Molecular characterization of invasive and non-invasive *Streptococcus pyogenes* isolates from Romania

Bogdan Luca-Harari,¹ Monica Straut,² Silvia Cretoiu,² Maria Surdeanu,² Vasilica Ungureanu,² Mark van der Linden³ and Aftab Jasir⁴

Correspondence

Aftab Jasir

aftab.jasir@med.lu.se

¹Institute of Laboratory Medicine, Section of Medical Microbiology, Lund University, Lund, Sweden

²National Institute of Research and Development for Microbiology and Immunology, Cantacuzino, Bucharest, Romania

³German National Reference Center for Streptococci, Department of Medical Microbiology, University Hospital RWTH Aachen, Aachen, Germany

⁴Department of Clinical Microbiology and Immunology, Lund University Hospital (USIL), Lund, Sweden

In 2002, the Romanian National Reference Laboratory was invited to join the Strep-EURO project to study invasive *Streptococcus pyogenes* infections. During 2003 and 2004, a total of 33 isolates recovered from invasive disease were received from eight Romanian counties. For comparison, 102 isolates from non-invasive disease, as well as a collection of 12 old invasive strains (isolated between 1967 and 1980) were included. All isolates were characterized by several methods: T and *emm* typing, presence of the fibronectin-binding protein F1 gene (*prtF1*), serum opacity factor (*sof*), and superantigen (SAG) genes (*speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *ssa* and *smeZ*). The recent invasive isolates exhibited 19 *emm*-types, of which *emm1*, *emm81*, *emm76*, *emm49* and *emm78* covered 57 % of the strains. Furthermore, multilocus sequence typing analysis revealed nine new sequence types, corresponding to *emm* types 1, 12, 49, 81, 92, 100, 106 and 119. The non-invasive isolates comprised 24 different *emm* types with a predominance of *emm1* and 12; the old invasive strains were of eight *emm* types, of which four were unique for this group. All isolates harboured *speB* and *speF*; *smeZ* was detected in all invasive strains, except for the *emm49* and *emm81* isolates. The majority of isolates from carriers, and patients with pharyngitis were *prtF1* positive, most of these (14 strains) being *emm12*. High tetracycline resistance rates were noted among both invasive and control isolates (54 % and 35 %, respectively), whereas macrolide resistance rates were low (3 % and 5 %, respectively). Active and continuing surveillance is required to provide an accurate assessment of the disease burden and to provide epidemiological data on the character of isolates in Romania.

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INTRODUCTION

Group A streptococci (GAS, *Streptococcus pyogenes*) is one of the major human pathogens (Carapetis *et al.*, 2005), 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,

pneumonia, septicaemia, necrotizing fasciitis (NF) and streptococcal toxic shock syndrome (STSS) (Cunningham, 2000). In addition, GAS infections can give rise to non-suppurative sequelae, such as acute rheumatic fever and acute glomerulonephritis (Cunningham, 2000).

The heterogeneity exhibited by the N terminus of the M-protein, the major GAS virulence factor (Bisno *et al.*, 2003), is used in the M-serotyping technique (Lancefield, 1962). The *emm* gene (encoding the M-protein) is successfully targeted in sequence typing (Johnson *et al.*, 2006). Another GAS surface protein, T-protein, was used as a basis for a crude, but widely used subdivision of strains (Moody *et al.*, 1965). The variable presence of an apoproteinase serum opacity factor (SOF) enabled separation of

Abbreviations: CSF, cerebrospinal fluid; GAS, group A streptococci; iGAS, invasive group A streptococci; MLST, multilocus sequence typing; NF, necrotizing fasciitis; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; SAGs, superantigens; SOF, serum opacity factor; ST, sequence type; STSS, streptococcal toxic shock syndrome; VT, *vir* type.

A supplementary figure is available with the online version of this paper.

GAS strains into SOF-positive or -negative. Several other molecular methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to characterize GAS isolates epidemiologically (Bruneau *et al.*, 1994; Enright *et al.*, 2001). Finally, *vir* typing is also used to type GAS; this is based on the structural and sequence heterogeneity of the *vir* regulon (renamed the *mga* regulon) encoding some key virulence factors, including the M-protein family (Caparon & Scott, 1987; Gardiner *et al.*, 1995).

Many extracellular virulence factors are also produced by GAS (Bisno *et al.*, 2003), including several superantigens (SAGs), such as streptococcal pyrogenic exotoxins. These molecules have the capacity to activate a high number of T-cells unspecifically, leading to the release of excessive amounts of inflammatory cytokines and serious systemic effects (Bisno *et al.*, 2003; Sriskandan *et al.*, 2007).

GAS are known often to persist in the host after antibiotic treatment, potentially giving rise to recurrent infections or disseminating into tissues (Kaplan *et al.*, 2006). This feature is linked to the ability of the bacteria to enter and persist within eukaryotic cells; one factor involved in this is fibronectin-binding protein (PrtF1/SfbI) (Neeman *et al.*, 1998; Schwarz-Linek *et al.*, 2006). The possible genetic linkage between this protein and macrolide resistance remains the subject of discussion (Facinelli *et al.*, 2001).

Knowledge regarding epidemiology of invasive GAS (iGAS) disease in Romania is very limited. As part of the Strep-EURO initiative, the Romanian National Reference Laboratory for Streptococcal Infections started to collect isolates and clinical data from invasive infections during 2003–2004. The aim of the present national report was to compare microbiological and epidemiological characteristics of iGAS isolates with those of non-invasive GAS isolates obtained during the same period.

METHODS

Collection of isolates. On behalf of the EC Fifth Framework Program project Strep-EURO, the Romanian Ministry of Health – General Directory of Public Health implemented the inclusion of iGAS in the national infectious diseases surveillance system. The clinical laboratories from all the Romanian administrative counties were thus encouraged to report cases and submit corresponding isolates, as well as contemporary non-invasive control isolates, to the National Reference Laboratory INCDMI ‘Cantacuzino’. Invasive cases were defined by the isolation of GAS from normally sterile sites [blood, cerebrospinal fluid (CSF), pleurae, peritoneal or joint fluid or deep tissue], or from a superficial site, in association with NF or STSS. The Working Group on Severe Streptococcal Infections (1993) definition of STSS was used, as follows: hypotensive shock in conjunction with two or more specified clinical indicators (renal impairment, abnormal liver function, respiratory distress, erythematous rash, disseminated intravascular coagulopathy, soft tissue necrosis), with cases of ‘definite’ (those with sterile-site isolates) and ‘probable’ (those with non-sterile-site isolates) STSS combined (The Working Group on Severe Streptococcal Infections, 1993). All control isolates were from non-invasive (superficial) infections (excluding NF and STSS).

A detailed questionnaire, requesting data regarding demographic characteristics, clinical manifestation, predisposing factors, treatment and outcome after 7 days was distributed to clinicians. Information regarding antibiotic susceptibility to penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol, ofloxacin, ciprofloxacin, spiramycin, vancomycin and quinupristin/dalfopristin was also requested.

In addition, a collection of 12 invasive GAS isolates recovered from 1967 to 1980 was included. The strains were from blood (six bacteraemia and one septic abortion isolate), peritoneum (three isolates) and CSF (one isolate), and four were from other sterile sites. Data on clinical presentation, risk factors and outcome for the corresponding cases were not available.

T- and *emm*-typing. Isolates were T-typed using commercial poly- and mono-specific T-antisera, according to the manufacturer’s recommendation (Sevapharma) (Moody *et al.*, 1965). For *emm*-typing, the N-terminal hypervariable portion of the *emm* gene was sequenced, as described previously (Beall *et al.*, 1996). DNA sequence alignment and *emm* type assignment was performed using BLAST (<http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm>) and sequences listed in the streptococcal *emm* sequence database at the Center for Disease Control and Prevention (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm).

***vir* typing.** *vir* regulon typing was performed on invasive strains using a previously described protocol, with a forward primer that anneals upstream of the regulatory gene *virR* and a reverse primer that anneals in the *scpA* gene (Surdeanu *et al.*, 2000). PCR products were digested with *Hae*II and *Hinf*I (Boehringer Mannheim and GIBCO). Restriction fragment length polymorphism (RFLP) profiles were examined using the Taxotron software package (Institut Pasteur, Paris, France); patterns were clustered by the single-linkage method with a fixed tolerance of 3.5 %. A Dice coefficient of 1 corresponds to 100 % similarity of RFLP profiles.

PFGE. All invasive isolates, as well as T1 and T12 non-invasive strains (determined using T-typing), were subjected to PFGE. Cultures in Brain Heart Infusion broth (Merck) were incubated for 18 h at 37 °C. DNA was extracted using techniques previously described (Stanley *et al.*, 1995). Chromosomal DNA was digested with 40 U *Sma*I (Promega) and fragments were separated in a CHEF Mapper XA System (Bio-Rad) by electrophoresis at 200 V for 23 h at 13.5 °C with an initial pulse time of 10 s and a final pulse time of 35 s. Results were analysed using the Fingerprinting II software version 3.0 (Bio-Rad). Values for position tolerance and optimization were 1 % and 0.5 %, respectively.

MLST. All recent invasive strains ($n=33$) were typed by MLST as described previously (Enright *et al.*, 2001); alleles and sequence types (STs) were identified using the MLST database (<http://spyogenes.mlst.net/>).

Toxin gene detection. The isolates were tested for the presence of the *speA*, *speB*, *speC*, *speF*, *speG*, *speH* and *ssa* genes by PCR using primers described by Luca-Harari *et al.* (2008). All seven pairs of primers were used in an initial multiplex PCR, using the following conditions: denaturing for 2 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, followed by a final elongation step for 7 min at 72 °C. In addition, presence of the gene of streptococcal mitogenic exotoxin, *smeZ*, was traced by a single PCR, using primers covering its known allelic variation (Darenberg *et al.*, 2007).

***sof* and *prtF1* gene detection.** The *sof* gene was detected using methods and primers described previously (Beall *et al.*, 2000), yielding amplicons of 560–700 bp. These were further digested with *Dde*I (Promega) and restriction fragments were separated on a 4 %

agarose gel (MetaPhor, FMC Bio Products). Amplification of *prtF1* was performed using published primers (Neeman *et al.*, 1998), resulting in amplicons of 100–600 bp which were visualized on a 3 % agarose gel (MetaPhor, FMC Bio Products).

Antimicrobial susceptibility testing. *In vitro* susceptibility to antibiotics was tested by disk diffusion on 5 % blood agar, following the instructions provided by the Clinical and Laboratory Standards Institute (CLSI). Minimal inhibitory concentrations were determined by broth microdilution, on Mueller–Hinton agar plates supplemented with 5 % (v/v) defibrinated sheep blood according to the guidelines of the CLSI (2005). Erythromycin-resistant strains were assigned the constitutive (cMLS), inducible (iMLS) or efflux-mediated (M) macrolide-resistance phenotype on the basis of the double-disk (erythromycin and clindamycin) test, as described by Seppala *et al.* (1993).

Antibiotic resistance gene detection. Multiplex PCRs were used for detection of tetracycline and macrolide resistance genes. Tetracycline-resistant isolates were tested for the presence of the ribosome protection genes *tetM* and *tetO*, using previously described primers (Olsvik *et al.*, 1995; Roberts *et al.*, 1993). Macrolide-resistant isolates were tested for the *ermA*, *ermB* and *mefA* genes, using published primers (Nielsen *et al.*, 2004; Sutcliffe *et al.*, 1996).

RESULTS AND DISCUSSION

Site of isolation, patient data and clinical presentations

One hundred and thirty-five GAS isolates from invasive and non-invasive infections were collected during the study period. The invasive isolates ($n=33$) were from blood ($n=12$), CSF ($n=1$), synovial fluid ($n=2$), wounds ($n=4$) or other normally sterile sites ($n=14$). The non-invasive isolates ($n=102$) were isolated from superficial infections or carriers: 79 from the throat (42 cases of pharyngotonsillitis, 1 of pneumonia, 1 of parotitis, 6 cases of scarlet fever and 19 of their contacts and 10 from asymptomatic carriers), 16 from skin lesions (7 from wounds, 4 from impetigo, 2 from pyoderma, 2 from abscesses and 1 from cellulitis), four from the middle ear and one isolate each from vagina (endometritis), urine (pyelonephritis) and conjunctiva (eye infection).

The age of the patients with invasive GAS infections varied between 2 months and 83 years. However, two age groups prevailed: 0–9 years ($n=11$, 33 %) and 40–49 years ($n=9$, 27 %). There was no gender preponderance. The age of carriers and patients with non-invasive GAS varied from 2 months to 69 years, with a male to female ratio of 1.2 : 1.

Clinical presentations in patients with invasive infections included cellulitis ($n=10$), NF ($n=4$), bacteraemia without focal symptoms ($n=3$) and osteomyelitis ($n=2$); the remaining 14 patients had arthritis, meningitis, bronchopneumonia, peritonitis, cervical adenitis, erysipelas or abscess. In total, nine STSS cases were reported (three of which were fatal); eight of these were adults and one was a 2-month-old baby with septic infection of the umbilical cord stump.

Underlying conditions were reported for 21 of the total 33 invasive cases (64 %); these included skin lesions (11 patients, 5 of whom developed STSS), immunosuppressive therapy (3 cases), hospital-acquired infection (one patient who also developed STSS), drug abuse (one case) and chickenpox (one case). In four cases, the patients had underlying conditions other than those listed in the questionnaire (malignancy, alcoholism, traumatism of superior right limb and malnutrition).

All fatal cases ($n=3$) occurred in patients with STSS, giving a case fatality rate (CFR) of 33.3 among this group.

Distribution of T and *emm* types

A large number of T and *emm* types was found (27 and 34, respectively) (Table 1). Predominant T types among the invasive strains were T12, T1 and T14, and among non-invasive isolates T1 and T12 were predominant, with these two types covering 54 % of the control isolates.

Although *emm* types 1, 3 and 28 are usually associated with invasive disease (Ekelund *et al.*, 2005), our small sample of isolates comprised a range of 19 *emm* types, of which *emm1*, *emm81*, *emm76*, *emm49*, *emm75* and *emm95* accounted for 61 % of the strains. Notably, *emm28* was absent from this collection and *emm1* was identified in only 15 % of isolates. Furthermore, several new *emm* types (*emm91*, 92, 95, 100, 106 and 119) were found among those isolates causing invasive disease; high prevalence of new types was also recently reported in a study from Sweden (Darenberg *et al.*, 2007). A striking type diversity of invasive GAS isolates was also observed in two recent studies from Hungary and Poland (Krucso *et al.*, 2007; Szczypa *et al.*, 2006). STSS cases are commonly associated with *emm* types 1 and 3 (Cunningham, 2000). However, it is interesting to note that there is also high diversity among STSS cases in our study (two cases in each of *emm1* and *emm95*; the remaining five cases were in *emm8*, *emm49*, *emm76*, *emm106* and *emm119*); the three fatal STSS cases were caused by *emm8*, *emm106* and *emm95*.

Among non-invasive isolates, 24 *emm* types were detected; *emm1* accounted for 25 % of the cases, followed by *emm12* (23 %). Other common types were *emm75*, 76, 81 and 95, representing around 20 % of the isolates. The old invasive strains exhibited eight *emm* types, of which *emm5*, *emm23*, *emm50/62* and *emm77* were exclusively found among these isolates.

Toxin gene detection

Overall, 20 patterns of SAg genes were found (Table 2). All tested strains harboured the chromosomally encoded genes *speB* and *speF*. Similarly, *smeZ* was detected in all invasive strains, except for the *emm49* and *emm81* isolates. The other tested genes were found at different frequencies, ranging from 0 to 4 per isolate. In particular, *speA* was detected in 20 % of the invasive isolates and in 38 % of

Table 1. T and *emm* type distribution of invasive (recent and old) and superficial (control) Romanian GAS isolates, and the occurrence of the *sof* and *prtF1* genes

Isolates are recent invasive (RI), old invasive (OI) or superficial control (C). Data regarding the presence of *sof* and *prtF1* genes were not available for 25 control strains. NT, Strains were not T-typable. NA, Data not available.

<i>emm</i> type	<i>sof</i>	<i>prtF1</i>	No. of isolates (RI/OI/C)	T type (no. of isolates)
1	—	—	5/2/5	1 (38)
2	—	—	0/0/1	2
3	—	—	0/0/2	3/B3264
4	+	+	1/0/2	4 (2); 28
5	—	—	0/1/0	5/11/12/27
6	—	+	0/2/1	6; 11/12; 3/13
8	+	+	1/0/2	8 (2); NT
9	+	+	1/0/1	9
12	+	+	1/2/21	12 (22); 5/11/12 (1); NT
22	+	+	0/0/1	8
23	—	+	0/1/0	8/25
25	+	+	1/0/0	25
28	+	+	0/0/1	28
33	—	—	0/0/1	3
44/61	+	+	0/0/2	8; 27
49	+	—	3/1/3	14 (6); B3264
50/62	+	+	0/1/0	12
64	—	—	1/0/0	3
65/69	—	—	1/0/1	3/13/B3264
	—	+	0/0/1	8
74	—	—	0/1/0	12
75	+	+	2/0/4	4; 11; 13; 25 (2); 8/25/Imp19
	+	—	0/0/1	8
76	+	+	4/0/5	5; 12 (8)
77	NA	NA	0/0/1	25
78	+	+	1/0/0	NT
81	+	+	4/0/4	5 (2); 8; 12 (3); 3/13; 27
84	+	—	0/0/1	5/11
87	+	+	0/0/1	28
91	—	—	1/0/0	25/Imp19
92	+	+	1/0/1	8; Imp19
95	—	+	2/0/4	5; 6; 8 (2); 25; NT
100	—	—	1/0/0	3/13
102	+	+	0/0/2	4
106	+	+	1/0/3	13 (3); NT
119	—	—	1/0/0	NT
NA	+	+	0/1/0	12
	—	—	0/0/1	28
	—	+	0/0/1	3/13
	NA	NA	0/0/1	NT

controls. This gene was common in *emm1* strains (five of seven invasive isolates and all controls). In contrast, *speC*, present in 57 % of recent invasive and 45 % of control isolates, was frequently detected among *emm12* strains

(77 %). In addition, all *emm12* strains were found to harbour *speH*, whereas only approximately 10 % were *ssa* positive.

SAg gene detection, in combination with other typing methods, is useful for epidemiological purposes. Present results, in line with previous reports, showed a high prevalence of *speA* in both *emm1* and *emm49* isolates (Sriskandan *et al.*, 2007; Szczypa *et al.*, 2006), whereas *speC* was associated with *emm12* (Jing *et al.*, 2006). The *smeZ* gene was detected in 79 % of recent invasive isolates, but was consistently absent from isolates of *emm* types 49 and 81; the only old invasive isolate lacking the gene was an *emm49* strain. This chromosomally encoded gene is known to be highly prevalent among GAS isolates (>90 %) (Sriskandan *et al.*, 2007).

sof and *prtF1* gene detection typing

In total, 21 of 33 new invasive strains (64 %), 5 old invasive (42 %) and 56 non-invasive (59 %) strains were positive for the *sof* gene. In general, the restriction profiles of *DdeI* amplicons correlated with the *emm* type, though with some exceptions. Thus, one *emm81* isolate, distinct from the other *emm81* strains, exhibited a profile identical to that of an *emm84* strain. The two *emm92* and the single *emm22* strain showed identical profiles (Fig. 1a), whereas the single *emm25* strain had the same restriction profile as three of the *emm75* isolates (Fig. 1b).

Of the recent and old invasive isolates, 64 % and 58 %, respectively, harboured the *prtF1* gene (Table 1). Eighty per cent of control strains, of 18 different *emm* types, were *prtF1* positive, whereas those of eight other *emm* types were negative. The majority of isolates from patients with pharyngitis and from throat carriers were *prtF1*-positive (Table 3), most of these (14 strains) being *emm12*. This might account for the ability of these isolates to adhere to and internalize into pharyngeal epithelial cells (Hanski *et al.*, 1992; Molinari *et al.*, 1997) and is supported by a recent study which demonstrated that *PrtF1/SfbI* was seldom encoded by isolates causing invasive disease but was encoded by 35 % of those from pharyngotonsillitis (Bianco *et al.*, 2006). In the present study, many of the invasive isolates, as well as superficial isolates, were of type T12; the presence of *prtF1* may thus be primarily correlated with the type rather than disease localization.

vir typing

All invasive strains were *vir*-typed. Resulting amplicons varied in size between 4200 bp (two *emm6* isolates) and about 8000 bp (one old invasive *emm1* isolate), though mostly they were about 6800 bp. Amplicons were further digested with *HinfI*, yielding 26 distinct profiles [each profile was considered a *vir* type (VT)]. *HaeIII* restriction of *vir* amplicon gave rise to between two and eight fragments varying in size from 100 bp to approximately 4200 bp. All strains examined shared a common 1200 bp

Table 2. SAg patterns of the Romanian strains, as related to *emm* type

Isolates are recent invasive (RI), old invasive (OI) or superficial control (C). NA, Data not available.

<i>emm</i> type (no. of isolates)	No. of isolates (RI/OI/C)	<i>speA</i>	<i>speC</i>	<i>speH</i>	<i>smeZ</i>	<i>ssa</i>
81 (1)	0/0/1	+	+	+	+	+
1 (7)	1/0/6	+	+	–	+	–
49 (1)	1/0/0	+	+	–	–	–
4 (1), 23 (1)	0/1/1	+	+	–	+	+
1 (1), 3 (1), 49 (5)	1/1/5	+	–	–	–	–
87 (1)	0/0/1	+	–	–	–	+
1 (25), 28 (1)	4/0/22	+	–	–	+	–
1 (2), 3 (1)	0/0/3	+	–	–	+	+
12 (1), 81 (7)	5/0/2	–	+	+	–	–
1 (1), 12 (20), 75 (2), 91 (1), 119 (1)	3/1/8	–	+	+	+	–
2 (1), 6 (1), 77 (1), 102 (1), 106 (1), NA (2)	0/0/8	–	+	–	–	–
4 (1)	0/0/1	–	+	–	–	+
5 (1), 6, (2), 12 (1), 25 (1), 50/62 (1), 65/69 (1), 75 (3), 76 (3), 78 (2), 95 (1), 102 (1), 106 (2), NA (1)	7/5/8	–	+	–	+	–
4 (1), 78 (1), 100 (1)	3/0/0	–	+	–	+	+
8 (3), 12 (4), 44/61 (2), 76 (1), 95 (2)	2/0/10	–	–	+	+	–
33 (1), 92 (2)	1/0/2	–	–	+	+	+
2 (1), 49 (1), 65/69 (1), 81 (1), 84 (1), 95 (1), NA (1)	1/0/6	–	–	–	–	–
1 (1), 9 (2), 64 (1), 65/69 (1), 74 (1), 75 (2), 76 (5), 95 (2)	7/3/6	–	–	–	+	–
22 (1)	0/0/1	–	–	–	+	+
81 (1)	0/0/1	–	–	+	–	–

'core' fragment. Among the 44 isolates tested, 18 distinct *HaeIII* *vir* profiles were found (data not shown). *HinfI* generated between 5 and 12 fragments varying in size from 100 bp to approximately 2100 bp and was more useful in further discriminating the RFLP patterns obtained by *HaeIII* digestion than when used as the primary restriction enzyme in *vir* typing. Thus, four *HaeIII* types were subtyped by *HinfI*. Eight isolates (Rou13–*emm9*; Rou4, Rou10, Rou29, 4347–*emm49*; Rou15–*emm25*; Rou3, Rou18–*emm75*) shared a single *HaeIII* VT. After restriction with *HinfI*, this *HaeIII* *vir* type was subtyped into two *HinfI* types (VT9/49 and VT25/75) (Fig. 2). Each of three other *HaeIII* VTs were divided into two *HinfI* types (VT119 and VT64; VT81 and VT69; VT6a and VT6b). Generally, there was a better agreement between PFGE, *emm* types and VTs determined using combined *HinfI* and *HaeIII*, rather than only *HaeIII* RFLP of the *vir* amplicon. Since the restriction profiles appeared to be *emm*-type specific, VTs were assigned corresponding to *emm* types (Fig. 2). However, as mentioned above, in two cases, the same VT was obtained for isolates of different *emm*-types (VT9/49 was common to one *emm9* and four *emm49* isolates, and VT25/75 was shared by one *emm25* and two *emm75* strains). The region targeted in *vir*-typing contains some important virulence genes, among them *emm*, *scpA* and *sic* (Akeson *et al.*, 1996; Caparon & Scott, 1987; Gardiner *et al.*, 1995). The technique, though not widely used, was shown to be discriminatory and, with few exceptions, provides a close correlation to M/*emm* typing (Gardiner *et al.*, 1998).

PFGE

All invasive and T-type 1 and 12 non-invasive isolates (54 % of control strains) were characterized by PFGE. The analysis revealed 19 distinct patterns among the invasive isolates, which generally grouped according to the *emm* type, with some exceptions (Table 4, Fig. 2). Thus, one *emm9* and four *emm81* isolates showed pattern C, whereas both *emm25* and *emm75* isolates grouped under pattern D. Interestingly, recent isolates of *emm1* and *emm49* showed different PFGE patterns compared with old isolates; however, they were still about 52 and 83 % related, respectively, within the same general clade. The two recent *emm95* isolates were unrelated, grouping under different PFGE patterns. The invasive T12 isolates were of three different *emm* types (*emm12*, 76 and 81); the two 'old' *emm12* invasive strains present 65 % similarity whereas the recent *emm12* invasive isolate (Rou6) is more closely related to the old invasive isolate 629 (similarity 84 %); the last two types grouped accordingly into two PFGE patterns. All T1 control strains were *emm1*. They presented PFGE pattern A, with the exception of two strains that presented pattern A1 and one remaining strain that presented pattern A2. Pattern A1 lacked a single band of pattern A. Pattern A2 had three bands that were different from pattern A. According to the criteria for interpreting DNA restriction patterns produced by PFGE, the three patterns shared by all contemporary T1 *emm1* isolates, irrespective of their origin, were closely related. Control isolates of T12 were *emm12*, but four isolates were *emm76*. PFGE patterns of T12 *emm12* isolates belonged to the

Fig. 1. (a) *Ddel* restriction profiles of *sof* amplicons illustrating some exceptions from the correlation with corresponding *emm* types. Lanes: 1, *emm*8; 2 and 3, *emm*9; 4, *emm*22; 5 and 6, *emm*92; 7, *emm*87; 8, *emm*84; 9–12, *emm*81; M, 50 bp DNA Step Ladder (sizes in bp). (b) *Ddel* restriction profiles of *sof* amplicons illustrating some exceptions from the correlation with corresponding *emm* types. Lanes: 1, *emm*25; 2–5, *emm*75; M, 50 bp DNA Step Ladder (sizes in bp).

pattern G and closely related subtypes G1–G4 (clustered at 86 % similarity); a single control strain belonged to subtype G5 (clustered at 76 % similarity with G and G1–G4). Five T12 *emm*12 strains were *Sma*I resistant, as they had macrolide resistance M phenotype. The use of isoschizomer *Xma*I failed to give a digestion profile. The T12 *emm*76 isolates presented PFGE pattern B, as did the majority of *emm*76 invasive isolates, with the exception of a single isolate which presented a pattern that differed by two bands from pattern B, designated pattern B2 (data not shown).

Due to the small number and geographical dispersion of the source of the isolates, any conclusions on clonal spread would be hazardous, although differences in PFGE patterns between recent and old strains of the same *emm* type (e.g. *emm*1 and *emm*49) were notable. A continuous change in circulating GAS populations, probably with acquisition of new virulence features, could conceivably explain this finding.

MLST

MLST is the most recently developed typing method, which allows distinctions among and within GAS popula-

tions. Compared to *emm*-typing, this method has the ability to index variation that has accumulated relatively slowly (McGregor *et al.*, 2004). Notably, despite the small number of invasive isolates (33), 23 different STs were detected; of those, 5 new allelic variants of housekeeping genes (Table 4), as well as 9 new sequence types (ST 434–442) were identified. As expected, the same *emm* type yielded similar STs; thus, four *emm*1 isolates were of ST28, whereas the remaining one was a single-locus variant of ST28 (recently designated ST440). Similarly, out of four *emm*81 isolates, three showed ST341 and the remaining one was a single locus variant, designated ST434. Though STs are often *emm* type-restricted, there are studies showing that some particular STs can occur in more than one *emm* type (McGregor *et al.*, 2004). However, since the numbers of isolates with the same *emm* type were limited in our study, it is difficult to draw conclusions regarding the discriminatory power of MLST within a single *emm* type.

Among different methods used for characterization of *S. pyogenes*, T- and *emm*-typing together will provide a good, quick and relatively inexpensive pool for the identification of isolates. However, for clonal identification and relationship analysis, PFGE and MLST will be required. While PFGE can reveal differences in total DNA, MLST targets specific genes with allelic variation. As stated above, *vir* typing might offer additional information but is not recommended for routine epidemiology. Since the number of isolates in our study was limited, any conclusion on the role of *prtFI* as an epidemiological marker will require further study.

Antimicrobial susceptibility

All invasive isolates were fully susceptible to penicillin, clindamycin, chloramphenicol, ofloxacin, ciprofloxacin, spiramycin, vancomycin and quinupristin/dalfopristin. Only one invasive and five non-invasive isolates were macrolide-resistant (3 % and 5 %, respectively). The invasive isolate showed the iMLS phenotype (and harboured *ermA*), whereas four out of five non-invasive strains were of M phenotype (*mefA*) and one showed cMLS resistance (*ermB*). These low macrolide resistance rates were similar to those found in Scandinavia (Littauer *et al.*, 2006; Luca-Harari *et al.*, 2008), but different from those in other European countries (Bingen *et al.*, 2004; Creti *et al.*, 2007).

In contrast, tetracycline resistance was noted among 20 (54 %) invasive and 36 (35 %) control isolates, comprising 24 different *emm* types. The determinant *tetM* was detected in 83 % and 97 % of resistant invasive and control strains, respectively. None of the five *emm*1 invasive strains and only one out of 30 *emm*1 non-invasive strains was resistant. A low percentage (10 %) of tetracycline-resistance was also found among *emm*12 non-invasive isolates. Since tetracycline is not the drug of choice in the treatment of GAS infections, the high rate of resistance among Romanian

Table 3. Presence of the fibronectin-binding protein gene (*prtF1*) in invasive and non-invasive strains isolated from patients with different clinical presentations during 2003 and 2004 in Romania

NA, Data not available.

Invasive			Control		
Clinical presentation	<i>prtF1</i>		Clinical presentation	<i>prtF1</i>	
	Positive	Negative		Positive	Negative
Abscess	2	0	Abscess	1	0
Bacteraemia	1	2	Asymptomatic carrier	13	4
Bronchopneumonia	1	0	Cellulitis	1	0
Cellulitis	6	3	Endometritis	1	0
Cervical adenitis	0	1	Eye infection	1	0
Erysipelas	0	1	HIV-infected patient	1	0
Meningitis	0	1	Impetigo	2	2
Necrotizing fasciitis	3	1	Otitis media	3	0
Osteomyelitis	2	0	Parotitis	1	0
Peritonitis	1	0	Pharyngotonsillitis	27	3
Septic arthritis	0	1	Pneumonia	1	0
STSS	0	1	Pyelonephritis	1	0
Other	2	0	Pyoderma	0	2
NA	2	2	Scarlet fever	1	0
			Wound	3	4
Total	20	13	Total	57	15

Fig. 2. Dendrogram based on PFGE patterns of the 45 new and old invasive *S. pyogenes* isolates in Romania, as determined by *emm*- and *vir*-typing (*emm*ST and VT, respectively). ND, Not done. PFGE patterns of non-invasive Romanian T12 and T1 isolates are shown in Supplementary Fig. S1, available in JMM Online.

Table 4. Molecular characterization of the 33 Romanian recent invasive GAS strains collected during 2003–2004

T type	emm type	PFGE pattern	No. of strains	ST*	Allele no. (similarity with other alleles)†						
					<i>gki</i>	<i>gtr</i>	<i>murL</i>	<i>mutS</i>	<i>recP</i>	<i>xpt</i>	<i>yqiL</i>
1	1	A	4	28	4	3	4	4	4	2	4
1	1	A	1	440 (SLV28)	4	3	4	55 (99.4 %)	4	2	4
4	4	S	1	38	5	7	8	5	15	2	1
8	8	NT	1	59	13	2	8	19	1	3	4
9	9	C1	1	75	15	14	7	18	19	3	1
12	12	G	1	436 (SLV36)	1	2	2	6	6	2	2
25	25	D	1	150	11	2	1	3	50	8	7
14	49	J	1	30	4	6	2	7	21	7	1
14	49	J1	1	437 (SLV30)	4	6	2	7	21	7	2
14	49	J2	1	441 (DLV29)	4	6	2	7	7	3	70 (99.5 %)
3	64	L	1	164	2	2	8	3	5	2	29
3/13/B3264	65/69	R	1	255	16	42	34	7	1	2	17
8/25/Imp19	75	D	1	150	11	2	1	3	50	8	7
25	75	F	1	150	11	2	1	3	50	8	7
12	76	B	2	378	11	6	3	6	6	27	46
12	76	B1	1	378	11	6	3	6	6	27	46
12	76	B	1	50	11	6	3	6	6	27	4
NT	78	N	1	253	16	2	11	3	1	13	1
12	81	C	2	341	91	2	65	7	1	3	60
3/13	81	C	1	341	91	2	65	7	1	3	60
12	81	C	1	434 (SLV341)	91	2	65	7	1	3	2
25/Imp19	91	Q	1	13	2	6	2	5	22	3	2
Imp19	92	P	1	435	63	2	7	5	2	2	2
NT	95	K	1	14	2	6	8	3	9	3	1
6	95	O	1	14	2	6	8	3	9	3	1
3/13	100	M	1	442 (DLV340)	101 (99.2 %)	2	8	3	2	3	71 (99.2 %)
NT	106	E	1	438 (DLV140)	51	2	14	26	43	35	2
NT	119	H	1	439 (SLV13)	2	6	2	5	87 (99.22 %)	3	2

*Single-locus variants (SLV) and double-locus variants (DLV) differ from another ST at only one or two of the seven MLST loci, respectively. This change represents new alleles or a change to another known allele and the strains are considered to be closely related (<http://spyogenes.mlst.net>; McGregor & Spratt, 2005).

†New STs/alleles or changed alleles resulting in new STs are indicated by bold type.

isolates is not readily explainable. However, the extensive use of tetracycline in the treatment of a variety of human and veterinary infections might have contributed to the dissemination of resistance among GAS isolates.

Since changes in the epidemiology of GAS infections, in particular the increase of severe cases, are noted worldwide, active surveillance is of high priority in the process of improving prophylactic measures and treatment strategies. However, the small number of isolates collected in the present study precludes an exact picture of the actual situation of invasive GAS disease in Romania. The limitations of surveillance (e.g. low clinical awareness, lack of network of laboratories and insufficient reporting strategies) need to be addressed in order to obtain a real image of the national incidence of these diseases. Epidemiological studies analysing the distribution of circulating *emm* types have clearly become increasingly important for the development of multivalent M-protein vaccines. This is one of few reports from Eastern Europe

that combines clinical information and microbiological findings of invasive and non-invasive GAS infections.

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