Replacement of methicillin-resistant Staphylococcus aureus clones in Hungary over time: a 10-year surveillance study

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ABSTRACT

The prevalence of methicillin-resistant Staphylococcus aureus (MRSA) in Hungary has been increasing and is now close to 20% among invasive isolates of S. aureus. In order to understand the evolution of MRSA in Hungary, two collections of isolates were studied: 22 representatives of a collection of 238 MRSA isolates recovered between 1994 and 1998, and a collection of 299 MRSA isolates recovered between 2001 and 2004. The isolates were first characterised by pulsed-field gel electrophoresis (PFGE) and were distributed into 19 different PFGE patterns. Representatives of each pattern were further characterised by spa typing, multilocus sequence typing (MLST) and staphylococcal cassette chromosome mec (SCCmec) typing. The Hungarian clone that was predominant in 1994–1998 (PFGE E, ST239-III) had almost disappeared in 2003–2004, being replaced by the Southern German clone (PFGE B, ST228-I) and the New York/Japan epidemic clone (PFGE A, ST5-II), which represented c. 85% of the 2001–2004 isolates. Thus, this study describes, for the first time, the co-dominance and extensive spread of the New York/Japan clone in a European country.

Keywords Clonal evolution, epidemiology, Hungary, molecular typing, MRSA, Staphylococcus aureus

INTRODUCTION

During the past two decades, methicillin-resistant Staphylococcus aureus (MRSA) has become the most prevalent and important nosocomial pathogen, causing serious infections such as skin abscesses and wound infections, osteomyelitis, endocarditis, pneumonia, meningitis, bacteraemia and toxic shock syndrome [1]. More recently, MRSA infections have been reported in the community among patients with and without traditional risk-factors for MRSA infection. These latter infections are associated predominantly with skin and soft-tissue abscesses and cellulitis [2].

In Europe, the prevalence of MRSA varies widely among nations and is consistently higher in southern countries, e.g., Portugal, Spain, Italy, France and Greece, which report a prevalence of >30%, as compared with <2% in Scandinavian countries, Switzerland and The Netherlands (http://www.earss.rivm.nl). In central and eastern Europe, the prevalence of MRSA has been estimated at c. 15% in Austria, the Czech Republic and Slovenia, between 20% and 37% in Hungary, Slovakia, Poland, Bulgaria and Croatia, and >60% in Romania (http://www.earss.rivm.nl) [3].

Epidemiological studies using molecular typing methods have indicated that the massive geographical spread of MRSA results from the

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dissemination of a few highly epidemic clones. These major epidemic clones belong to sequence type (ST) 5 (SCCmecII—New York/Japan, and SCCmecIV—Pediatric), ST239 (SCCmecII/III—Brazilian), ST247 (SCCmecIA—Iberian), ST22 (SCCmecIV—EMRSA-15), ST36 (SCCmecII—EMRSA-16) and ST45 (SCCmecIV—Berlin) [4–6].

Another epidemic MRSA clone, sharing ST239 with the Brazilian clone, was described for the first time in Hungary, and was reported to be widely dispersed in Hungarian hospitals between 1993 and 1998 [7,8]. Further studies reported the local predominance of this clone in other regions of the world, namely Taiwan and China [9], and more recently in India [10], Poland [11] and Norway [12]. Norway has one of the lowest rates of MRSA in Europe, which indicates the high capacity for dissemination of this particular clone.

Besides the limited number of MRSA clones circulating in Europe, the phenomenon of clonal replacement has been observed; i.e., clones that were disseminated widely during the beginning of the last decade have become less common and have been replaced by other epidemic clones. In Portuguese hospitals, the prevalent Portuguese clone (ST239-III variant) in the mid-1980s was replaced by the Iberian clone (ST247-IA) in 1992–1993, and later, by the Brazilian clone (ST239-III/IIIA) [5]. Another example of clone replacement was observed in German hospitals between 1994 and 2002. The Northern German (ST247-I) and the Hannover (ST254-IV) clones, which were prevalent at the beginning of the 1990s, were replaced in 2000 by the Berlin (ST45-IV), the Southern German and the Barnim (ST22-IV) clones, and 1 year later by the Rhine-Hesse (ST5-II) MRSA epidemic clone [13]. Also, in the Czech Republic, Melter et al. [14] reported the arrival of the epidemic EMRSA-15 (ST22-IV) clone, replacing the previously prevalent clones in the country, i.e., the Brazilian or ST239-related clones and the Iberian (ST247-IA) clone.

The incidence of MRSA in Hungary is currently c. 20% among invasive isolates of *S. aureus* (http://www.earss.rivm.nl). However, since the late 1990s, there have been no molecular data available from Hungary. Therefore, the aims of the present study were, first, to provide an update of the MRSA clonal types circulating in Hungary between 2001 and 2004, and second, to trace the temporal evolution of the epidemic clones in Hungary during the last decade.

**MATERIALS AND METHODS**

**Bacteria**

The study investigated two collections of isolates: (i) 299 isolates selected from among 3539 MRSA isolates, recovered between 2001 and 2004 from different provincial hospitals located in 18 of the 19 Hungarian counties (Fig. 1), sent to the Hungarian National Center for Epidemiology as part of routine MRSA surveillance—the 299 isolates were all single patient invasive isolates (from blood cultures or cerebrospinal fluid), with 15 isolates from 2001, 48 isolates from 2002, 103 isolates from 2003, and 133 isolates from 2004; and (ii) 22 clinical MRSA isolates selected from among 238 MRSA isolates recovered between 1994 and 1998 in six Hungarian counties (Fig. 1) [8]—these 22 isolates represented all of the pulsed-field gel electrophoresis (PFGE) profiles described by Oliveira et al. [8],
and were included in the present study to enable a more detailed characterisation in order to trace the evolution of the clonal types circulating in Hungary between 1994 and 2004.

**Antimicrobial susceptibility testing**

Susceptibility to oxacillin, vancomycin, ciprofloxacin, erythromycin, clindamycin, gentamicin, trimethoprim–sulphamethoxazole, tetracycline, rifampicin, teicoplanin, quinupristin–dalfopristin and linezolid was tested by the disk-diffusion method according to CLSI guidelines [15]. Antimicrobial susceptibility testing for oxacillin and vancomycin was also performed using Etests (AB Biodisk, Solna, Sweden) according to the manufacturer’s instructions. *S. aureus* ATCC 25923 was used as a quality control strain for antimicrobial susceptibility testing.

**Phage typing**

The isolates recovered between 2001 and 2004 were typed using the international basic set of phages for *S. aureus* isolates of human origin, and a set of ten phages selected by Richardson et al. [16] that were developed in certain countries in response to local problems in typing MRSA strains. All phages were used at 100× the routine test dilution.

**PFGE**

PFGE was performed for all isolates as described by Chung et al. [17]. In brief, genomic DNA samples immobilised in agarose were digested overnight with *Sma*I (New England BioLabs, Beverly, MA, USA), followed by resolution of the restriction fragments in a CHEF-DRIII contour-clamped homogeneous electric field apparatus (Bio-Rad, Hemel Hempstead, UK). PFGE patterns were analysed visually and interpreted using the criteria of McDougal et al. [18], and were also analysed using BioNumerics v.4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Concatamerised phage *i* DNA (New England BioLabs) and a *Sma*I genomic digest of *S. aureus* NCTC 8325 were used as internal markers to normalise the gels. Percentage similarities were determined from a dendrogram that was constructed using the unweighted pair group with arithmetic averages (UPGMA) method and the Dice coefficient, with band position tolerance and optimisation set at 1%. According to Carrico et al. [19], and after reviewing the epidemiological data associated with each of the clusters, a similarity coefficient of 80% was used to define the PFGE type clusters.

**spa typing**

Analysis of polymorphism in the X region of the protein A gene (spa) was performed as described previously [20] for at least one representative of each PFGE type (*n* = 40). The spa types were determined by assignment according to the Ridom web server (http://www.ridom.de/spaserver/), using Ridom StaphType v.1.4.10 software (Ridom GmbH, Wurzburg, Germany) and the eGenomics web server (http://tools.egenomics.com/). As observed previously [20,21], spa types with similar repeat profiles were grouped together in spa lineages, which were identified in the present study by numbers.

**Multilocus sequence typing (MLST)**

MLST was performed on at least one representative of each PFGE pattern (*n* = 49) by PCR amplification of internal fragments of seven housekeeping genes as described by Enright et al. [22], except that primer arcF2 (5'-CTTTATT-GATTCCAAGGCG) was used [23], or according to van Leeuwen et al. [24]. The allelic profile, and hence the ST, of each isolate was identified using the MLST database (http://www.mlst.net).

**SCCmec and ccrAB typing**

The staphylococcal cassette chromosome *mec* (SCCmec) type was determined for at least one representative of each PFGE pattern/spa lineage (*n* = 66) using the multiplex PCR strategy developed by Oliveira and de Lencastre [25], which assembles a specific amplification profile for each cassette type. A ccrAB (cassette chromosome recombinase)-specific PCR [26] was performed to confirm the presence of SCCmec type IV, and also when a non-conclusive result was obtained using the multiplex strategy. Amplification of ccrB [27] was also performed for one isolate that was not typeable using the above approaches.

**Panton–Valentine leukocidin (PVL) gene analysis**

The presence of the PVL *lukS/lukF* genes was investigated by PCR [28] for representatives of each PFGE pattern.

**RESULTS**

**Antibiotic profiles and phage typing**

The 321 isolates were assigned to 12 antibiotypes (Table 1). All isolates were resistant to oxacillin, and high proportions were resistant to erythromycin (95.6%), ciprofloxacin (95.3%), clindamycin (87.9%) and gentamicin (58.9%). None of the isolates was resistant to linezolid, teicoplanin, quinupristin–dalfopristin or vancomycin.

Among the 299 isolates collected between 2001 and 2004, 193 (64.5%) were typeable with both sets of phages: 154 belonged to phage group III, 13 to group 83A, ten to group I, nine to group 95, and seven to group 80. The remaining 106 isolates, which could not be assigned to a defined group, included 58 non-typeable isolates and 48 belonging to mixed groups. These 48 isolates included 22 isolates from mixed groups III + 96, nine isolates from groups III + I, six isolates from groups III + II, and five isolates from groups III + 94/96. Mixed groups I + 83A, I + 94/96 and II + 83A each contained a single isolate, and the remaining three isolates belonged to groups 83A + 94/96.
Table 1. Clonal distribution of methicillin-resistant *Staphylococcus aureus* isolates recovered during 1994 - 1998 and 2001 - 2004 in Hungarian hospitals

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year of isolation</th>
<th>PFGE pattern</th>
<th>No. of isolates</th>
<th>Phage group</th>
<th>spa lineages</th>
<th>SC finally type</th>
<th>Antibiotyp</th>
<th>Oxa</th>
<th>Cip</th>
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* aIsolates selected correspond to representatives of each pulsed-field gel electrophoresis (PFGE) pattern.
* bThe isolates are representatives of the major phage group within each PFGE pattern. NT, not typeable; ND, not determined; Mixed, the isolates belong to mixed phage groups.
* cThe nomenclature adopted was that of the RIDOM web server, according to Harmsen et al. [48].
* dThe nomenclature adopted was that of the eGenomics web server, according to Shopsin et al. [20].

ST, sequence type; CC, clonal complex; R, resistance; I, intermediate susceptibility; S, susceptibility; Oxa, oxacillin; Gp, ciprofloxacin; Gen, gentamicin; Van, vancomycin; Tei, teicoplanin; Rif, rifampicin; Tet, tetracycline; SXT, trimethoprim-sulphamethoxazole; Ery, erythromycin; Cli, clindamycin; Lin, linezolid; Q-D, quinupristin-dalfopristin.
Macrorestriction analysis

All 321 MRSA isolates were studied by PFGE. Although the isolates were distributed into 19 PFGE patterns (types A–C, E–L, P–T, V, X and Z) differing by six or more bands, two major clonal groups could be distinguished: pattern B, accounting for 141 (44%) of the isolates, and pattern A, accounting for 113 (35.2%) isolates. With the exception of pattern E (n = 13, 4.5%), all remaining clones were represented by <4% of the isolates, and were considered to be minor clones (Table 1).

spa typing, MLST and SCCmec typing

Representatives of the 19 PFGE patterns were characterised further by spa typing (n = 40), MLST (n = 49) and SCCmec type (n = 66) (Table 1). According to spa typing, the isolates were assigned to seven spa lineages. MLST identified 11 different sequence types (ST5, ST228, ST239, ST36, ST254, ST247, ST8, ST1, ST45, ST22 and ST875), which belonged to seven clonal complexes (CC5, CC8, CC1, CC30, CC45, CC22 and CC12).

All four of the typical SCCmec types I–IV, as well as variants IA, IIA and IVA, were detected among the isolates investigated. One isolate, belonging to CC12, was mecA-positive, but was not typeable by the PCR multiplex strategy [26], or by ccrAB [27] and ccrB [28] typing. In the two predominant clonal complexes, CC5 appeared to be associated with both SCCmecI and SCCmecII, while SCCmecIII and SCCmecIIIA were detected only in CC8, in association with ST239.

Although PFGE patterns A and B, which included most (n = 254) of the isolates, were assigned to a specific spa type, i.e., spa type t002/2 or spa type t041/388 (RIDOM/eGenomics nomenclature), respectively, they shared the same spa motif r20r17r12r17r16 (eGenomics nomenclature: DMGMK) and were both associated with spa lineage 1. Lineage 2, motif r16r02r25r17r24 (eGenomics nomenclature: KAOMQ), included nine PFGE patterns that, with the exception of pattern L, all belonged to ST239-SCCmec type III (or IIIA).

Of the 11 STs identified, two predominated (>113 isolates each) and belonged to CC5: (i) ST5, associated with PFGE patterns A and J, and with SCCmecII; and (ii) ST228, a double-locus variant of ST5 in the tpi and yqiL alleles, corresponding to PFGE pattern B and SCCmecI.

PVL screening

Representatives (n = 65) of each PFGE pattern were selected randomly and tested for the presence of the PVL determinant. None of the isolates tested positive for the genes encoding PVL.

Temporal and geographical clonal distribution

The evolution of MRSA clones in Hungarian hospitals between 1994 and 2004 is summarised in Fig. 2. Previous data [8] and the results obtained for the 22 representative isolates by spa typing, MLST and SCCmec typing showed that 99% of the isolates (i.e., all except two isolates) from 1994–1996 belonged to ST239-III. Several PFGE patterns were associated with the ST239-III isolates, but the Hungarian clone (PFGE E) was clearly predominant (74%). Over time, the prevalence of ST239-III clones, including the Hungarian clone, decreased progressively to a minimum of <0.5% in 2003–2004. In parallel, two other well-represented clones emerged in Hungary: (i) PFGE B–ST228-I (the Southern German clone) was introduced in 1997–1998, represented by 28% of the isolates; and (ii) PFGE A–ST5-II (the New York/Japan
clone) emerged in 2001–2002, with a prevalence of 30%. Over time, the prevalence of both clones increased, reaching 50% and 40%, respectively, in 2003–2004, at which time they became the two most prevalent clones isolated in Hungary. The two isolates recovered in 1994–1996 that did not share ST239 were found to belong to the Hannover clone (PFGE F–ST254-IV). This clone, absent during 1997–1998, re-emerged during 2001–2002 (6%), but remained at a relatively low prevalence (1%) during the following years.

The MRSA isolates from 1994–1998 were recovered from six of the 19 Hungarian counties, while the prevalent Hungarian clone ST239-III was found in five different counties throughout the country (Fig. 1). Despite the fact that the county in which the capital was located was over-represented among the 2001–2004 isolates, all except one of the remaining counties were represented in this collection (Fig. 1). During this second study period, the Hungarian clone (or ST239-related clones) was still present in four counties throughout Hungary, three of which were included in the 1994–1998 study, as well as in an additional southern county. The Southern German clone ST228-I, which was restricted to one county until 1999, was isolated subsequently in nine additional counties throughout Hungary. Moreover, the New York/Japan clone ST5-II, present in Hungary only since 2001, was widespread throughout 11 counties. The rapid dissemination of these clones might be linked to the transfer of Hungarian patients from hospital to hospital, and hence from county to county.

DISCUSSION

Few surveillance studies have described the evolution of MRSA in central and eastern Europe. Although there was a gap in the present study between 1999 and 2000, during which MRSA isolates from Hungary were not obtained, this report describes the evolution of the clonal types circulating in Hungary over the last decade.

The predominance of a single clone in Hungarian hospitals between 1993 and 1994 was reported by de Lencastre et al. [7]. This Hungarian clone, characterised by ST239-III, was related closely to the epidemic Brazilian clone, which is dispersed widely in Europe, South America and Asia [5]. Oliveira et al. [8] used a combination of spa typing and PFGE typing to reveal a significant decrease in the prevalence of the Hungarian clone, from c. 74% in 1994–1996 to 42% in 1997–1998. Although the Hungarian clone has also been described in Poland [11] and Norway [12], the present results show that it is currently almost non-existent in Hungary. Two other clones, both belonging to CC5, i.e., the Southern German (ST228-I) and the New York/Japan (ST5-II) clones, are now the predominant clones in Hungarian hospitals.

The Southern German clone was first described in the southern area of Germany in 1992, and spread throughout Germany during 1995–1996 [29]. About 1 year later, this clone was detected in Budapest, the Hungarian capital, with a prevalence of 28%, and then spread rapidly throughout Hungary, becoming the prevalent clone in 2003–2004. ST228-I has also been reported to be the predominant clone (86% of MRSA isolates) in a hospital in Slovenia [30]. In Croatia, which also borders Hungary, Budimir et al. [31] reported that ST111-I, a single-locus variant of ST228 with the same SCCmeC type, occurred at a prevalence of 52% in bloodstream isolates. Moreover, other minor clones associated with ST228-I have been reported in Italy [32], Denmark [33], Belgium [34] and Switzerland [35] (http://www.mlst.net), showing that clone ST228-I, or related STs, are relatively common in Europe.

After being isolated in northern European countries [5], ST5 was detected in Hungary soon after its double-locus variant, ST228. ST228 seems to have evolved from ST5 [36]. However, the appearance of these two clones in Hungary probably represents independent events, since ST228 was the first to be detected (Fig. 2). ST5 appeared almost simultaneously in 11 counties, whereas ST228 was first isolated in the restricted area of Budapest, and only spread later throughout the country. Interestingly, to our knowledge, the New York/Japan clone has never been described in countries bordering Hungary, and was therefore probably imported from countries in which it is highly prevalent, i.e., the USA, Japan, Korea and Mexico [37–39]. Nevertheless, clone ST5-II has been reported recently in some Belgium hospitals, as was the ST45-IV clone, which was predominant among 251 isolates recovered from 95 hospitals during 2003 [40]. ST45-IV was also isolated in Hungarian hospitals, but with a very low prevalence (three isolates). Other epidemic clones have been described in...
central Europe, e.g., EMRSA-15 (ST22-IV), which has been detected in hospitals throughout all regions of the Czech Republic, replacing both the Brazilian (ST239) and related clones, and the Iberian clone (ST247) [14]. Interestingly, one of the minor clones described in Hungary, PFGE pattern V (Table 1), belonged to ST22-IV and was represented by two isolates from a single county. Thus, ST5 and related clones, such as ST228 and ST111, as well as the non-multiresistant EMRSA-15 (ST22) clone, are becoming the major clones circulating in eastern European countries, in addition to the Iberian and the Brazilian or ST239-related clones.

Concerning the 2001–2004 MRSA collection, with the exception of phage groups 80 and 83A, which belonged only to PFGE pattern A, the other phage groups (I and III) included more than one PFGE pattern. In the two major PFGE patterns, A and B, most isolates belonged to phage group III. Phage typing is still used to characterise S. aureus [41], but this collection showed low typeability, with 35.4% of the isolates not being assigned to any group (i.e., belonging to mixed phage groups or not typeable). There was poor concordance between phage typing and the DNA-based approaches used, as has been observed previously [42].

Although the PVL determinant, a bicomponent leukotoxin virulence factor linked to severe necrotising fasciitis and necrotising pneumonia, has been detected previously in the hospital setting [43,44], it is associated more commonly with community-acquired MRSA strains harbouring SCCmec types IV or V [45]; it was not detected in any of the nosocomial Hungarian isolates in the present study. Voyich et al. [46] recently reported that strains lacking PVL were as virulent in mouse sepsis and abscess models as those that produce the leukotoxin, and therefore concluded that PVL is not the major virulence determinant of community-acquired MRSA.

In summary, the present results indicate that a temporal recycling of the prevalent clones circulating in Hungary occurred between 1994 and 2004. Although ST239-related clones are still isolated in central Europe, e.g., in Poland [11] and Romania [47], the prevalence of ST239-related clones is currently almost null in Hungary. A progressive replacement of the Hungarian clone by the Southern German and New York/Japan epidemic clones was observed in the hospital setting between 2001 and 2004, and the co-dominance and massive spread of the New York/Japan clone in a European country was observed for the first time.

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