotype was observed among clinical GAS isolates in Poland (27). In the present study, all the erythromycin-resistant isolates manifested the MLS_B phenotype. In light of this observation, the use of clindamycin as a therapeutic option requires more attention.

The M protein constitutes a major virulence factor of GAS; certain *emm* types, mainly types 1, 3, 11, 12, and 28, were associated with STSS and other severe GAS infections (4, 7, 22). Apart from the most common types, types *emm1* and *emm12*, which together accounted for 39% of the isolates, many other *emm* types were observed in our study. Such an *emm* type distribution is in general agreement with the type distribution of invasive GAS isolates in other European countries (6, 16). In addition, almost 15% of the isolates in this study represented *emm* types (types 64, 74, 84, 85, 95, and 117) which have rarely, if ever, been associated with severe GAS infections, while some other GAS types of increased invasiveness (*emm3*, *emm18*) were absent in this study. On the basis of epidemiological data demonstrating that the majority of noninvasive and invasive streptococcal infections are caused by a limited number of M types, a multivalent vaccine containing amino-terminal M-protein fragments from 26 different serotypes of GAS was recently developed in the United States (18). However, the vaccine would include the *emm* types of only 30.7% of the organisms identified in the present study, which reflects differences in the epidemiologies among various geographic locations.

Many reports on the pathogenesis of STSS and other invasive GAS diseases stressed the role of streptococcal toxins (pyrogenic exotoxins and superantigens), primarily SpeA, in the pathogenesis of GAS infections. The presence of toxin genes with different profiles and their relationships to *emm* types have been noted by many investigators (6, 9, 15, 30, 32). In our study, we observed the predominance of a single exotoxin gene profile in all eight *emm1* isolates: *speA* positive and *speC* and *ssa* negative. In contrast, the eight *emm12* isolates were characterized by two toxin gene profiles, and all lacked the *speA* gene. These findings are in accordance with the findings of previous studies from The Netherlands, Denmark, and Belgium (6, 9, 30) and support the strong association between invasive GAS isolates with the *emm1* type and the presence of the *speA* gene. Moreover, we observed an association between the severity of GAS infection and the *emm1* type, which was responsible for 50% of all STSS cases.

Until now, no data on the genetic diversity of Polish invasive GAS isolates have been available. This study revealed the presence of two widely distributed clones of *emm1*/ST28 and

emm12/ST36 that accounted for almost 40% of all cases of invasive GAS disease, while the remaining isolates were highly diverse and each was typically found only once or twice. The *emm1*/ST28 clone was highly homogeneous, while the *emm12*/ST36 clone was further differentiated by susceptibility testing, PFGE, and exotoxin gene profiling. However, isolates of *emm1* are significantly overrepresented in invasive disease; in the studies of pharyngeal GAS of Shulman et al., the prevalences of types 1 and 12 were comparable (23). Moreover, in a surveillance study conducted in the United States, infection with the *emm1* GAS strain was a statistically significant predictor of the patient's death (22). In our study, the *emm1*/ST28 clone represented the major cause of STSS (50% of all the cases, two of which were fatal), and this form of the disease clearly dominated for this clone (of the eight isolates, five were associated with cases of STSS), further supporting the increased virulence of this clone. In contrast, some other clones, such as *emm12*/ST36, *emm4*/ST39, and *emm28*/ST244, occur predominantly in uncomplicated throat infections rather than in invasive disease (8).

In summary, the results obtained in this pilot study provide useful comparative data for future research, and the study constitutes the first molecular study on Polish invasive GAS isolates. While many questions about the pathogenesis and epidemiology of invasive GAS infections remain to be answered, our data support the association between severe invasive GAS diseases and *emm1* GAS isolates bearing the *speA2* gene.

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