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ORIGINAL PAPER

# A New Rapid and Cost-Effective Method for Detection of Phages, ICEs and Virulence Factors Encoded by *Streptococcus pyogenes*

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Abstract

*Streptococcus pyogenes* (group A *Streptococcus*, GAS) is a human pathogen that causes diseases of various intensity, from mild strep throat to life threatening invasive infections and postinfectional sequelae. *S. pyogenes* encodes multiple, often phage encoded, virulence factors and their presence is related to severity of the disease. Acquisition of mobile genetic elements, carrying virulence factors, as phages or ICEs (integrative and cojugative elements) has been shown previously to promote selection of virulent clones. We designed the system of eight low volume multi- and one singleplex PCR reactions to detect genes encoding twenty virulence factors (*spd3*, *sdc*, *sdaB*, *sdaD*, *speB*, *spyCEP*, *scpA*, *mac*, *sic*, *speL*, *K*, *M*, *C*, *I*, *A*, *H*, *G*, *J*, *smeZ* and *ssa*) and twenty one phage and ICE integration sites described so far for *S. pyogenes*. Classification of strains based on the phage and virulence factors absence or presence, correlates with PFGE MLST and *emm* typing results. We developed a novel, fast and cost effective system that can be used to detect GAS virulence factors. Moreover, this system may become an alternative and effective system to differentiate between GAS strains.

Key words: *Streptococcus pyogenes*, GAS, superantigens, virulence factors, typing, phage

## Introduction

*Streptococcus pyogenes* (group A *Streptococcus*, GAS) is an important human pathogen that causes a broad spectrum of skin and mucosal surface infections. GAS diseases range from mild, such as streptococcal pharyngitis or impetigo, to severe toxin-mediated, among which are necrotizing fasciitis or toxic shock syndrome and postinfectious diseases (Cunningham, 2000). GAS is responsible for over 600 millions of new infections every year and causes half a million deaths as a result of infections and post-infectional sequelae (Carapetis *et al.*, 2005).

The success of GAS as a pathogen relies on the production of multiple virulence factors involved in various aspects of host-pathogen interactions (Tart *et al.*, 2007). Initial contact between bacteria and the host is achieved by the activity of multiple adhesins produced by GAS which bind host proteins and extracellular matrix proteins such as plasminogen, collagen, keratin (for a review see Courtney *et al.*, 2002; Cunningham, 2000; Oehmcke *et al.*, 2010; Smeesters *et al.*, 2010). After initial contact, bacteria invade host tissues and often disseminate causing systemic reaction (Tart *et al.*, 2007). Multiple classes of GAS virulence factors such as proteases, DNases and pyrogenic toxins (superantigens) are involved in interaction between bacteria and the host in post attachment phase.

Major surface adhesin – M protein, is involved in tissue invasion and interaction with human immune system (Perez-Caballero *et al.*, 2004). Other elements of the human immune system are inactivated by set of specialized proteases. ScpA a highly specific peptidase encoded by *scpA* gene degrades C5a factor of the complement (Cleary *et al.*, 1992). SpeB is a cysteine protease that can inactivate C3b factor of the complement (Terao *et al.*, 2008) and multiple other host factors involved in immune response such as interleukin-1b precursor and immunoglobulins (Chiang-Ni and Wu, 2008). In addition SpeB is involved in tissue destruction by activation of pro-matrix metallo-proteases and degradation of fibronectin, vitronectin, plasminogen and kininogen (Chiang-Ni and Wu, 2008). The protease MAC/IdeS cleaves specifically human IgG

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(von Pawel-Rammingen *et al.*, 2002). Recently discovered protease SpyCEP is involved in degradation of chemokines and chemotactic factors as interleukin-8, granulocyte chemotactic protein-2, growth related oncogene α and β and macrophage inflammatory protein 2-α (Edwards *et al.*, 2005; Kurupati *et al.*, 2010; Sumby *et al.*, 2008; Zinkernagel *et al.*, 2008).

DNases produced by GAS are involved in dissemination of bacteria and escape from neutrophil extracellular traps (Sumby *et al.*, 2005; Walker *et al.*, 2007). And finally, large group of toxins encoded by GAS genes (*speL*, *speK*, *speM*, *speC*, *speI*, *speA*, *speH*, *speG*, *speJ*, *smeZ* and *ssa*) is involved in systemic toxicity.

Some of the GAS virulence factors, *e.g.* SpeB, ScpA, SpyCEP, Mac, SdaB and SpeG, are chromosomally encoded, however, large fraction of virulence factors such as majority of DNAses and superantigens *e.g.* SpeA, SpeC , SpeH and SSA are encoded by mobile genetic elements – phages and conjugative mobile elements integrated into the chromosome (ICEs – integrative and cojugative elements) (Beres and Musser, 2007). Based on the comparison of genome sequences of multiple GAS strains, metagenome of GAS contains on average about 10% of exogenous elements (Beres and Musser, 2007; Ferretti *et al.*, 2001). So far, 67 mobile elements (55 prophages and 12 ICE elements) integrated at 21 distinct loci of the core chromosome in the 12 GAS genomes have been identified (Beres and Musser, 2007) (Table I).

Over the years, multiple serological, restriction fragment based and PCR based methods of GAS typing and virulence factors detection were used (Cleary *et al.*, 1988; Commons *et al.*, 2008; Hartas *et al.*, 1998; Koller *et al.*, 2010; Lintges *et al.*, 2007; Matsumoto *et al.*, 2003; Maxted *et al.*, 1973; Moody *et al.*, 1965; Nandi *et al.*, 2008; Schmitz *et al.*, 2003; Seppala *et al.*, 1994; Swift *et al.*, 1943). Each of the methods presents various advantages and disadvantages. Serological assays are usually less precise than molecular methods. Methods based on the analysis of restriction patterns, and methods based on random amplification, are often difficult for analysis and comparison. Multiple PCR assays utilizing specific targets were developed before the era of massive genome sequencing that allows including the knowledge of allelic variations between strains of various serotypes in the design of more specific systems. Also, multiple PCR based systems were designed mostly as singleplex reactions what increases screening costs in case of detection of multiple virulence factors.

Currently, to determine the relationships between GAS isolates and strains, three major methods are typically used. The first method, which is regarded as a golden standard by many laboratories, is pulsed field gel electrophoresis (PFGE) typing (Bert *et al.*, 1997). PFGE is often recommended as a reference method in outbreak investigations, especially for food borne diseases (http://www.cdc.gov/pulsenet/, http://www.medvetnet.org/cms/). PFGE is a method based on restriction fragment size polymorphism. Chromosomal DNA is released from bacteria and digested with rare cutting restriction enzyme directly in agarose gel and fragments are separated using alternating voltage gradient (for a review see (Herschleb *et al.*, 2007; Slater, 2009)). PFGE typing detects rather large and recent evolutionary changes in bacterial DNA such as insertion or excision of a phage, large insertions and deletions and mutations resulting in a loss or appearance of a new restriction site (Tenover *et al.*, 1995). Two strains are related to each other when the number of differences in restriction patterns is below 7 (Tenover *et al.*, 1995). PFGE is a technique with high discriminatory power, but it is time consuming and available protocols require from minimum two days to over one week to determine PFGE type of the strain (Herschleb *et al.*, 2007). What's equally important, the technique requires relatively expensive equipment, skilled labor and the results of PFGE are often difficult to compare between laboratories.

The second method used routinely in GAS epidemiology is *emm* typing. *Emm* typing, is a molecular equivalent of serotyping and allows grouping of GAS strains into serotypes/genotypes based on the type of the surface M protein (Facklam *et al.*, 1999; Hoe *et al.*, 1999). It is an easy and straightforward method that utilizes sequencing of a portion of the *emm* gene, which encodes hypervariable region of the M protein. The major advantage of molecular *emm* typing is rapid identification of novel variants of M protein responsible for new serotypes. The *emm* typing requires PCR amplification, purification of the amplicon and single sequencing reaction as a next step (Beall *et al.*, 2000).

The third method, multi-locus sequence typing (MLST), is based on sequencing of seven housekeeping loci to detect allelic changes. Differences in allelic profiles in isolates are assigned to known or new sequence types (STs) (Enright *et al.*, 2001). Similarly to *emm* typing the method requires PCR amplification, purification of the product and sequencing. The method is relatively fast and reliable but in case of GAS, requires 14 sequencing reactions per isolate, which significantly increases the costs of typing. Because of the cost of MLST, alternative approaches to detect allelic changes in genes included in MLST scheme are developed, such as PCR assays with high resolution melting curves named Mini-MLST (Richardson *et al.*, 2010).

In this report, we present a rapid and cost effective method to detect virulence factors encoded by GAS and phages/ICE elements integrated into genome using set of multiplex PCR reactions. Described system allows easy, simultaneous detection of 20 GAS virulence

Table I
Integration sites of phages and ICE elements in sequenced *S. pyogenes* genomes (Modified after Beres and Musser, 2007)

| Integration site | Strain | Exogenous Element | Virulence Gene(s) | CDS Start of the integrated element | CDS Stop of the integrated element |
|---|---|---|---|---|---|
| A | MGAS10394 | 10394.1 | sdn | 0020 | 0068 |
| B | MGAS8232 | 8232.1 | speA1 | 0336 | 0394 |
| C | SF370 | 370.1 | speC-spd1 | 0655 | 0712 |
| C | MGAS10270 | 10270.1 | speC-spd1 | 0536 | 0598 |
| C | MGAS10750 | 10750.1 | speC-spd1 | 0560 | 0622 |
| C | Manfredo | man.4 | speC-spd1 | 1263 | 1322 |
| C | MGAS2096 | 2096.1 | speC-spd1 | 0553 | 0602 |
| C | MGAS9429 | 9429.1 | speC-spd1 | 0532 | 0594 |
| C | MGAS8232 | 8232.2 | speC-spd1 | 0716 | 0779 |
| D | MGAS10394 | 10394.2 | speA4 | 0733 | 0741 |
| E | SF370 | 370.2 | speH-speI | 0937 | 1008 |
| E | MGAS10270 | 10270.2 | spd3 | 0796 | 0853 |
| E | MGAS10750 | 10750.2 | spd3 | 0831 | 0889 |
| E | Manfredo | man.3 | speH-speI | 1021 | 1070 |
| E | MGAS9429 | 9429.2 | speH-speI | 0795 | 0851 |
| F | MGAS315 | 315.1 | none | 0681 | 0736 |
| F | SSI | SPsP5 | none | 0877 | 0937 |
| G | SF370 | 370-RD.1 | srtA | 1075 | 1088 |
| G | MGAS5005 | 5005-RD.1 | srtA | 0797 | 0816 |
| G | MGAS10270 | 10270-RD.1 | srtA | 0910 | 0932 |
| G | MGAS10750 | 10750-RD.1 | srtA | 0945 | 0967 |
| G | MGAS2096 | 2096-RD.1 | srtA | 0869 | 0890 |
| G | MGAS9429 | 9429-RD.1 | srtA | 0911 | 0934 |
| G | MGAS6180 | 6180-RD.0 | srtA | 0771 | 0793 |
| H | MGAS5005 | 5005.1 | speA2 | 0995 | 1052 |
| H | MGAS315 | 315.2 | ssa | 0919 | 0978 |
| H | SSI | SPsP6 | ssa | 1118 | 1172 |
| H | MGAS10394 | 10394. 3 | speK-sla | 0982 | 1026 |
| H | MGAS8232 | 8232.3 | speL-speM | 1238 | 1309 |
| H | MGAS6180 | 6180.1 | speC-spd1 | 0967 | 1033 |
| I | MGAS2096 | 2096-RD.2 | tet (O) | 1103 | 1159 |
| I | MGAS6180 | 6180-RD.1 | none | 1079 | 1089 |
| J | MGAS10394 | 10394.4 | mef(A), R6 | 1123 | 1173 |
| K | SF370 | 370.3 | spd3 | 1436 | 1488 |
| K | MGAS5005 | 5005.2 | spd3 | 1168 | 1222 |
| K | MGAS315 | 315.3 | spd4 | 1094 | 1145 |
| K | SSI | SpsP4 | spd4 | 0717 | 0771 |
| K | MGAS10750 | 10750.3 | ssa | 1276 | 1328 |
| K | Manfredo | man.2 | spd4 | 0631 | 0692 |
| K | MGAS10394 | 10394.5 | speC-spd1 | 1194 | 1242 |
| K | MGAS8232 | 8232.4 | spd3 | 1444 | 1506 |
| L | MGAS10270 | 10270.3 | speK-sla | 1297 | 1361 |
| L | MGAS315 | 315.4 | speK-sla | 1203 | 1266 |
| L | SSI | SPsP3 | speK-sla | 0597 | 0659 |
| L | MGAS6180 | 6180.2 | speK-sla | 1220 | 1285 |
| M | MGAS10270 | 10270-RD.2 | R28 | 1378 | 1411 |

Table I continued

| Integration site | Strain | Exogenous Element | Virulence Gene(s) | CDS Start of the integrated element | CDS Stop of the integrated element |
|---|---|---|---|---|---|
| M | MGAS6180 | 6180-RD.2 | R28 | 1302 | 1337 |
| N | MGAS315 | 315.5 | speA3 | 1300 | 1354 |
| N | SSI | SPsP2 | speA3 | 0507 | 0561 |
| N | Manfredo | man.1 | spd3 | 0471 | 0535 |
| N | MGAS10394 | 10394.6 | sda | 1338 | 1366 |
| O | MGAS315 | 315.6 | sdn | 1408 | 1458 |
| O | SSI-1 | SPsP1 | sdn | 0408 | 0456 |
| P | MGAS5005 | 5005.3 | sda | 1414 | 1467 |
| P | MGAS2096 | 2096.2 | sda | 1440 | 1492 |
| P | MGAS9429 | 9429.3 | sda | 1415 | 1468 |
| P | MGAS8232 | 8232.5 | sda | 1745 | 1808 |
| R | MGAS10394 | 10394.7 | spd3 | 1540 | 1562 |
| S | MGAS10750 | 10750-RD.2 | erm(A) | 1679 | 1719 |
| T | SF370 | 370.4 | none | 2122 | 2147 |
| T | MGAS10270 | 10270.4 | none | 1874 | 1896 |
| T | MGAS10750 | 10750.4 | none | 1897 | 1921 |
| T | Manfredo | man.5 | none | 1764 | 1779 |
| T | MGAS10394 | 10394.8 | none | 1804 | 1824 |
| T | MGAS6180 | 6180.3 | none | 1789 | 1813 |
| U | MGAS10270 | 10270.5 | none | 1917 | 1951 |
| U | MGAS6180 | 6180.4 | none | 1840 | 1864 |

factors (VF) and screening of 21 phage and ICE integration sites. The described PCR based method combined with *emm* typing can be effectively used to differentiate between GAS strains.

## Experimental

### Materials and Methods

**Bacterial isolates.** Over 650 highly diverse GAS isolates analyzed in the study were sent to KORLD (National Reference Center for Antibiotic Resistance) and KOROUN (National Reference Center for Infections of Central Nervous System) as a part of routine reference activity and as a part of BiNet network for monitoring invasive infections (http://www.koroun.edu.pl/binet_info01.php). Bacterial strains were sent from over 60 laboratories located in multiple geographical areas of Poland and were isolated from various forms of the GAS diseases (throat, skin and invasive infections). *Emm* types of the strains were determined as routine part of diagnostic work according to (Beall *et al.*, 1996) and CDC's recommendations. In addition, we used highly clonal population of strains which PFGE patterns, *emm* and ST types were previously determined (Szczypa *et al.*, 2004).

**PFGE.** PFGE analysis was performed according to modified method by Stanley and co-workers (Stanley *et al.*, 1995). Briefly, agarose plugs containing bacteria were incubated for 4 h at 37°C in lysis buffer with lysozyme (100 µg/ml, Sigma) and mutanolysin (40 µg/ml, Sigma), followed by overnight treatment with proteinase K (1 mg/ml). DNA embedded in plugs was digested with *SmaI* (Fermentas) for 4h, and separated at 14°C for 22 h in CHEF-DR III system (Bio-Rad) in 0.5x TBE buffer, with 6V/cm, initial pulse 1 s., final pulse 30 s.

**Isolation of chromosomal DNA**. Chromosomal DNA was isolated from cells grown overnight on Columbia agar plates supplemented with 5% sheep blood (BioRad, BioMerieux) using the Genomic Mini AX BACTERIA kit (A&A Biotechnology) or the Genomic Mini kit (A&A Biotechnology) according to the manufacturer's protocol, with additional initial cell wall digestion with lysozyme (1 mg/ml, Sigma) and mutanolysin (500 U/ml, Sigma) for 30 min at 37°C, in the presence of RNAse. Chromosomal DNA used as a template for PCR reactions was diluted 10-fold.

**Primer design and specificity tests.** Primer pairs were designed using the modified Primer3 software, available as the Primer-BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed to conserved regions of detected genes (in case of virulence factors) or conserved regions

Table II
Primers used in this study

| Name | Sequence | Size (bp) | Position in reference sequence | Ref. sequence |
|---|---|---|---|---|
| Toxins MIX I | | | | |
| SpeL F | CCTGAGCCGTGAAATTCCCA | 657 | 1041734–1041753 | NC_003485 |
| SpeL R | ACACCAGAATTGTCGTTTGGT | | 1042370– 1042390 | |
| SpeK F | CCTTGTGTGTGTATCGCTTGC | 568 | 39278 – 39298 | NC_004587 |
| SpeK R | TTGCTGTCCCCCATCAAACT | | 39825 – 39844 | |
| SpeM F | ATCGCTCATCAAACTTTTCCT | 496 | 1042875–1042895 | NC_003485 |
| SpeM R | CCTTGTGTGTGTATCGCTTGC | | 1043350–1043370 | |
| SpeC F | GCCAATTTCGATTCTGCCGC | 405 | 617333–617352 | NC_003485 |
| SpeC R | TGCAGGGTAAATTTTTCAACGACA | | 617715–617737 | |
| SmeZ F | TTTCTCGTCCTGTGTTTGGA | 246 | 1662332–1662351 | NC_007297 |
| SmeZ R | TTCCAATCAAATGGGACGGAGAACA | | 1662554 – 1662578 | |
| SpeI F | TTCATAGACGGCGTTCAACAA | 176 | 819507–819527 | NC_002737 |
| SpeI R | TGAAATCTAGAGGAGCGGCCA | | 819662–819682 | |
| Toxins MIX II | | | | |
| ssa F | AAGAATACTCGTTGTAGCATGTGT | 678 | 39833–39856 | NC_004585 |
| ssa R | AATATTGCTCCAGGTGCGGG | | 40491–40510 | |
| SpeA F | AGGTAGACTTCAATTTGGCTTGTGT | 576 | 331570–331594 | NC_003485 |
| SpeA R | GGGTGACCCTGTTACTCACGA | | 332125–332145 | |
| SpeH F | TGAGATATAATTGTCGCTACTCACAT | 480 | 786364–786389 | NC_011375 |
| SpeH R | CCTGAGCGGTTACTTTCGGT | | 786824–786843 | |
| SpeG F | TGGAAGTCAATTAGCTTATGCAG | 384 | 183579 – 183601 | NC_004070 |
| SpeG R | GCGAACAACCTCAGAGGGCAAA | | 183942 – 183962 | |
| SpeJ F | TCCTTGTACTAGATGAGGTTGCAT | 286 | 364343 – 364366 | NC_007296 |
| SpeJ R | GGTGGGGTTACACCATCAGT | | 364609 – 364628 | |
| DNases | | | | |
| spd3 F | ATCGTCGTACTTGGCAAGGTT | 784 | 1146098–1146118 | NC_007297 |
| spd3 R | GCCGCTTCTTCAAACTCTTCG | | 1146861–1146881 | |
| sdc F | AAGCTTAGAAACTCTCTCGCCA | 600 | 49–70 | AF410852 |
| sdc R | AGTTCCAGTAATAGCGTTTTTCCGT | | 624–648 | |
| sdaB F | TATAGCGCATGCCGCCTTTT | 440 | 1700383–1700402 | NC_007297 |
| sdaB R | TGATGGCGCAAGCAAGTACC | | 1700803–1700822 | |
| sdaD F | TTTACGCTGAATCGGGCACT | 295 | 1385864–1385883 | NC_007297 |
| sdaD R | GGCTCTGGTTTGCTTTCCCA | | 1386139–1386158 | |
| Proteases/inhibitors | | | | |
| speB_F | AGACGGAAGAAGCCGTCAGA | 952 | 1698752– 1698771 | NC_002737 |
| speB_R | TCAAAGCAGGTGCACGAAGC | | 1699684–1699703 | |
| spyCEP F | GATCCGGCCCATCAAAGCAT | 786 | 344582–344601 | AE004092 |
| spyCEP R | AGCTGCCACTGATGTTGGTG | | 345348–345367 | |
| scpA F | GCTCGGTTACCTCACTTGTCC | 622 | 1669854 – 1669874 | NC_009332 |
| scpA R | CAATAGCAGCAAACAAGTCACC | | 1670453–1670477 | |
| Mac F | TCTTGCCCTGTTGAAAGTGT | 389 | 681947–681966 | NC_011375 |
| Mac R | CGAGGTGGTATTTTTGACGCC | | 682315–682335 | |
| sic F | TTACGTTGCTGATGGTGTATATGGT | 150 | 1682672–1682696 | NC_002737 |
| sic R | TTTGATAGAGGGTTTTCAGCTGGC | | 1682798–1682821 | |
| Phages MIX 1 | | | | |
| phageA_F | AGCTTCGTCAGTTCATTGATGAGT | 343 | 34380–34403 | NC_007297 |
| phageA_R | GGAGTTAATCTTTGTCTGATCACCGT | | 34723–34698 | |

Table II continued

| Name | Sequence | Size (bp) | Position in reference sequence | Ref. sequence |
|---|---|---|---|---|
| phageG_F | ACTTGAAGAAGCTGGAGCAACA | 477 | 809606–809627 | NC_004070 |
| phageG_R | AGGCAATAGCATCTGGCGTC | | 810052–810033 | |
| phageB_F | ATCAGTCGCGCCTACCGTAT | 636 | 301872–301891 | NC_007297 |
| phageB_R | TTACTAGAAGGGGCCTGCCG | | 302508–302489 | |
| phageE_F | TGAGACATGGTGGAAAGCAGA | 1022 | 739495–739515 | NC_007297 |
| phageE_R | TGGTCGAAATAACCAAGGGCA | | 740517–740497 | |
| phageD_F | ACGCTTGACTGACTTCGGTG | 1168 | 720561–720580 | NC_007297 |
| phageD_R | TGGGACTTATCCGTTGTCACG | | 721729–721709 | |
| Phages MIX2 | | | | |
| phageK_F | TGGTCTGCCATCCATTGTCT | 425 | 1195963–1195982 | NC_008022 |
| phageK_R | AGCCTTCAAAGCTGGTAAAGCT | | 1196377–1196356 | |
| phageJ_F | TGATCCATGGTGACCTGCTT | 563 | 1123319–1123338 | NC_007297 |
| phageJ_R | TCGACATTGGCCAGGGAGAT | | 1123881–1123862 | |
| phageC_F | ATTGCAACAGGTAGCCCAGC | 670 | 531240–531259 | NC_007297 |
| phageC_R | CTTCACGCGCAGAACGGATA | | 531910–531891 | |
| phageH_F | AGGCTTTTGAATTACGTTTTGTC | 870 | 1058226–1058248 | NC_008022 |
| phageH_R | TGAATCAGACGGTTGAGGCT | | 1059095–1059076 | |
| phageM_F | CCACAGCTGTTTCAACACTTTCA | 1143 | 1252437–1252459 | NC_007297 |
| phageM_R | AATTGGCGCTCGGACATGAT | | 1253579–1253560 | |
| Phages MIX3 | | | | |
| phageN_F | TCACCGTTAATTCCCATTCGCT | 349 | 1282773–1282794 | NC_007297 |
| phageN_R | CCGTAGGACAGTTGGGCAAA | | 1283121–1283102 | |
| phageO_F | TCACAAAAGCCAGTTGGTCGAT | 452 | 1341889–1341910 | NC_007297 |
| phageO_R | TATCGTCGTGACTACCGGCT | | 1342340–1342321 | |
| phageP_F | CTAAGGATGTAGTCACTACCCATTTTGTC | 544 | 1493473–1493501 | NC_004070 |
| phageP_R | TCTGGCTTGACTTACACGCT | | 1494016–1493997 | |
| phageI_F | GGTGCCACGTAATGATAACTTGTTC | 666 | 1069901–1069925 | NC_007297 |
| phageI_R | GTAGACCCGCCACGAAAAGG | | 1070566–1070547 | |
| phageL_F | GCCAACTGGCCATTTTCTGC | 899 | 1236869–1236888 | NC_007297 |
| phageL_R | AAGCAAGGAAATGATCGCGG | | 1237767–1237748 | |
| Phages MIX4 | | | | |
| phageQ_F | CCAGCCATAATCTCAGTTGAGACAGTTG | 364 | 1434160–1434187 | NC_007297 |
| phageQ_R | GGTTCCATCCAAATCAATGGCAATC | | 1434523–1434499 | |
| phageR_F | AACGACGTTGCCCTTCCGCA | 432 | 1512239–1512258 | NC_007297 |
| phageR_R | TCCAAGCTCCTGGCTCGAATGT | | 1512670–1512649 | |
| phageT_F | CGCTGGCCTTTCTACAACTTCACCA | 555 | 1772901–1772925 | NC_007297 |
| phageT_R | AGCAACGCTTGAAAAAGATGGCGAT | | 1773455–1773431 | |
| phageU_F | CTCTTCCCTTTTGTCTGCTAACGGT | 671 | 1796895–1796919 | NC_007297 |
| phageU_R | CCACGGTCACATCCTTGTTGACGG | | 1797565–1797542 | |
| phageS_F | ACACTGACCTTTGAAAAACTCATCCA | 917 | 1586507–1586532 | NC_007297 |
| phageS_R | ATGATAATAGTCGTAGGGATGCTTGTATTATAAAA | | 1587423–1587389 | |
| Primers to detect integration into F site | | | | |
| PhageF_F | CCCGAAGTGAAATCGATGATTGACA | ~1000 – | 778913–778937 | NC_007297 |
| | TCCCACGCTCACGCTCCAAA | ~3000 | 780465–780446 | NC_007297 |
| Control primers for phage detection | | | | |
| DnaA_F | TGCCGAAGCTATTCGCGCCA | 240 | 1227–1246 | NC_007297 |
| DnaA_R | ACTGTTGAATGGTCTCTGCCACCA | | 1466–1443 | |

Table III
PCR composition of the multiplex reactions

| Reagent | Toxins MIX I | Toxins MIX II and proteases | DNAses | Phages MIX 1,2 and 4 | Phages MIX 3 | Phage F |
|---|---|---|---|---|---|---|
| 100 µM or 10 µM primers mix | 0.6 µl | 0.5 µl | 0.4 µl | 0.6 µl | 0.7 µl | 0.2 µl |
| 10x Taq polymerase buffer with $(NH_4)_2SO_4$ (Fermentas) | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| 25 mM $MgCl_2$ | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| 1 mM dNTP | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl | 0.5 µl |
| water | – – – | – – – | – – – | – – – | – – – | 2.15 µl |
| 10x diluted chromosomal DNA template | 2.8 µl | 2.9µl | 3.0 µl | 2.8 µl | 2.7 µl | 1 µl |
| Taq polymerase (Fermentas) | 0.1 µl (0.5 U) | 0.1 µl (0.5 U) | 0.1 µl (0.5 U) | 0.1 µl (0.5 U) | 0.1 µl (0.5 U) | 0.1 5µl (0.75 U) |

of genes surrounding phage integration site. Primer sequences, their chromosomal location and accession number of reference sequence are listed in Table II.

**Composition of the reaction and PCR conditions.** To detect 20 virulence factors, four multiplex reactions were designed. To detect 21 mobile genetic element integration sites, four multiplex and one singleplex reaction were designed. Composition of all primer mixes and size of the amplicons generated in multiplex reactions are listed in Table II. For the ease of use, equal volumes of 100 µM primer stocks were mixed into appropriate mixes, namely: "Toxins MIX I", "Toxins MIX II", "proteases", "DNAses". For the case of use, equal volumes of 10 µM stocks were mixed into: "Phages MIX 1", "Phages MIX 2", "Phages MIX 3" and "Phages MIX 4". To avoid degradation, primers premixes were aliquoted, so the single portion was sufficient to run the whole 96 well PCR plate without multiple freezing-thawing cycles. Final composition of each PCR reaction is presented in Table III. All PCR reactions were carried out in a total volume of 5 µl in a Veriti thermocycler (Applied Biosystems); conditions of PCR reaction are presented in Table IV.

**Statistical Analysis.** Simpson's Index of Diversity, and the Wallace Coefficient were calculated using online tool http://darwin.phyloviz.net/ (Carrico *et al.*, 2006; Pinto *et al.*, 2008). Analysis of strains was performed with Bionumerics package (Applied Maths).

Table IV
PCR reaction conditions used to amplify products in multiplex reactions

| | Toxins MIX I and II Dnases | | Proteases mix | | Phages 1–4 and phage F | |
|---|---|---|---|---|---|---|
| | T | t | T | t | T | t |
| Denaturation | 95°C | 0:15 | 95°C | 0:15 | 95°C | 0:15 |
| Annealing | 60°C | 0:20 | 52.5°C | 0:45 | 64°C | 0:30 |
| Elongation | 72°C | 2:00 | 72°C | 3:00 | 72°C | 3:30 |

All reactions were amplified for 40 cycles with initial denaturation was carried out for 3 min at 95°C, and final elongation for 7 min at 72°C
T – temperature; t – time

## Results and Discussion

**Detection of phages.** Phages/ICEs of group A *Streptococcus* are major sources of genetic diversity in this group of organisms, carriers of antibiotic resistance genes and multiple proven and putative virulence factors (Beres and Musser, 2007). Detection of integrated mobile elements can distinguish between GAS strains with closely related genetic backgrounds and with addition of other typing methods can be used in detailed epidemiological investigations (Beres *et al.*, 2010; Beres *et al.*, 2004).

Comparison of multiple GAS genomic sequences revealed 21 potential integration sites for phages and ICE elements (Beres and Musser, 2007) and Table I. To screen all 21 integration sites (named from A through U as in (Beres and Musser, 2007)), we designed set of four multiplex PCR reactions that are able to amplify products only when no element is integrated between open reading frames flanking integration site. Detection of the integrated phages and ICE elements is based on the assumption that in the standard PCR reaction, large (above 10 kb) element integrated into the chromosome cannot be efficiently amplified and furthermore detected. The designed primer pairs within single multiplex reaction had equal annealing temperatures and 100 bp or more size difference between products for easy product tracing. Primers were tested individually using an annealing gradient of temperatures from 55 to 72°C to select the optimal annealing temperature for multiplex PCR. Only primers that generated single amplicons were selected for composition of multiplex reactions (data not shown).

Because the lack of the PCR product denotes positive detection of large integrated element into particular integration site, to avoid PCR errors resulting from negative PCR amplification, in all multiplex reactions positive control of amplification (240 bp fragment of *dnaA* gene) is included. Examples of phage profile (PP) typing of randomly chosen strains from our GAS collection are

Table V
Simpson's Index of Diversity (SDI) and Wallace's coefficient (WC), calculated for strains analyzed by phage profiling (PP) and virulence factor profiling (VF)

**A.**

| Typing Method | # partitions | Simpson's ID | C.I. (95%) |
|---|---|---|---|
| Phage profile (PP) | 185 | 0.965 | (0.960–0.971) |
| Virulence factor profile (VF) | 95 | 0.943 | (0.936–0.951) |
| Virulence factor profile (VF) without "proteases mix" | 94 | 0.944 | (0.936–0.952) |
| *emm* type | 40 | 0.908 | (0.899–0.917) |

**B.**

| | PFGE | ST | *emm* | VF | PP |
|---|---|---|---|---|---|
| PFGE | | 1.000 (1.000–1.000) | 1.000 (1.000–1.000) | 0.990 (0.979–1.000) | 0.986 (0.974–0.997) |
| ST | 0.564 (0.379–0.749) | | 1.000 (1.000–1.000) | 0.899 (0.768–1.000) | 0.648 (0.472–0.824) |
| *emm* | 0.564 (0.379–0.749) | 1.000 (1.000–1.000) | | 0.899 (0.768–1.000) | 0.648 (0.472–0.824) |
| VF | 0.622 (0.430–0.813) | 1.000 (1.000–1.000) | 1.000 (1.000–1.000) | | 0.721 (0.556–0.886) |
| PP | 0.858 (0.703–1.000) | 1.000 (1.000–1.000) | 1.000 (1.000–1.000) | 1.000 (1.000–1.000) | |

Information about absence (0)/presence (1) of particular virulence factor or integrated element was concatenated into binary sequence of 20 or 21 digits and used for calculations with http://darwin.phyloviz.net/ComparingPartitions/. **A** SDI calculated for group of 656 divergent strains; **B.** WC calculated for group of highly clonal strains (PFGE pattern A from Szczypa, *et al.*, 2004)

presented in Figure 1. To test primer specificity, we preformed PP typing using two reference strains of known genomic sequence: MGAS6180 (NC_007296.1 (Green *et al.*, 2005)) and MGAS10270 (NC_008022 (Beres and Musser, 2007)). Based on the genomic sequence , strain MGAS6180 carries 7 elements integrated into sites G, H, I, L, M, T, U and MGAS10270 carries 7 elements integrated into sites C, E, G, L, M, T, U (Table I) ( Beres and Musser, 2007). In concordance with the predicted product presence and size, we were able to detect fragments that denote putative integration sites without inserted element, and we were not able to detect products that amplified large integrated element (Fig. 2). In some cases, such as for site G in strain MGAS10270 and site L in strain MGAS6180, very weak bands are observed and are probably a signal derived from a DNA isolated from fraction of GAS cells where the element was excised from the chromosome.

Two mobile elements 315.1 and SPsP5 are integrated into integration site "F" in M3 strain MGAS315 (between ORFs SpyM3_0680 and SpyM3_0737) and SSI-1 (between ORFs SPs0876 and SPs0938), respectively (Beres and Musser, 2007). However, based on the BLAST searches in all sequenced GAS genomes, the region encompassed by ORFs flanking prophage integration site varies in length and gene content in different strains. Therefore, primers detecting integrated element F amplify fragment of varying size, from about 1 kb to over 3 kb in the absence of integrated prophage. We decided to exclude primers detecting integration in the F site from multiplex reaction to increase PCR specificity and efficiency and run the reaction separately (Fig. 1E). With additional optimization of the reaction, primers detecting F integration site can be included in "phage mix 3", however detected bands are often weaker and gels more difficult for interpretation (Fig. 2).

To determine resolution of the phage profile detection as a typing method, we calculated Simpson's Index of Diversity (SID) based on analysis of highly diverse 656 GAS strains (Table VA). Among 40 *emm* types, we detected 185 distinct phage profiles with Simpson's Index of Diversity of 0.965 (CI 95% 0.960–0.971). Phage profile is also good predictor of *emm* type, with PP→*emm* Wallace's coefficient (WC) equal 0.953 (CI 95% 0.926–0.980).

Insertion or excision of large DNA fragments such as phages/ICEs from the chromosome usually is reflected in PFGE analysis. To test if the presence of integrated elements correlates with PFGE pattern, we analyzed homogenous population of strains previously described by Szczypa and co-workers (Szczypa *et al.*, 2004). Performed PP analysis showed that detection of elements inserted into putative integration sites correlates with PFGE patterns, *emm* type and ST (Fig. 3AB).

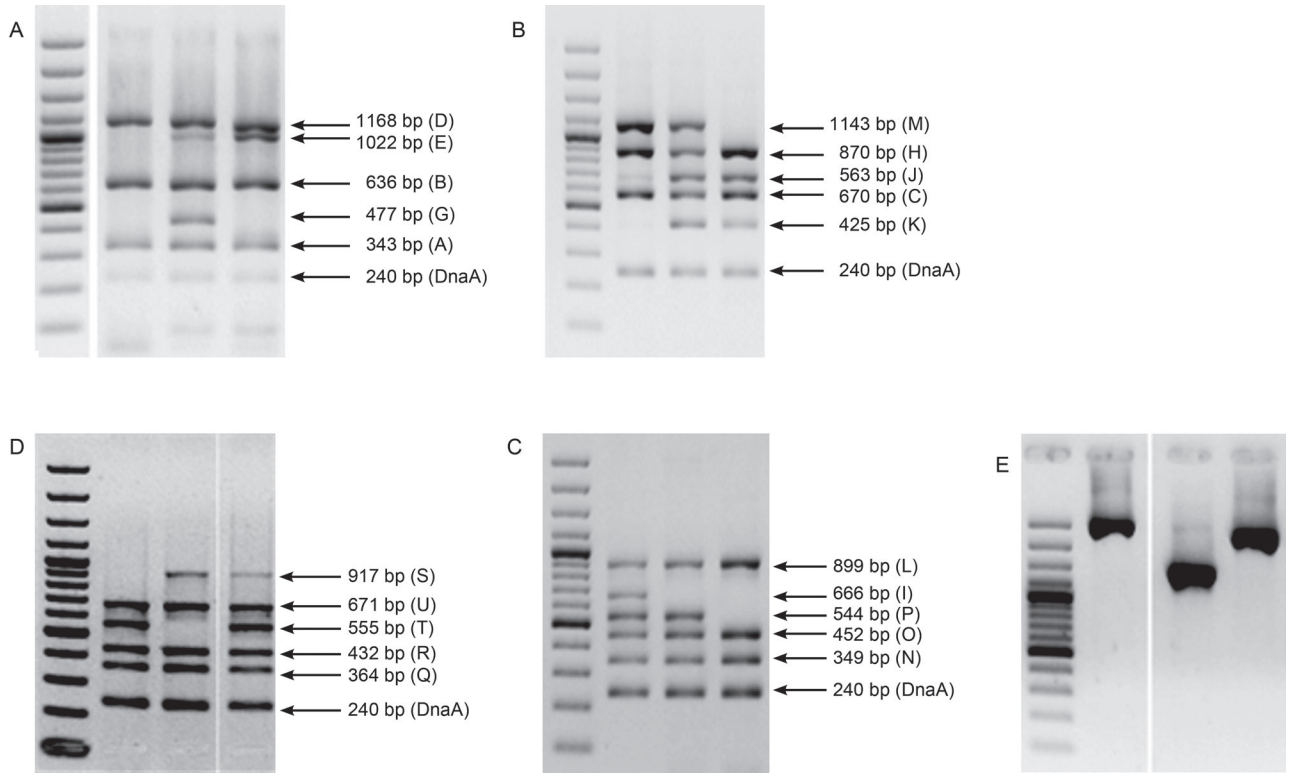


Fig. 1. Detection of twenty one GAS phage and ICE integration sites in randomly chosen GAS strains.

Each panel represents multiplex PCR reaction: A: Phage MIX1, B: Phage MIX2, C: Phage MIX3, D: Phage MIX4, E. Phage F Amplification of a product denotes lack of integrated element at the chromosomal location. Arrows denote expected product size based on the GAS genomic sequences, letters in parentheses denote the mobile element integration sites after (Beres and Musser, 2007) and Table I, 1.5% agarose/TBE, marker: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

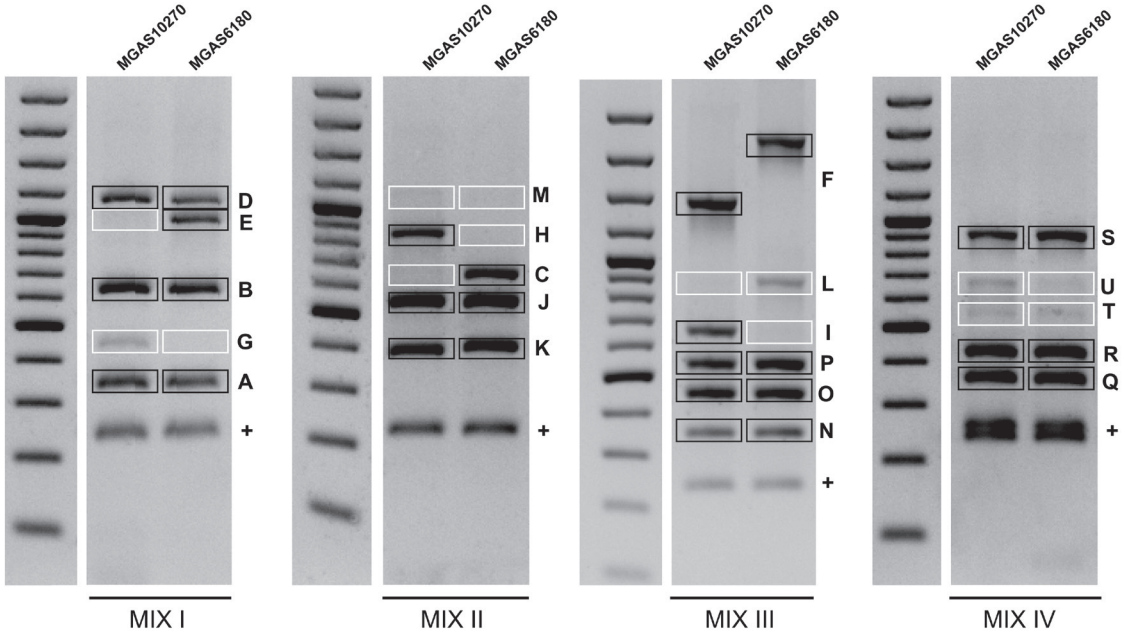


Fig. 2. Detection of integrated mobile genetic elements in reference strains MGAS6180 and MGAS10270.

Capital letters denote the mobile element integration sites (after (Beres and Musser, 2007) and Table I) detected by each multiplex reaction and "+" denotes positive control – amplification of *dnaA* fragment. Amplification of a product denotes lack of integrated element at the chromosomal location. Black boxes denote locations without integrated element and white boxes denote chromosomal locations with integrated mobile elements as annotated for the genomic sequences of MGAS6180 (sites G, H, I, L, M, T, U) (Green, *et al.*, 2005) and MGAS10270 (sites C, E, G, L, M, T, U). 1.5% agarose in TBE buffer, marker: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

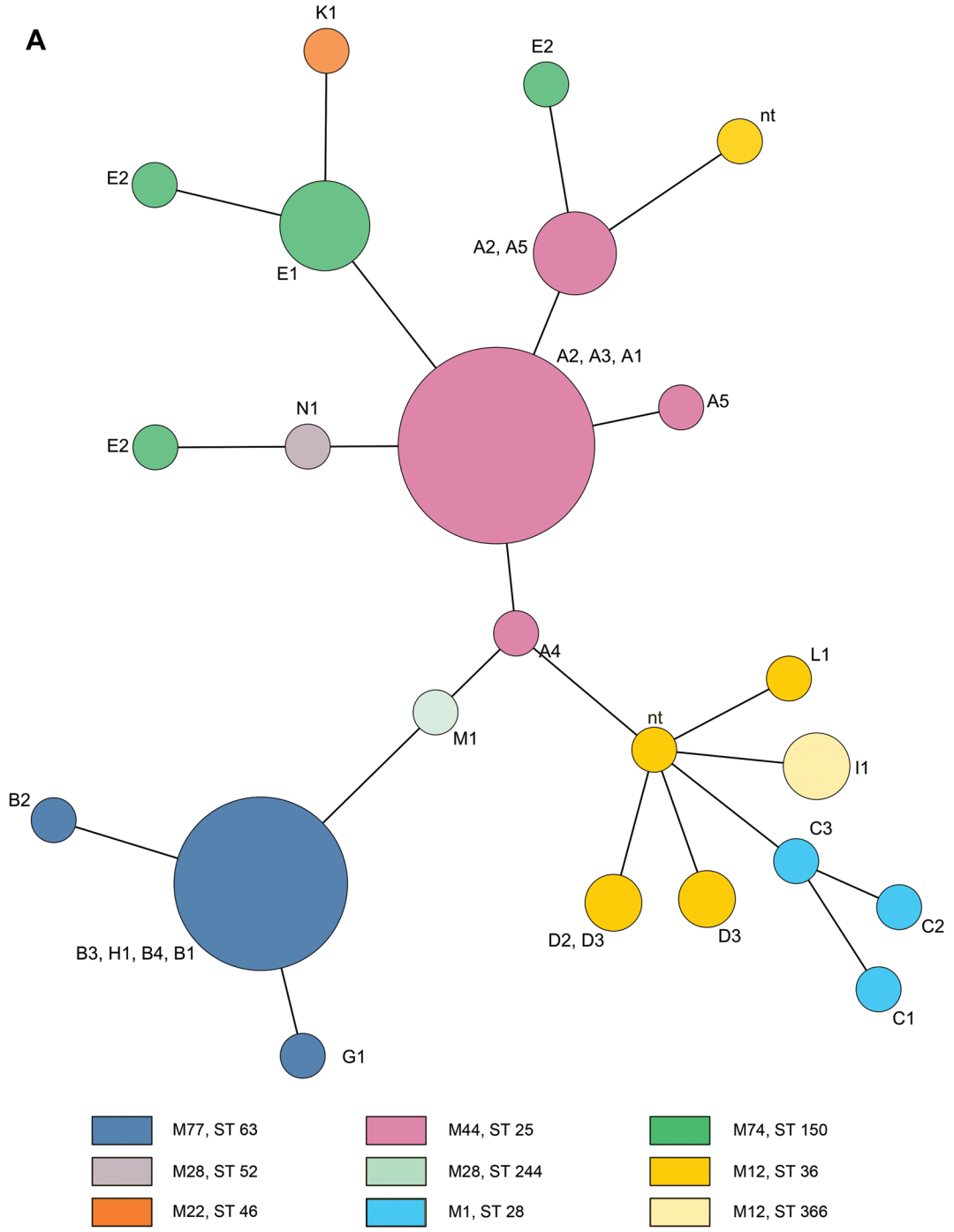


Fig. 3A.

We detected differences in phage content within single *emm*/ST groups that was reflected in described previously subtype of PFGE (Fig. 3). Although PFGE subtyping is the best predictor of phage content ($WC_{PFGE \rightarrow PP}$= 0.986; CI 95% 0.974–0.997), conversely, PP typing can detect variants that reflect PFGE subtypes with over 85% probablity ($WC_{PP \rightarrow PFGE}$=0.858; CI 95% 0.703–1.000) (Table VB).

**Detection of virulence factors.** Multiple virulence factors produced by GAS such as superantigens, proteases and DNAses are linked to disease severity and clinical manifestations of infection (Bernal *et al.*, 1999; Fraser *et al.*, 2000; Proft *et al.*, 2000). In particular, presence of *speA* gene is associated with streptococcal toxic like shock syndrome and scarlet fever (Hauser *et al.*, 1991; Musser *et al.*, 1991; Stevens *et al.*, 1989; Yu and Ferretti, 1989) and *smeZ* participates in repression of cognate anti-streptococcal responses (Unnikrishnan *et al.*, 2002). Therefore, the detection of virulence factors can be used as a predictor of disease severity and as a diagnostic marker.

We designed set of four, low volume, multiplex reactions that allow simultaneous detection of 20 GAS virulence factors. Two multiplex reactions detect genes

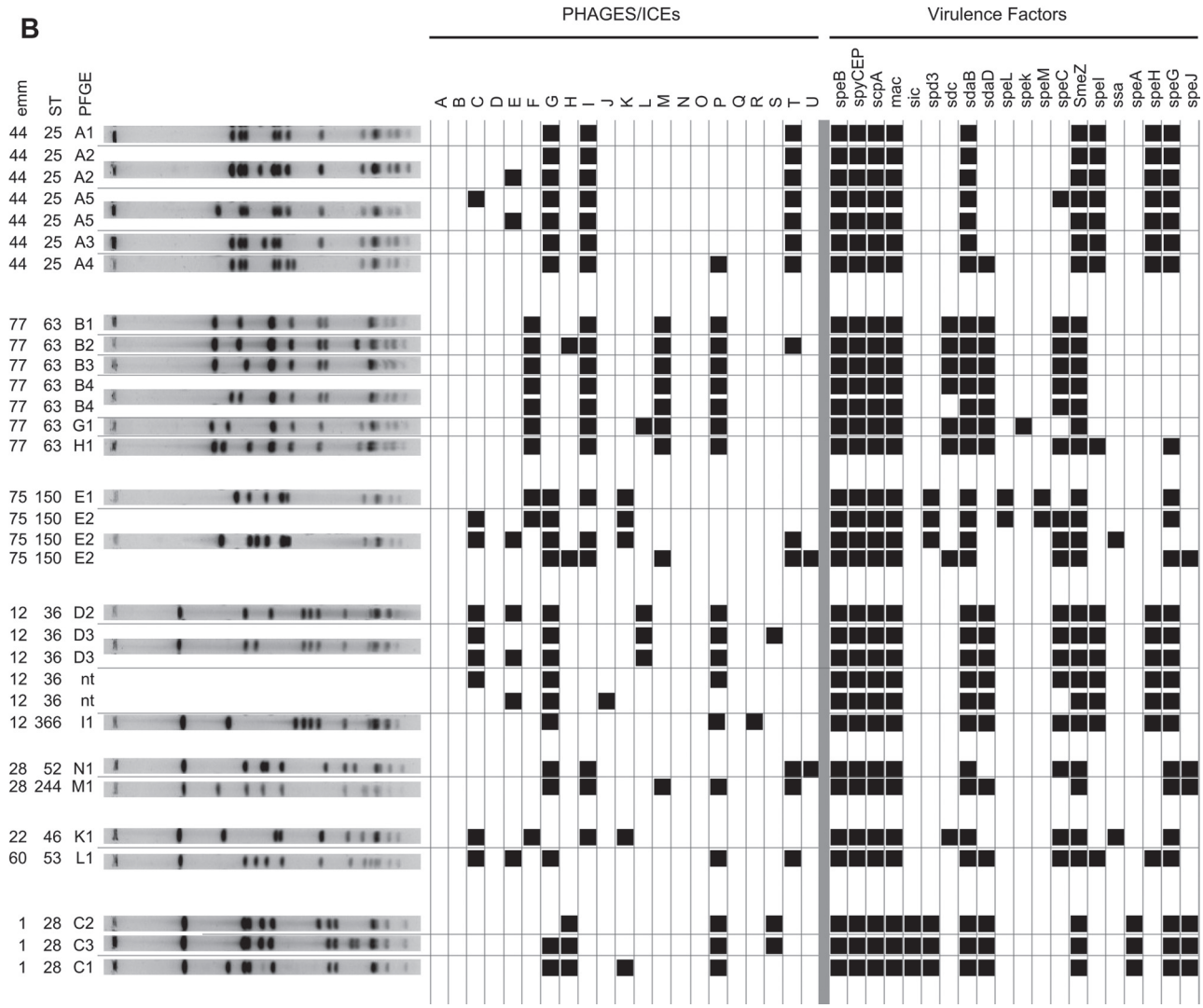


Fig. 3. Correlation between detected phage/ICE integration sites and virulence factors with M type (*emm*), sequence type (ST) and PFGE pattern (after (Szczypa *et al.*, 2004)).

A. A through K designations (with subtypes marked with arabic numerals) denote PFGE patterns detected by (Szczypa *et al.*, 2004). Clusters and relationship between them are based on detected phages and ICE elements and were determined using Minimum Spanning Tree method of BioNumerics package by Applied Maths. Circle size indicates number of isolates in each PFGE group. B. Black rectangles denote phages/ICEs and virulence factors detected in analyzed strains. Strips of PFGE gels represent detected patterns and sub-patterns.

encoding 11 superantigens: *speL*, *speK*, *speM*, *speC*, *smeZ*, *speI* and *ssa*, *speA*, *speH*, *speG*, *speJ*; one multiplex PCR detects DNases: chromosomal *sdaB* (named also streptodornase B, *speF*, MF, designated M5005_1738 in strain MGAS5005) and phage encoded *spd3* (M5005_Spy1169), *sdc*, (sdalpha, SpyM3_1409), *sdaD* (M5005_1415); fourth multiplex reaction detects genes encoding proteases *scpA*, *speB*, *mac*, *spyCEP* and streptococcal inhibitor of complement *sic*. An example of the PCR products separation after detection of virulence factors in four multiplex reactions is presented in Fig. 4 A-D.

To assure that the possible negative result of amplification of multiplex reactions "Toxins MIX I" and "Toxins MIX II" was not caused by poor quality of DNA, results of the reactions were always cross-checked with the results of other reactions and the detection of chromosomally located genes served as positive control of DNA amplification.

Distribution of phage encoded virulence factors could be in majority of cases attributed to the detected integrated elements known to encode particular virulence factor (Beres and Musser, 2007). Therefore, detection of particular superantigens was routinely compared with detected phage profiles. Example of such comparison can be seen in Fig. 5. Lack of detected products in multiplex reaction "Toxins MIX I" correlates with detection of elements integrated into sites F, G and T that do not encode superantigens. In case of the same strain, detection *spd3* gene correlates with the detection of the mobile element integrated into R site that can carry this type of DNAse. Detection of virulence factors was validated using reference strains of

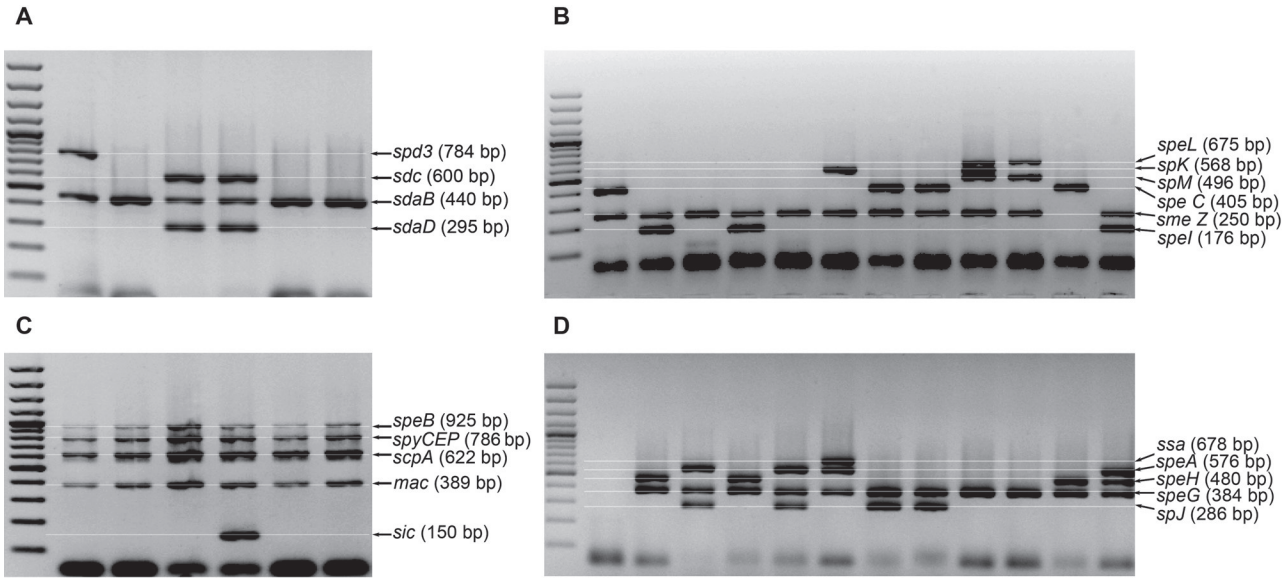


Fig. 4. Detection of twenty GAS virulence factors in randomly chosen strains.

Each panel represents multiplex PCR reactions: A: DNAses, B: toxins I, C: proteases and sic, D: toxins II. 1.5% agarose/TBE, marker: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

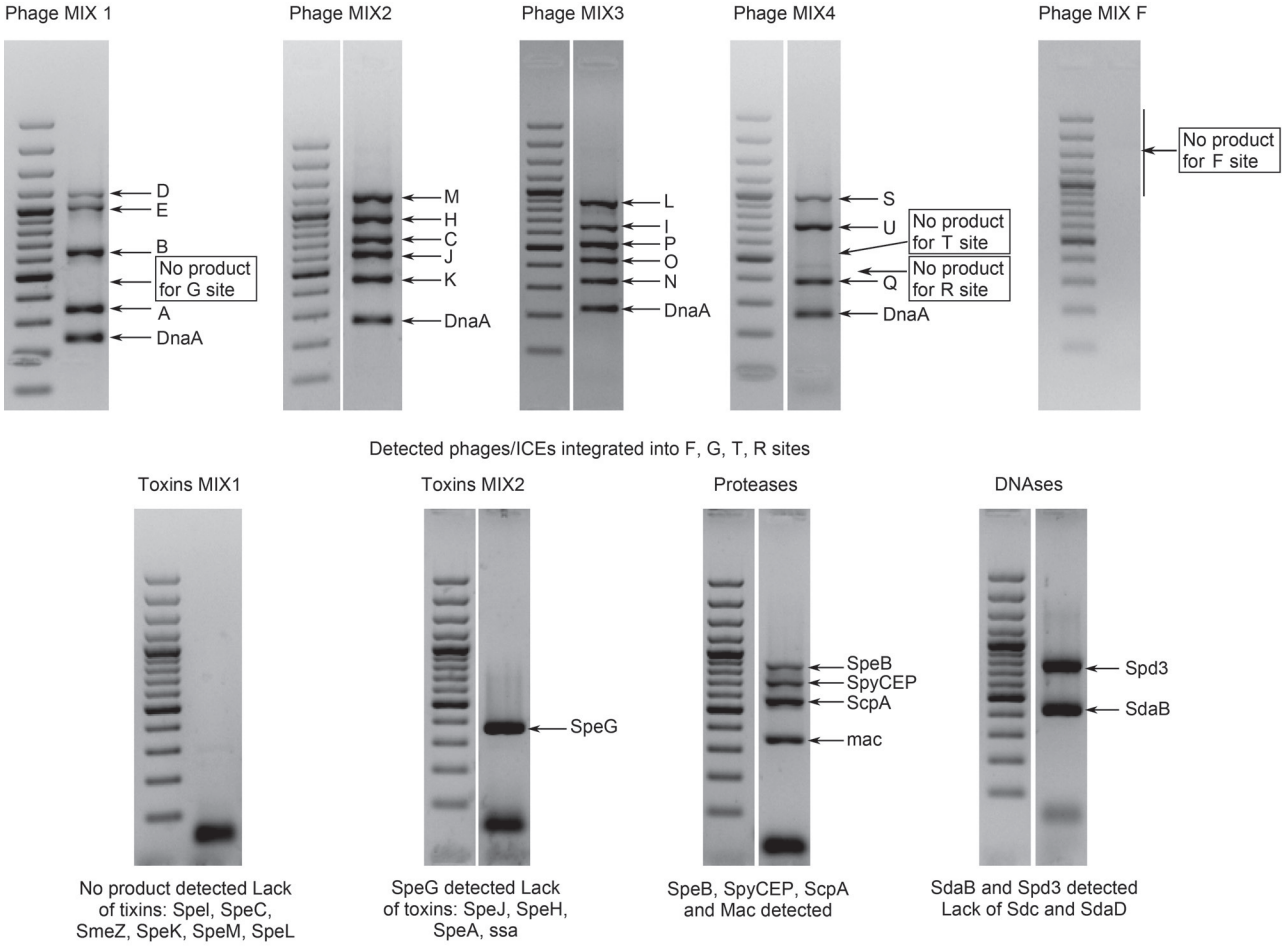


Fig. 5. Analysis of phage and virulence factors presence in a single M81 strain.

Analysis of phage integration sites detected elements integrated into F, G, T and R chromosomal locations. Based on the genome sequences, the integration sites correspond with the elements not carrying any virulence factors (sites F, G and T) and encoding Spd3 DNase (site R) (Beres and Musser, 2007). During the analysis of virulence factors, phage encoded s*pd3* DNAse was detected, as well as chromosomally encoded *speG*, *speB*, *spyCEP*, *scpA*, *mac* and *sdaB*.Marker: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

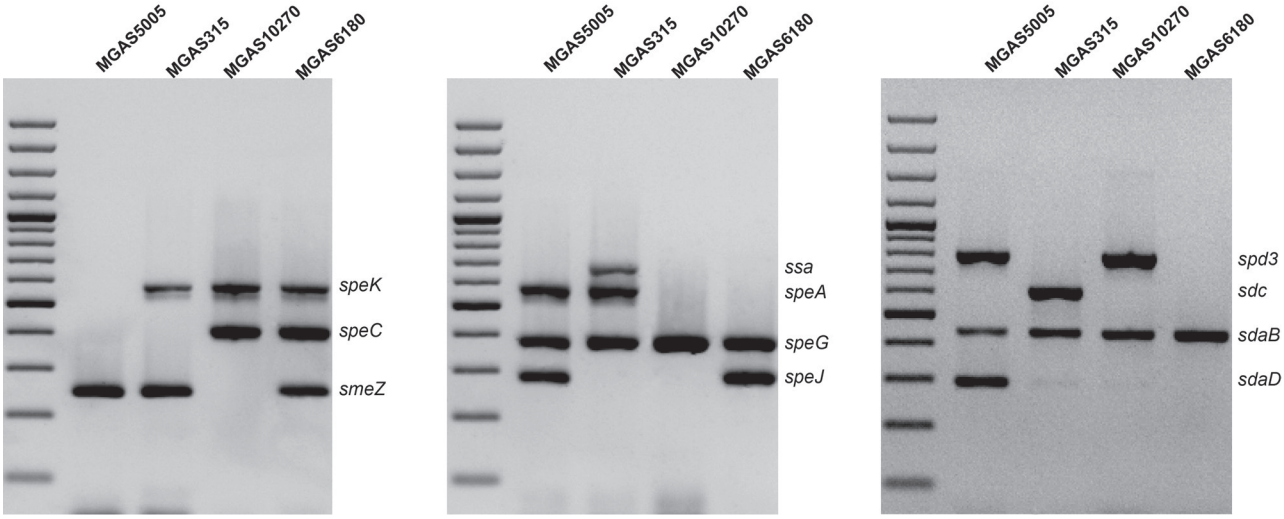


Fig. 6. Detection of toxins and DNAses in sequenced reference GAS strains MGAS5005 (NC_007297.1), MGAS315 (NC_004070.1), MGAS10270 (NC_008022.1) and MGAS6180 (NC_007296.1).

Each panel represents multiplex PCR reaction: **A**: toxins I , **B**:, toxins II **C**: DNAses. Chromosomally located *speB, mac, spyCEP* were detected in all cases *sic* gene was detected in MGAS5005 (data not shown). 1.5% agarose/TBE, marker: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas).

known genomic sequence and virulence factor profiles; the detected profile matched predicted profiles (Fig. 6).

Analysis of 656 diverse GAS strains detected 95 virulence factor profiles among 40 *emm* types and 185 phage profiles (SID = 0.943; CI 95% 0.936–0.951). The number of detected VF profiles is lower than phage profiles because phages encoding certain virulence factors, such as SpeC or SpeK can be carried by phages integrated in various sites (Beres and Musser, 2007), so single virulence factor profiles can mach different phage profiles.

Based on SID calculations (Table V) and the fact that chromosomally encoded proteases SpeB, SpyCEP, ScpA and Mac are detected in virtually all strains, the detection of virulence factors can be simplified. Abbreviated method (without "proteases mix") has identical resolution as not abbreviated method (Table V). The mix, however, can be used for the analysis of *emm* type 1 strains to detect variants of *sic* gene. As an alternative approach, primers detecting *sic* gene can be added to mixes "toxins II" or "DNases".

The group of strains chosen to further test the method of virulence factor detection, was highly clonal based on previous analyses (Szczypa *et al.*, 2004) and this work). Analysis of genes encoding virulence factors shows that these strains have potential to produce almost identical virulence factors within each PFGE group. In addition particular virulence factors within each group seem to be encoded by the same phage and differences in virulence factor profiles are reflected by subgroups of PFGE patterns (Fig. 3B).

In a conclusion, we developed two inexpensive methods that allow easy differentiation between *S. pyogenes* strains. In addition, detection of superantigens and other virulence factors in clinical strains can provide invaluable information for further epidemiological investigations. Comparing with PFGE and MLST, the method is fast (2–3 h of PCR amplification with additional time for electrophoresis) and cost of multiplex PCR reactions is much lower than sequencing. The discriminatory power of the system used as typing method is comparable with PFGE, and it can be used when rapid strain comparison is required.

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