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Characteristics of *Streptococcus pyogenes* causing invasive infections among adults in Portugal, 2016–2019: Pre-COVID-19 expansion of the $M1_{UK}$ sublineage

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A B S T R A C T

Background: Genome-based epidemiological surveillance of *Streptococcus pyogenes* (Lancefield Group A *Streptococcus*, GAS) infections facilitated the detection of emergent successful lineages, such as the $M1_{UK}$ sublineage. This sublineage dominated the post-COVID-19 upsurge of invasive GAS infections (iGAS) in multiple countries, including Portugal. Here, we characterized the genetic lineages causing iGAS in Portugal during 2016–2019 to evaluate possible temporal trends and compare them with internationally circulating lineages.

Methods: Whole-genome sequencing and antimicrobial susceptibility testing were performed for 273 iGAS isolates.

Results: The dominant *emm* types were *emm*1 ($n = 87$), *emm*3 ($n = 37$), and *emm*89 ($n = 26$), collectively comprising 55 % of all isolates ($n = 273$). Throughout the study, the $M1_{UK}$ sublineage increased in prevalence, accounting for 48 % of all *emm*1 isolates. Core-genome multilocus sequence typing supports multiple introductions of $M1_{UK}$ in Portugal pre-COVID-19, and a limited relatedness to the $M1_{UK}$ isolates recovered during the post-COVID-19 surge in pediatric iGAS. Several internationally disseminated lineages expressing various *emm* types were identified. Mutations inactivating key regulators of virulence (CovRS and RopB) and in the capsule locus were found in a significant fraction of isolates. Macrolide resistance was primarily associated with the *erm*(A) and *erm*(B) genes and remained low (4 %), highlighting differences between Europe and North America.

Conclusions: Despite adult iGAS in Portugal being caused by geographically widespread, successful GAS lineages that may be repeatedly introduced in the country, including $M1_{UK}$, there was no apparent increase in disease. This is consistent with upsurges of iGAS post-COVID-19 not being driven primarily by the emergence or introduction of novel GAS clones.

1. Introduction

The sequence of the *emm* gene region encoding the hypervariable portion of the M protein (*emm* type) is the most widely used molecular marker for identifying lineages of *Streptococcus pyogenes* (Lancefield Group A *Streptococcus*, GAS). However, in recent years the employment of whole-genome sequencing (WGS)-based approaches in epidemiological surveillance provided evidence for the existence of intra-*emm* type

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sublineages with particular molecular and epidemiologic characteristics.[1–4]

The $M1_{UK}$ sublineage has been in the spotlight due to its association with a post-COVID-19 multi-country upsurge in invasive infections (iGAS), also found among pediatric patients in Portugal.[5–9] This sublineage carries 27 characteristic single nucleotide polymorphisms (SNPs) and overexpresses the superantigenic toxin SpeA.[2] It expanded in the UK in the twenty-tens, simultaneously with a rise of scarlet fever and iGAS, but has since been reported in several continents.[2,5,6,9–15] The lack of pre-COVID-19 information on the circulating GAS lineages in most countries does not allow us to discard the possibility that a post-COVID-19 introduction of the $M1_{UK}$ sublineage could have contributed to the rise in the number of iGAS infections.

Although mutations in penicillin-binding proteins (PBPs) associated with increased β-lactam minimal inhibitory concentrations (MICs) have been documented,[16–18] GAS remains uniformly susceptible to β-lactams. Still, macrolides and lincosamides are important alternatives for allergic patients and as adjunctive therapy in severe iGAS. Macrolide resistance, frequently conferring cross-resistance to lincosamides, has been significantly increasing in the US,[19] and macrolide-resistant GAS was recently added to the WHO Bacterial Priority Pathogens List.[20]

Continued molecular surveillance of iGAS isolates is essential to identify emergent lineages with antimicrobial resistance or with increased invasive or dissemination capacity. In Portugal, we described fluctuations in the clonal composition of iGAS isolates throughout 2000–2015 using conventional typing methods.[21–23] Here, we used WGS to comprehensively characterize iGAS isolates recovered from adults (≥18 years old) throughout Portugal during 2016–2019 and compare them to internationally disseminated lineages.

2. Methods

2.1. Bacterial isolates

Clinical microbiology laboratories from 38 hospital centers across Portugal were invited to voluntarily contribute all GAS isolated from normally sterile sites of adult patients (≥18 years old) between January 1st, 2016 and December 31st, 2019. Approval for the study was granted by the Institutional Review Board of the Centro Académico de Medicina de Lisboa (Ref. 258/22-AD/24).

2.2. Whole genome sequencing

Genomic DNA extraction was performed with the PureLink™ genomic DNA minikit (Invitrogen, Carlsbad, CA, USA), followed by library preparation with the Nextera DNA library preparation kit (Illumina, San Diego, CA, USA), and sequencing with an Illumina MiSeq or NextSeq instrument.[24] Raw sequencing data and sample metadata were deposited in the European Nucleotide Archive (ENA) under project accession number PRJEB78617.

2.3. Sequencing data analysis

Besides the WGS dataset generated in this study (Supplemental Table S1), other datasets were used for comparison (Supplemental Table S2), namely: (i) 30 iGAS isolates recovered from children (<18 years) in Portugal, September 1, 2022–31 May 2, 023[5]; (ii) 135 non-invasive GAS isolates recovered in London, UK, 2009–2016[2]; 802 *emm*4 isolates recovered in the UK, USA, Canada, and the Netherlands between 1992 and 2022,[3,4,25] 268 genetically, temporally, and geographically diverse isolates of *emm*3, *emm*89, *emm*6, *emm*12, and *emm*28 selected from the Davies et al., 2019 dataset[26] according to previously detailed criteria.[24]

Draft genomes were *de novo* assembled and annotated, followed by determination of multilocus sequence type (ST), *emm* type, $M1_{UK}$-characteristic SNPs, NAD-glycohydrolase promoter variant (P*nga*), whole-genome multilocus sequence typing (wgMLST) profile, and antimicrobial resistance genes, as previously described[5,24] (detailed methods, tools, and settings are available as Supplemental material). Minimum spanning trees were created with PHYLOViZ online[27] based on the allelic profiles of the loci shared by all isolates under analysis [core-genome multilocus sequence typing (cgMLST) profiles].[24]

The sequences of genes of interest (*gyrA*, *parC*, *covR*, *covS*, *ropB*, and *pbp2x*) were obtained from the GAS wgMLST schema[24] and aligned with reference sequences for identification of mutations, as detailed in Supplemental Materials and Methods.

2.4. Antimicrobial susceptibility testing

Susceptibility testing was performed for all isolates according to the guidelines and interpretative criteria of the Clinical and Laboratory Standards Institute (CLSI),[28] using the following disks (Oxoid, Basingstoke, UK): penicillin, vancomycin, erythromycin, tetracycline, levofloxacin, chloramphenicol, clindamycin, and linezolid. E-test strips (BioMérieux, Marcy l'Etoile, France) were used to determine MICs in cases of intermediate susceptibility and in isolates with mutations in PBP2X.

3. Results

3.1. Demographic data and isolate sources

Between January 2016 and December 2019, 273 non-duplicate GAS isolates (Supplemental Table S1) were received from patients ≥18 years (median 66 years, range 18–97 years), of which 151 (55 %) were males. The yearly distribution of the isolates was: 74 isolates in 2016, 77 in 2017, 72 in 2018, and 50 in 2019.

Isolates were recovered from normally sterile sites, mostly from blood ($n = 253$), followed by synovial fluid ($n = 9$), pleural fluid ($n = 5$), ascitic fluid ($n = 5$), and cerebrospinal fluid ($n = 1$).

3.2. Molecular typing

A total of 36 *emm* types [Simpson's Index of Diversity (SID) = 0.858 ($CI_{95\ \%}$ 0.828–0.889)] and 55 STs [SID = 0.884 ($CI_{95\ \%}$ 0.854–0.914)] were identified (Table 1). The top three *emm* types were *emm*1 (31.2 %), *emm*3 (13.6 %), and *emm*89 (9.5 %). One isolate was classified as *emm*-non-typable due to a complete deletion of the *emm* gene (Supplemental Table S1).

In general, isolates sharing the same *emm* type were grouped together in the minimum spanning tree-like (MST) representation based on the cgMLST profiles, with links between isolates of different *emm* types ranging between 639 and 999 allelic differences and links between isolates of the same *emm* type ranging between 0 and 482 allelic differences, from a total of 1111 compared core loci (Fig. 1). There were a few exceptions of isolates that were not linked to the remaining isolates sharing the same *emm* type (*emm*68, *emm*18, *emm*77, and *emm*28), but carrying unrelated STs. One isolate presenting *emm*228 was grouped together with *emm*12 isolates (link distance of 32 allelic differences), sharing the same ST (ST36). An alignment with the *emm*12.0 type sequence showed that *emm*228 likely emerged from *emm*12.0 due to a deletion event encompassing 42bp in the *emm* type-determining region.

The cgMLST profiles of the 87 *emm*1 isolates identified in this study were compared with those of isolates recovered in Portugal during 2022-2023[5], and in London during 2009–2016, previously classified as $M1_{global}$, $M1_{inter}$, or $M1^2_{UK}$ (Fig. 2A, Supplemental Table S2). Isolate clustering agreed with the number of $M1_{UK}$-characteristic SNPs determined for the isolates. Isolates with 26 SNPs, which occur due to the loss of the Φ5005.3 prophage carrying one of the SNPs,[6] were also grouped with those carrying the 27 SNPs and were therefore classified as $M1_{UK}$. These 26-SNP isolates were not linked in the MST, suggesting multiple independent prophage losses. The $M1_{UK}$ isolates from Portugal were not grouped into a single cluster, consistent with multiple introductions of

Table 1
emm types, sequence types, antimicrobial resistance profiles, and antimicrobial resistance genes of 273 invasive group A *Streptococcus* isolates recovered from adults in Portugal, 2016–2019.

emm type (no. isolates)	ST (no. isolates)	Antimicrobial resistance (no. isolates)	Antimicrobial resistance genes
1 (87)	28 (83) 785 (1) 1299 (1) 1300 (1) 1304 (1)	S (87)	–
3 (37)	15 (22) 315 (15)	S (37)	–
89 (26)	101 (17) 1303 (4) 824 (3) 1285 (1) 1295 (1)	S (26)	–
6 (16)	382 (15) 1310 (1)	S (14), Lev (1) S (1)	– –
12 (16)	36 (15) 1291 (1)	S (16)	–
4 (15)	39 (15)	S (15)	–
28 (14)	52 (7) 458 (4) 456 (2) 1220 (1)	S (13) Tet (1)	– *tet*(M)
22 (8)	46 (7) 1309 (1)	Tet (7) S (1)	*tet*(M) –
87 (8)	62 (8)	S (8)	–
75 (5)	150 (4) 49 (1)	S (4) M (1)	– *mef*(A)-*msr*(D)
77 (5)	63 (4) 399 (1)	$iMLS_B$ + Tet (4) $iMLS_B$ + Tet (1)	*erm*(A) + *tet*(O) *erm*(T) + *tet*(M)
Other[a] (36)	a	$cMLS_B$ + Tet (4)[b] Tet (16)[c] S (16)	*erm*(B) + *tet*(M) *tet*(M)[d] –

ST, sequence type; S, susceptibility to all antimicrobials tested; Lev, intermediate resistance to levofloxacin; M, resistance to erythromycin and susceptibility to clindamycin; $iMLS_B$, resistance to erythromycin and inducible resistance to clindamycin; $cMLS_B$, resistance to erythromycin and constitutive resistance to clindamycin; Tet, resistance to tetracycline.

[a] *emm* types represented by < 5 isolates (*emm*-ST combinations are available in Supplemental Table S1).

[b] The $cMLS_B$ + Tet phenotype was identified in isolates presenting *emm*68-ST247 ($n = 1$), *emm*102-ST60 ($n = 1$), and *emm*169-ST53 ($n = 2$).

[c] The Tet phenotype was identified in isolates presenting *emm*18-ST402 ($n = 1$), *emm*18-ST1216 ($n = 2$), *emm*23-ST1286 ($n = 1$), *emm*33-ST3 ($n = 2$), *emm*34-ST1100 ($n = 1$), *emm*55-ST100 ($n = 1$), *emm*68-ST1287 ($n = 1$), *emm*74-ST120 ($n = 1$), *emm*76-ST978 ($n = 1$), *emm*93-ST17 ($n = 1$), *emm*116-ST227 ($n = 1$), *emm*118-ST834, and *emm*119-ST239 ($n = 1$).

[d] Four isolates carried the *tet*(L) gene in addition to *tet*(M).

this sublineage, instead of expansion from a single initial introduction.

Overall, the $M1_{UK}$ sublineage accounted for 42/87 *emm*1 isolates (48.3 %), presenting a significant increasing trend ($p < 0.001$), which was also noted for overall *emm*1 isolates independently of lineage ($p < 0.001$), despite a notable but non-significant decrease of *emm*1 in 2019, associated with a reduction in $M1_{global}$ isolates ($p = 0.007$) (Fig. 2B). In contrast, *emm*3, *emm*6, and *emm*89 presented a decreasing trend throughout the study ($p < 0.010$) (Supplemental Fig. S1).

Comparison of the *emm*4 isolates with those of 802 *emm*4 isolates from previously published datasets[3,4,25] (Supplemental Table S2), showed that 8 of the isolates from Portugal belong to the MEW427-like sublineage, presenting the typical *emm/enn* gene fusion,[25] while the remaining 7 *emm*4 isolates grouped with the MGAS1050-like sublineage and did not present the gene fusion (Supplemental Fig. S2). Both sublineages were isolated in all study years.

For contextualization within the global molecular epidemiology of GAS, isolates belonging to the remaining *emm* types comprising >10 isolates were compared to assemblies of the same *emm* type previously selected to represent the genetic, geographic and temporal diversity of GAS[26] (Supplemental Table S2). The majority of *emm*3 isolates from Portugal were closely related to isolates from the UK or North America (MST links ≤17 allelic differences from a total of 1308 compared loci) (Supplemental Fig. S3). The exception was a cluster of 9 closely related isolates (MST links of 0–4 allelic differences) recovered in 2016 and 2017 that had 44 allelic differences from the closest international isolate (a strain from the UK), possibly representing a local sublineage.

All *emm*89 isolates carried the P*nga*-3.1 variant of the *nga-ifs-slo* operon promoter[29] and lacked the *hasA* and *hasB* genes, therefore corresponding to the acapsular sublineage that expanded during the two-thousands.[30] Accordingly, they were grouped together with international isolates carrying the same promoter variant in the MST (Supplemental Fig. S4).

Most isolates of *emm*6, *emm*12, and *emm*28 were grouped with internationally disseminated lineages (Supplemental Figs. S5–S7). The most remarkable exceptions were an *emm*12-ST36 isolate that differed at 113 alleles (from a total of 1333 compared loci) from the closest isolate (a strain from India) (Supplemental Fig. S6), and an *emm*28-ST1220 isolate, which closest isolate (a strain from Kenya) had 1116 allelic differences (from a total of 1299 compared loci) (Supplemental Fig. S7).

3.3. Other genomic characteristics

Using allele calling data, draft assemblies were screened for the presence of mutations in the loci encoding the CovRS two-component regulator and the RopB stand-alone regulator.

The identified *covR* variants presented only missense mutations, whose phenotypic impact is difficult to predict.[31] In contrast, a total of 30 isolates (11.0 %) did not have a *covS* allele identified using the wgMLST schema, or presented an allele with indels, frameshifts, or nonsense mutations predicted to result in non-functional CovS proteins.[31,32] These isolates were genetically diverse, comprising 19 *emm* types and 22 STs. The occurrence of null alleles in *covS* was not associated with any *emm* type, but *emm* types represented by < 5 isolates were more likely to carry a null *covS* allele ($p < 0.001$, significant after FDR) (Supplemental Table S3). In contrast, *emm*1 isolates were more likely to carry full-length *covS* alleles ($p < 0.001$, significant after FDR).

Null *ropB* alleles were found in 11 isolates (4.0 %) belonging to 7 *emm* types and 7 STs. No significant associations were observed between null *ropB* alleles and individual *emm* types (Supplemental Table S4).

A total of 88 isolates (32.2 %) lacked a full-length allele of at least one of the essential genes for hyaluronic acid capsule production (*hasA* and *hasB*), of which 67 (76.1 %) carried a high-expression variant of the *nga-ifs-slo* promoter (P*nga*-3.1 or P*nga*-3.2) (Table 2). The great majority ($n = 80$) belonged to *emm* types previously known for frequently being unencapsulated, namely *emm*4, *emm*11, *emm*22, *emm*28, *emm*68, *emm*77, *emm*87, *emm*89, *emm*94, and *emm*102.[29] Of the remaining 8 isolates, 6 belonged to *emm* types where truncation of either *hasA* or *hasB* was detected in a minority of isolates (*emm*1, *emm*12, *emm*18), as reported previously.[29] Additionally, both *emm*169 isolates carried truncated *hasA* and *hasB* genes.

3.4. Antimicrobial resistance

All isolates were susceptible to penicillin, vancomycin, chloramphenicol, and linezolid.

Tetracycline resistance was observed in 32 isolates (11.7 %), of which 24 carried the *tet*(M) gene, 4 carried both *tet*(M) and *tet*(L), and 4 harbored *tet*(O) (Table 1). Tetracycline-resistant isolates were highly diverse, comprising 17 distinct *emm* types and 20 STs.

Of the 10 erythromycin-resistant isolates (3.7 %), 5 presented

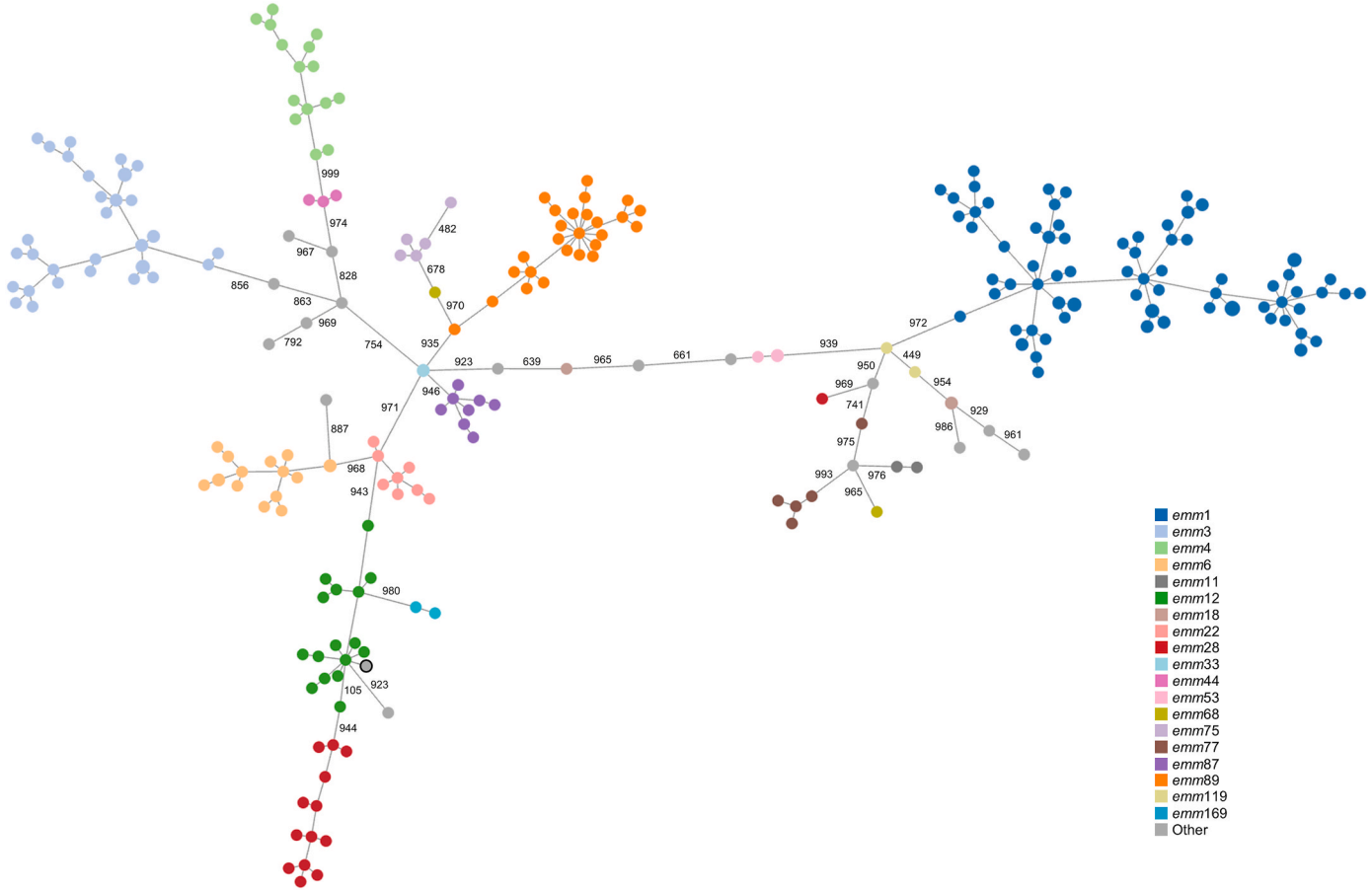


Fig. 1. Minimum spanning tree generated with the cgMLST profiles of invasive group A *Streptococcus* isolates recovered from adults, Portugal, 2016–2019 ($n = 273$). The size of each node is proportional to the number of isolates with that particular cgMLST profile, on a logarithmic scale. Nodes are colored according to *emm* type. Highlighted with a black outline circle is an *emm*228-ST36 isolate that is grouped with *emm*12-ST36 isolates. Link distances >100 allelic differences between nodes are labeled (from a total of 1111 compared loci).

inducible clindamycin resistance and carried the *erm*(A) ($n = 4$) or *erm*(T) ($n = 1$) genes, 4 presented constitutive clindamycin resistance and harbored *erm*(B), and 1 was clindamycin-susceptible, carrying *mef*(A) and *msr*(D) (Table 1). All isolates presenting *erm* genes also harbored a tetracycline resistance gene, either *tet*(M) ($n = 5$) or *tet*(O) ($n = 4$), and were phenotypically resistant to tetracycline. Erythromycin resistance was found in isolates of 5 *emm* types and 6 STs, with the dominance of a lineage characterized as *emm*77-ST63 carrying the *erm*(A) and *tet*(O) genes.

One *emm*6 isolate presented intermediate susceptibility to levofloxacin (MIC = 3 μg/mL). Analysis of the quinolone resistance determining regions (QRDRs) of the *gyrA* and *parC* genes indicated the presence of mutations D142N and S79A, respectively, the latter associated with the clonal spread of *emm*6 isolates with increased fluoroquinolone MICs.[33]

Three isolates encoded PBP2X variants previously associated with increased β-lactam MICs, namely an *emm*89 isolate with the F274L mutation, and two *emm*12 isolates carrying different amino acid changes (M593T and G600D).[16–18] These three isolates presented a penicillin MIC of 0.016 μg/mL.

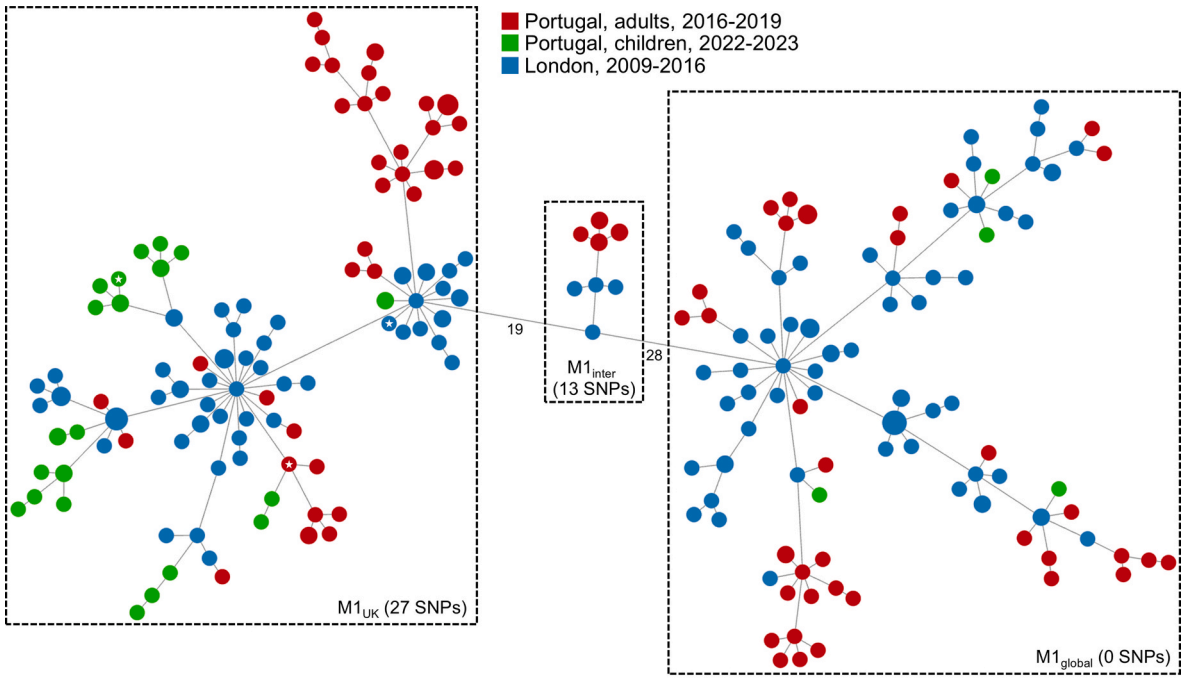
4. Discussion

During 2022–2023 multiple countries reported a rise in iGAS, in most cases dominated by the $M1_{UK}$ sublineage.[6–9,13,34] In Portugal, from September 2022 to May 2023 there was a ~4-fold increase in pediatric iGAS relative to pre-pandemic seasons, and 47 % of the available isolates were typed as $M1^{5}_{UK}$. Here we show that the $M1_{UK}$ sublineage was already expanding among adult iGAS in Portugal prior to the COVID-19 pandemic, accounting for 42/273 isolates (15 %) recovered throughout the country during 2016–2019, with an increasing trend.

Interestingly, most of the isolates from the post-pandemic pediatric iGAS surge in Portugal were more closely related to $M1_{UK}$ isolates recovered in London during 2009–2016 than to the $M1_{UK}$ isolates from pre-pandemic adult iGAS in Portugal (Fig. 2-A). This suggests that the post-pandemic surge of $M1_{UK}$ in Portugal may have resulted from the expansion of subclusters different from the ones previously causing infection in our country. Genomic analysis of *emm*1 isolates recovered from both adults and children during the pandemic and post-pandemic years and comparison with the clades recently identified within $M1_{UK}$ in England[6] is warranted to clarify these aspects.

Based on cgMLST analysis, most isolates of this study were assigned to internationally disseminated lineages, despite the identification of a few local subclusters. An *emm*4 sublineage carrying cryptic prophages and an *emm/enn* gene fusion has disseminated by multiple continents.[3,4,15,25] This sublineage, designated as MEW427-like,[25] has been associated with increased virulence and progressively outcompeted the historical *emm*4 strains in North America.[4] Our data indicates the presence of this emergent sublineage among invasive isolates in Portugal, although complete replacement of the historical MGAS10750-like sublineage did not occur, with both MEW427- and MGAS10750-like isolates co-existing until 2019.

Comparison of the *emm* type distribution in this study with a dataset of previously characterized 295 iGAS isolates recovered from adults in Portugal during 2010–2015[23] showed a reduction in *emm* type diversity [$SID_{2010\text{-}2015} = 0.897$ ($CI_{95\,\%}$ 0.877–0.917), $SID_{2016\text{-}2019} = 0.858$ ($CI_{95\,\%}$



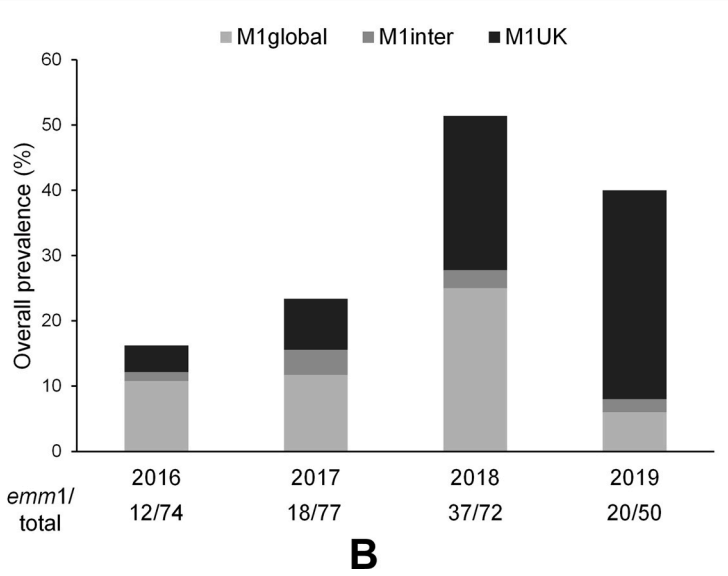


Fig. 2. Distribution of *emm*1 sublineages among invasive group A *Streptococcus* (iGAS) isolates recovered from adults, Portugal, 2016–2019. (A) Minimum spanning tree generated with the cgMLST profiles of: iGAS isolates recovered from adults, Portugal, 2016–2019 (n = 87; red); iGAS isolates recovered from children (<18 years), Portugal, September 2022–May 2023 (n = 30; green)[5]; non-invasive GAS isolates, London, UK, 2009–2016 (n = 135; blue).[2] The size of each node is proportional to the number of isolates with that particular cgMLST profile, on a logarithmic scale. Link distances separating the previously identified sublineages are labeled as the number of allelic differences between nodes (from a total of 1290 compared loci). Link distances in the minimum spanning tree vary from 1 to 17 allelic differences between nodes included in the $M1_{UK}$ box [all carrying the 27 $M1_{UK}$-characteristic SNPs, except for the nodes marked with a white star, which carry 26 SNPs (n = 2) or 23 SNPs (n = 1)], from 2 to 10 allelic differences between nodes included in the $M1_{inter}$ box (all carrying 13 of the 27 $M1_{UK}$-characteristic SNPs), and from 1 to 50 SNPs between nodes included in the $M1_{global}$ box (carrying none of the $M1_{UK}$-characteristic SNPs). (B) Prevalence of $M1_{global}$ (0 SNPs), $M1_{inter}$ (13 SNPs), and $M1_{UK}$ (26 or 27 SNPs) isolates among iGAS isolates recovered from adults, Portugal, 2016–2019 (n = 273), per study year. Absolute numbers for *emm*1 and total isolates are presented below each year.

0.828–0.889), p = 0.035], along with an increase in the prevalence of the top three *emm* types from 46 % to 55 % (p = 0.044). However, the leading *emm* types remained the same and there were no significant changes in individual *emm* type prevalence after FDR correction (Supplemental Table S5). Therefore, our results point to a stability in the *emm* types causing adult iGAS in Portugal during the past decade, which does not exclude the occurrence of important intra-*emm* type changes that can only be detected using additional molecular markers, such as the expansion of the $M1_{UK}$ sublineage. However, since there was no increase in the number of isolates received in each year, this expansion did not result in iGAS increases in the pre-COVID-19 period. This argues that other factors beyond the emergence of specific clones underly the

Table 2
Isolates lacking full-length *hasA* or *hasB* genes among 273 invasive group A *Streptococcus* isolates recovered from adults in Portugal, 2016–2019.

emm-ST	No. isolates	Full-length *hasA*	Full-length *hasB*	P*nga*
*emm*1-ST28	2[a]	–	+	3.2
*emm*1-ST28/1299	2[b]	+	–	3.1
*emm*4-ST39	15	–	–	3.2
*emm*11-ST403	1	–	–	1.1
*emm*11-ST403	1	–	+	1.1
*emm*12-ST36	1	+	–	3.1
*emm*18-ST1216	1	–	+	2.1
*emm*22-ST46[c]	8	–	–	3.1
*emm*28-ST52[d]	13	–	+	1.1
*emm*68-ST247	1	–	+	2.1
*emm*77-ST63	4	–	+	3.2
*emm*77-ST399	1	–	+	1.1
*emm*87-ST62	8	–	+	3.2
*emm*89-ST101[e]	26	–	–	3.1
*emm*94-ST1294	1	+	–	3.1
*emm*102-ST60	1	–	+	2.1
*emm*169-ST53	2	–	–	1.1

ST, sequence type; P*nga*, promoter variant of the *nga-ifs-slo* operon.[28]
[a] One isolate was assigned to $M1_{inter}$ and the other to $M1_{global}$.
[b] One isolate was assigned to $M1_{UK}$ and the other to $M1_{global}$.
[c] One isolate presented ST1309.
[d] Other STs were: ST456 ($n = 2$) and ST458 ($n = 4$).
[e] Other STs were: ST824 ($n = 3$), ST1285 ($n = 1$), ST1295 ($n = 1$), and ST1303 ($n = 4$).

post-COVID upsurge in iGAS.

The tetracycline and erythromycin resistance rates determined herein (12 % and 4 %, respectively) were also in line with those recorded for adult iGAS in 2010–2015 (10 % and 4 %, respectively),[23] with erythromycin resistance remaining mostly associated with the presence of the *erm*(A) or *erm*(B) genes conferring the $iMLS_B$ and $cMLS_B$ phenotypes, respectively. This macrolide resistance rate is lower than the 7 % reported in neighboring Spain during 2016–2020[35] and is in sharp contrast to the marked increase documented in the US during 2015–2019, reaching 25 % non-susceptibility in 2018–2019.[19] Similarly to data from Barcelona concerning invasive and non-invasive isolates from 2016-2018,[36] the leading macrolide-resistant lineage in our study was *emm*77-ST63, presenting the $iMLS_B$ phenotype and tetracycline resistance due to the presence of the *erm*(A) and *tet*(O) genes.

Three different amino acid substitutions previously associated with increased β-lactam MICs were identified. Each of them was detected in a single isolate, with no clonal expansion, contrarily to observations from Iceland, where the M593T mutation was detected in virtually all *emm*12 also carrying the *mef*(A)-*msr*(D) genes encoding macrolide efflux.[16] The penicillin MICs determined for our three isolates (0.016 μg/mL) were in line with the small increases in MIC previously associated with these PBP2X mutations, but still well below the accepted susceptibility cutoff value (0.12 μg/mL).[16–18,28]

The two-component system CovRS directly or indirectly regulates multiple factors implicated in GAS virulence and immune evasion, and acquisition of mutations impairing CovS sensor function are thought to promote a transition from local to disseminated infection and are overrepresented among iGAS isolates.[31,37] In line with previous observations,[31] 11 % of our isolates had no *covS* locus or carried a *covS* variant predicted to result in null CovS activity. Although *emm*1 is dominant among iGAS, in the current dataset null *covS* alleles were underrepresented in *emm*1 isolates. The only *emm*1 isolate presenting a null *covS* belonged to the $M1_{UK}$ sublineage, despite recent data indicating a lower prevalence of *covRS* mutations in this sublineage when compared with $M1_{global}$ isolates.[6] In contrast, infrequent *emm* types were significantly associated with the presence of null *covS* alleles. This suggests that abrogation of CovS function may play a particularly important role in the pathogenesis of GAS lineages with potentially lower virulence, while highly virulent lineages, such as the contemporary *emm*1, are less dependent on the acquisition of CovS-inactivating mutations to establish invasive disease.

Although the hyaluronic acid capsule has historically been considered a major virulence factor, multiple lineages causing invasive and non-invasive infections have recently been shown to lack a functional capsule operon.[1,29,38,39] Nearly one third of the isolates in the current study lacked a full-length copy of at least one of the two essential genes for capsule production, most of which (76 %) carried a high-expression variant of the promoter of the operon encoding NAD-glycohydrolase (NADase) and streptolysin-O (SLO). These data further support a non-essential role for the capsule in the establishment of invasive infections by certain GAS lineages, in particular when associated with increased expression of NADase and SLO.[29]

A limitation of this study is the voluntary nature of isolate submission. Although this prevents us from calculating incidences, the stability of our surveillance network and the participation of laboratories from 38 hospital centers distributed across the country guarantees the representativeness of the collection. Given that decreased susceptibility to β-lactams was not the focus of this work, MIC determination was performed only for penicillin and for isolates carrying PBP2X mutations previously associated with increased β-lactam MICs. However, this strategy may have prevented us from identifying novel mutations in PBP-encoding genes potentially associated with decreased β-lactam susceptibility. Another limitation is that identification of isolates with abrogated CovS function was based solely on the presence of truncated or absent *covS* alleles, and not on any phenotypic analysis. Although some amino acid changes may also impact CovS activity, the prediction of the phenotypic consequence of missense mutations is complex. Therefore, we took a conservative approach and considered as null *covS* alleles only those that carried indels and nonsense mutations similar to those previously linked with an altered virulence factor activity profile and associated with invasive infections.[31,32]

This study provides a characterization of the molecular epidemiology and antimicrobial resistance profiles of iGAS during the pre-COVID-19 period in Portugal. Although there were no major changes in the dominant *emm* types and antimicrobial resistance relative to a previous study, the use of WGS data allowed the identification of internationally disseminated intra-*emm* sublineages, including the $M1_{UK}$, which has been associated with a post-pandemic upsurge of iGAS and was already expanding in our country during 2016–2019. Continued molecular and epidemiologic surveillance of iGAS is warranted to monitor the progression of such lineages after the bottleneck imposed during the COVID-19 pandemic, as well as to evaluate if the local sublineages identified during 2016–2019 persisted and expanded among iGAS in Portugal, or if they were associated with temporally restricted outbreaks.

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CRediT authorship contribution statement

Ana Friães: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rafael Mamede:** Software, Methodology, Formal analysis. **Beatriz Santos:** Methodology. **José Melo-Cristino:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Mario Ramirez:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Portuguese Group for the Study of Streptococcal Infections:** Methodology, Investigation.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.

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