ST913-IVa-t991 Methicillin-Resistant *Staphylococcus aureus* among Pediatric Patients, Israel

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In Israel, prevalence of sequence type 913, staphylococcal cassette chromosome *mec*IVa, *spa* type t991 methicillin-resistant *Staphylococcus aureus* lineage has surged among pediatric populations, predominantly in Arab and Orthodox Jewish communities. Antimicrobial resistance patterns vary by demographics. This lineage's spread and microevolution in the Middle East underscore the need for ongoing surveillance.

In 2010, a new methicillin-resistant *Staphylococcus aureus* (MRSA) clone, belonging to the clonal complex (CC) 913, Panton-Valentine leukocidin (PVL)–negative, staphylococcal cassette chromosome *mec* type IV, was isolated from Bedouin children in Israel (*1*). In 2012, isolates of CC913 were further analyzed, and their *spa* type was revealed as t991. Four t991 isolates were identified in hospitals across Israel, indicating the spread of the clone to communities beyond the Bedouin population in southern Israel (*2*). In 2015, a total of 12 t991 isolates were obtained from 280 patients (*3*), and in 2019, a total of 6 t991 isolates were obtained from 112 patients (*4*), mainly from children.

Since 2015, MRSA isolates of *spa* type t991 have emerged to become one of the main lineages in hospitals and health maintenance organizations in Israel. However, despite its significance, comprehensive characterization of *spa* type t991 clone is lacking. We explore its genomic context and antibiotic profile in this study.

The Study

During 2012–2020, the *S. aureus* national reference laboratory of Israel received a total of 4,646 MRSA isolates, obtained from skin and soft tissue infections (SSTIs), that were classified into 284 different *spa* types. Types t002, t008, and t032 were the most prevalent; t002 comprised 25% of total MRSA SSTI isolates, t008 comprised 15%, and t032, 5%. During that period, the proportion of t991 MRSA gradually increased to 13% of total MRSA SSTI isolates, whereas the leading *spa* types in MRSA SSTIs (t002 and t008) remained stable (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/30/8/23-0981-App1.pdf). During that period, 689 *S. aureus* samples of *spa* type t991, *mecA*-positive, PVL-negative, were received at the *S. aureus* national reference laboratory (Appendix). Most of the samples (406, 70%) were isolated from SSTIs; 66% isolates were from patients <5 years of age (p = 0.0001), whereas 4% were isolated from patients >60 years of age. In addition, most patients resided in localities associated with Arab and Orthodox Jewish populations (*5*). The number of t991 MRSA isolates from SSTI and blood increased dramatically, from 5 in 2012 to 180 in 2019 and 146 in 2020 (https://microreact.org/project/r4dFwJGXudh3gfyWtE1f87-t991final) (Figure 1).

We conducted whole-genome sequencing on 20 t991 MRSA isolates that were selected (Appendix Table), along with 3 t991 MRSA isolates from Germany (*6*,*7*). The isolates clustered into 4 separate clades (Figure 2). Clade A consisted of the 3 t991 isolates from Germany and is 130 whole-genome multilocus sequence typing (wgMLST) alleles distant from the first isolate of clade B, which consisted of 7 isolates from patients who lived in the Negev and were admitted to the same hospital. Clade C consisted of 5 isolates from patients residing in the Jerusalem district. Clade D consisted of 8 isolates, 7 of which were from Orthodox Jewish patients.

We tested 116 t991 MRSA isolates for phenotypic susceptibility by using the broth microdilution method (Figure 3). Isolates from patients living in Arab localities were more resistant to erythromycin and chloramphenicol, whereas those isolated from patients living in Jewish localities showed higher resistance to gentamicin, ciprofloxacin, levofloxacin, and

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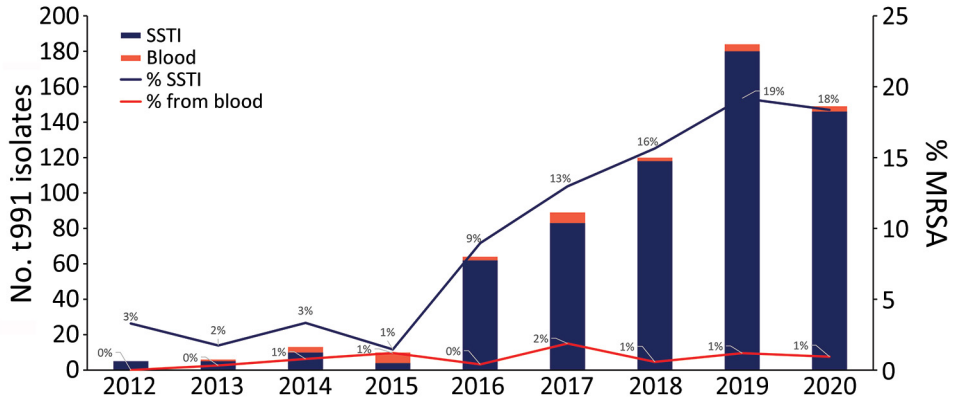

Figure 1. t991 MRSA isolates isolated from blood and SSTIs among patients in Israel during 2012–2020. Left y-axis represents number of t991 isolates isolated and right y-axis represents the relative part from total MRSA SSTI or blood isolates. MRSA, methicillin-resistant *Staphylococcus aureus*; SSTI, skin and soft tissue infection.

moxifloxacin (Appendix Figure 2). That tendency was statistically significant for chloramphenicol (p = 0.01) and gentamicin (p = 0.01).

We found 9 antimicrobial resistance (AMR) determinants, 8 AMR genes and 1 point mutation, among the 20 t991 MRSA sequences (Figure 4). Overall,

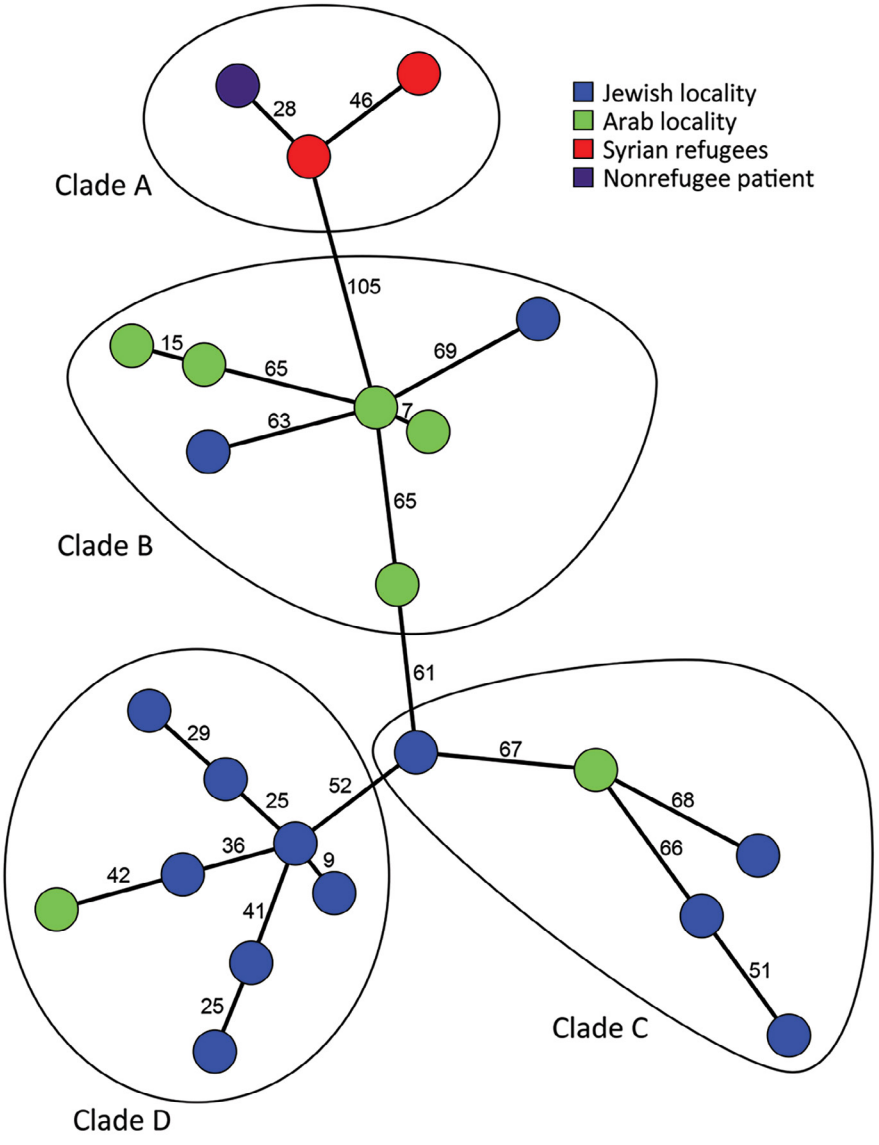

Figure 2. Phylogenetic relationships between 23 t991 MRSA genomes isolated in Israel and Germany. The figure shows a minimum spanning tree, created in Bionumerics software (https://www.bionumerics.com), based on 3,904 wgMLST allele IDs of sequenced t991 MRSA isolates. Each node represents an isolate; numbers along branches connecting nodes indicate the numbers of allelic differences between isolates. The isolates are further divided into 4 clades (A–D). MRSA, methicillin-resistant *Staphylococcus aureus;* wgMLST whole-genome multilocus sequence typing.

correlation between genotype prediction based on WGS and phenotypic AMR was 99% with a sensitivity of 94% and specificity of 100%. All discrepancies were associated with an absence of resistance determinant among phenotypically resistant isolates. No data for quinolone resistance genes or mutational resistance were predicted by the BioNumerics (https://www.bionumerics.com) or AMRFinder (https://github.com/ncbi/amr/releases/tag/amrfinder_v3.10.21) algorithms. In addition, we did not test phenotypic resistance against mupirocin.

We compared virulence profiles of 20 WGS t991 isolates with 3 t991 isolates from Germany, community-acquired MRSA USA300, and USA400 (*8*) as a reference using the functional genotyping tool of Bionumerics version 8.0. The main difference in virulence gene profile is reflected in the group of genes associated with adherence (Appendix Figure 3). In addition, virulence profiles can be grouped into 6 patterns on the basis of the presence of specific adherence factor genes in the genomes (Appendix Figure 4). We found no correlation between virulence profile and AMR, age, sex, residence location, or association with 1 of the genomic clades.

Next, to assess the genetic relationship of t991 isolates to other MRSA strains circulating worldwide, we created GrapeTree on the pubMLST site (https://pubmlst.org/organisms/staphylococcus-aureus) based on wgMLST data of representative local MRSA t991 strain (SA14675) along with 37,883 *S. aureus* global isolates (Appendix Figure 5) (*9*). The closest node to strain SA14675 is at a distance of 74 wgMLST alleles and is composed of 7 isolates. The next closest node is at a distance of 1,486 wgMLST alleles and composed of isolates that belong mainly to CC1.

Conclusions

Most t991 cases were isolated from young patients who live in strictly Orthodox and Arab localities. A possible explanation for this phenomenon is a similar lifestyle of the 2 sectors, characterized by overcrowding and large families. Regarding the evolution of this clone and its spread into the population, this strain appears to have evolved by multiple different genetic events. This assumption is supported by several findings. First, t991 MRSA isolates demonstrated classification into 4 distinct clades on the basis of geographic location and sectoral association; we noted genetic variation and weak clonality evident from the considerable distances between nodes, even within the same clade. Second, antibiotic resistance patterns vary between isolates obtained from patients who live in Jewish and Arab localities (Appendix Figure 2). Finally, *spa* type t991 composition is very short; it consists of 3 repeats (07-33-23) and can be formed as a result of genetic rearrangement of numerous MRSA strains harboring longer *spa* type repeats in which the repeats of *spa* type t991 from a part of their repeat succession. Worldwide phylogenetic analysis indicates that t991 MRSA stands out as a distinct emerging lineage because it appears considerably distant from most strains included in the GrapeTree (Appendix Figure 5).

Phenotypic AMR data for global isolates were available for 2 isolates (*7*,*10*). One isolate obtained in Kuwait was resistant to erythromycin, clindamycin, trimethoprim, and fusidic acid (*10*), and the

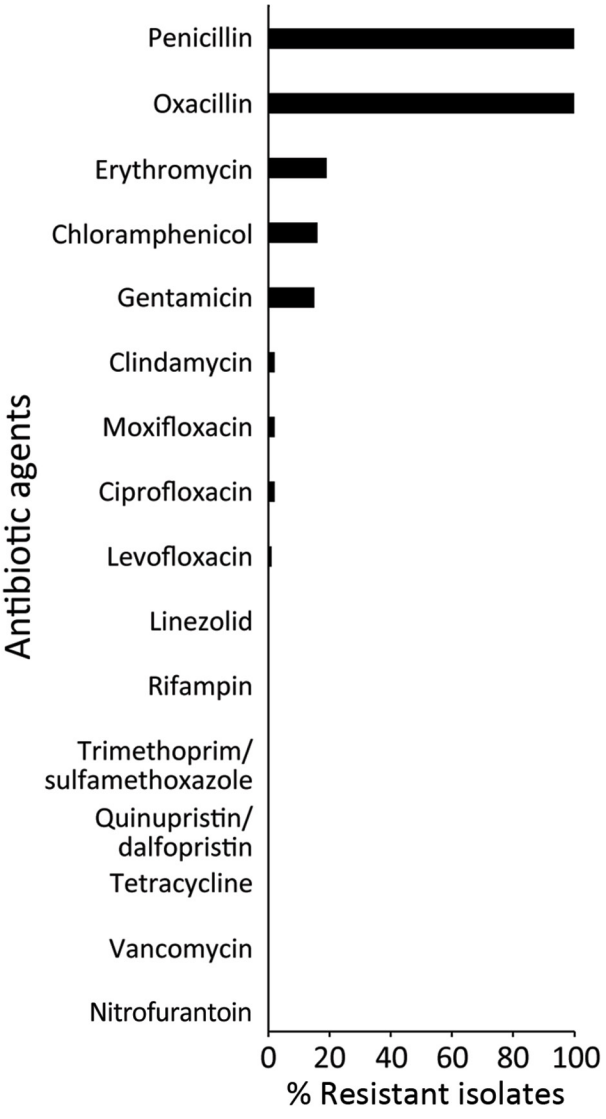

Figure 3. Percentage of resistant isolates to antibacterial agents among 116 t991 MRSA isolates from Israel tested for antimicrobial susceptibility using the broth microdilution method. MRSA, methicillin-resistant *Staphylococcus aureus.*

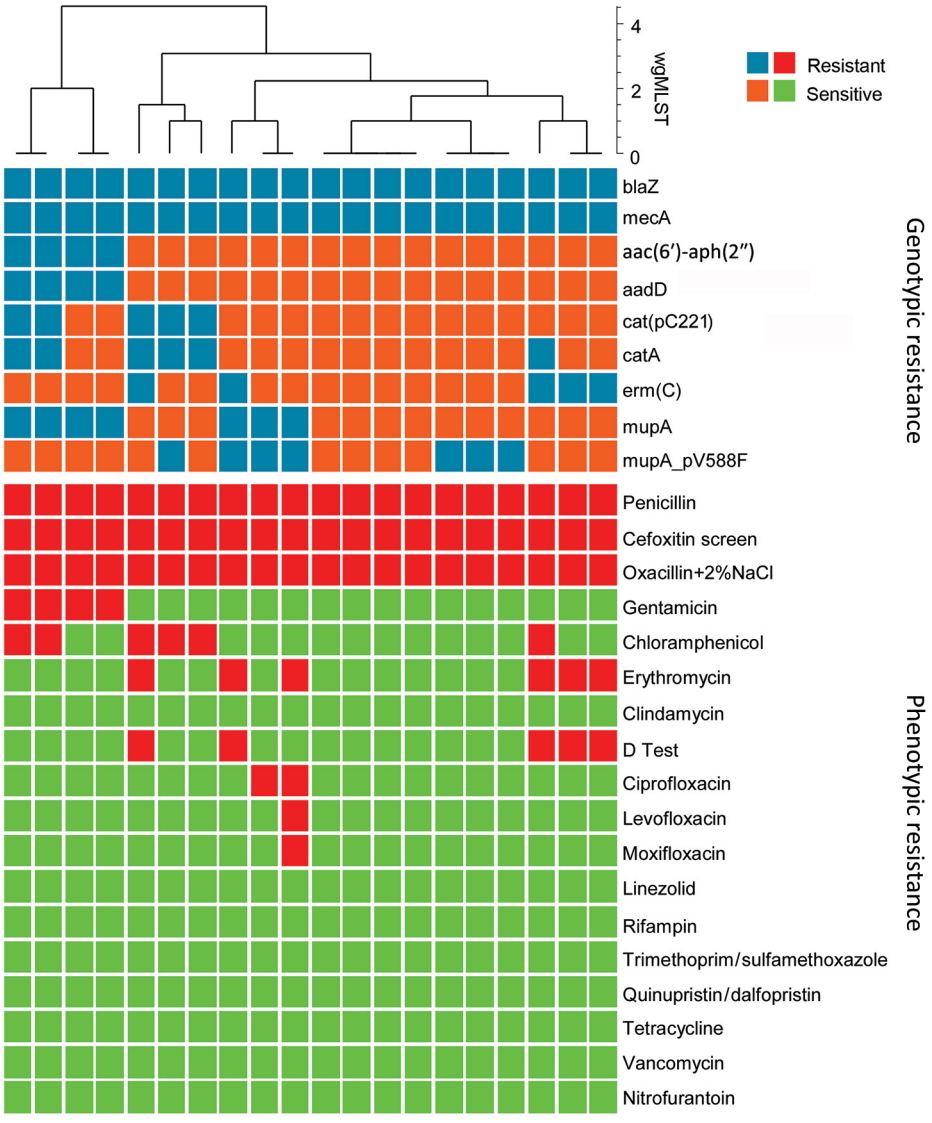

Figure 4. Comparison of genotypic and phenotypic resistance patterns of 20 t991 MRSA isolates from Israel tested using whole-genome sequencing. Blue tiles represent presence of resistance gene and orange tiles absence of resistance gene; red tiles represent antimicrobial resistance and green tiles antimicrobial sensitivity. Clustering is based on wgMLST data and generated by BioNumerics software (https://www.bionumerics.com). wgMLST, whole-genome multilocus sequence typing.

other was isolated in Germany from a refugee from Syria (*7*) and was resistant to erythromycin, clindamycin, and tetracycline. Out of the 116 tested t991 isolates, none showed resistance to tetracycline by antimicrobial susceptibility. For 2 isolates, we observed phenotypic resistance for erythromycin and chloramphenicol without prediction of AMR determinant. Close inspection of those isolates revealed they were actually positive for *ermC* and *cat*, and their sequences were fragmented into multiple contigs. Consistent with previous publications (*10*–*12*), all isolates tested, except for the strain from the Syria refugee (*7*), were positive for *eta*, a toxin responsible for skin infections seen mainly among young patients (*3*,*4*,*7*). Those findings are in accordance with the observation that t991 MRSA is predominantly isolated from children.

In conclusion, our study shows the emergence of t991 MRSA in Israel. These strains affect mainly pediatric populations, and a geographic distribution is limited mainly to the Middle East. The epidemiologic and genomic information our research provides will assist further investigation on the origin and dissemination of this clone.

About the Author

Dr. Baum is head of the National Staphylococcus Reference Laboratory at the Ministry of Health, Israel. His interests include genomic epidemiology and infectious diseases.

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Appendix

Materials and Methods

Bacterial isolates, media and lysate preparation – *S. aureus* isolates of *spa* type t991 originated mainly from SSTI between 2012 to 2020. The samples were submitted to the National *S. aureus* reference laboratory (NRL) from clinical labs located across the State of Israel for bacteriological characterization. In Israel clinical labs are required to send MRSA and MSSA isolates from bacteremia, along with MRSA from wound infections, to the national center for further analysis and storage in our strain bank. All strains are sent along with demographic data and isolation source information. Strains NCTC 13552 and NCTC 14245 were used as positive control in *mecA*, *mecC* and PVL real time PCR reaction. Strain SA104 (*mecA* and PVL positive, *spa* type t008) was used as a positive control in *spa* PCR. ATCC strain 29213 was used an Internal quality control in the broth microdilution assay. All strains were cultured at 37ºC for 16-24 hours.

The strains were cultured on CHROMagar MRSA/MSSA plates (DD066, hylabs, Israel) and a single colony was transferred to Nutrient Agar (PD040, hylabs, Israel). Lysis of bacterial cells was performed by suspending a single colony in 100µl lysis buffer (lysozyme 50.8 units; lysostaphin 2.7 units; TRIS 0.1M pH=8; EDTA 0.01M; DDW to a final volume of 100 µl) and incubation at 37 ºC for 30 min following 10 min of boiling. The lysates were diluted 1:40 and 2 µl of the dilution was used as a template in real time and PCR reactions.

Real time PCR and *spa* PCR: multiplex real time PCR for the simultaneous detection of *mecA*, *mecC*, PVL and *nuc* gene, which serve as an internal amplification control, was performed

as previously described by Pichon et al (PMID: 22687894), Fosheim et al (PMID: 21697325)and CDC document No ARC.TE.C.0055. In the NRL *spa* type analysis is performed on all MRSA and PVL positive MSSA isolates Molecular typing by *spa* type was performed as previously described by Kahl et al (PMID: 15635028). Analysis of *spa* sequences was performed using BioNumerics 8.0 software (Applied Maths, Belgium).

Antimicrobial susceptibility testing: antimicrobial susceptibility testing was performed using the broth microdilution method. Sensititre susceptibility plates (gram positive GPALL1F AST Plate) was used according to the manufacturer's instructions. Briefly, ~ 5 colonies were suspended in ddw to a turbidity of 0.5 McFarland. Then, 10 µl of the suspension were transferred into 11 ml of cation-adjusted Mueller-Hinton broth (cat. Number T3462). The plate was inoculated using the Sensititre AutoInoculator / AIM. Following 24 hours incubation, results were read using the VIZION platform (Sensititre). Minimum inhibitory concentrations (MIC) were determined according to CLSI guidelines (M100 2020). Correlation, sensitivity and specificity calculation were performed as previously described by Rokney et al (PMID: 32903472).

Genomic DNA extraction, WGS analysis and Bioinformatics analysis Genomic DNA extraction was performed by using the QIAsymphony platform (QIAGEN, Hombrechtikon, Switzerland). DNA library was prepared using Nextera XT kit (Illumina Inc. San Diego, CA, USA). *De novo* assembly by SPAdes and whole genome MLST (wgMLST) was performed on the BioNumerics 8.0 software (Applied Maths, Belgium) by using the default settings. Genomic comparison were visualized using minimum spanning tree (MST) based on 3,904 wgMLST allele IDs by using the BioNumerics 8.0, with alleles difference annotated on the branches. Resistance genes and virulence factor detection was carried out on the BioNumerics 8.0 by using the *S. aureus* functional genotyping tool. In addition, antimicrobial resistance genes presence was predicted by the AMRfinder software.

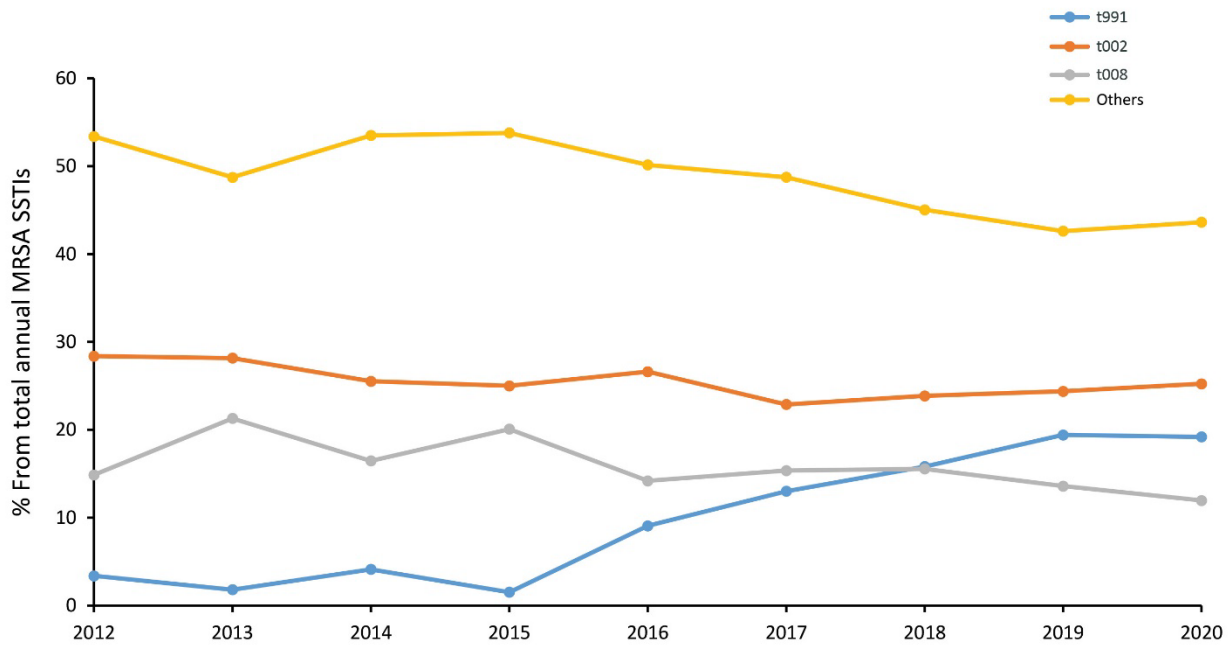
Grapetree creations GrapeTree was created on the pubMLST site. In short, the genome collection database was filtered by sequence bin (total length > 2.5 Mbp) and GrapTree was created using cgMLST of 37,883 *Staphylococcus aureus* isolates using the default settings and colored by clonal complex (MLST).

Statistical analysis: Statistical analyses were performed with GraphPad (https://www.graphpad.com). Data was considered as statistically significant if $p < 0.05$.

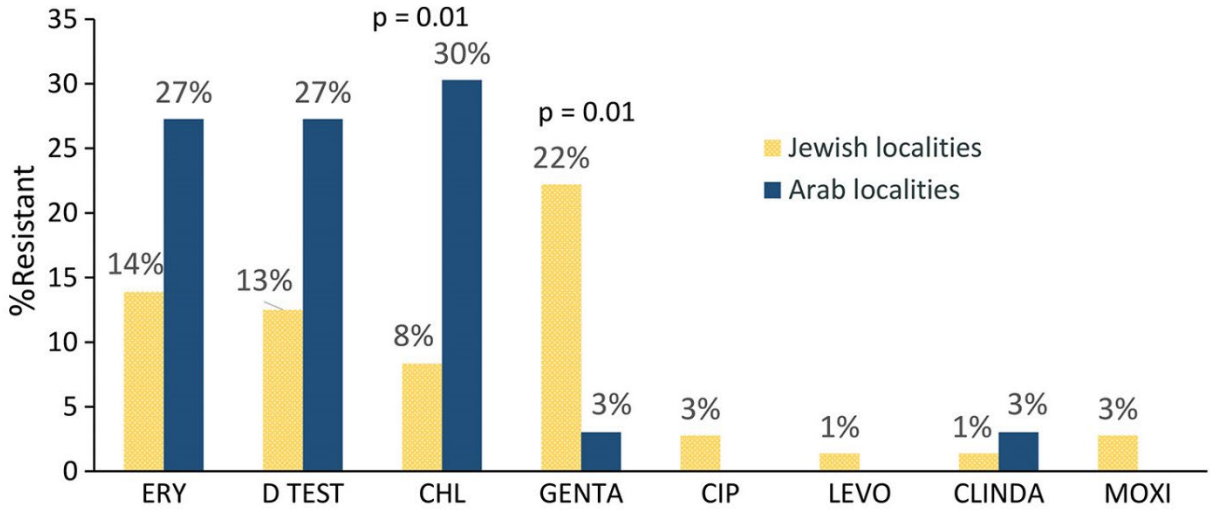
The following genomes were used for WGS analysis comparison: t991 isolate from Syrian refugee in Germany in 2018 (PMID: 34785377). T991 isolates from Syrian refugee and non-refugee patient in Germany in 2015 and 2016 respectively (PMID: 29851962). Strain SA14675 was deposited in the pubMLST database under the ID 36889.

Appendix Table. Demographic characteristics and antibiotic resistance profiles of 20 MRSA strains analyzed by whole-genome sequencing

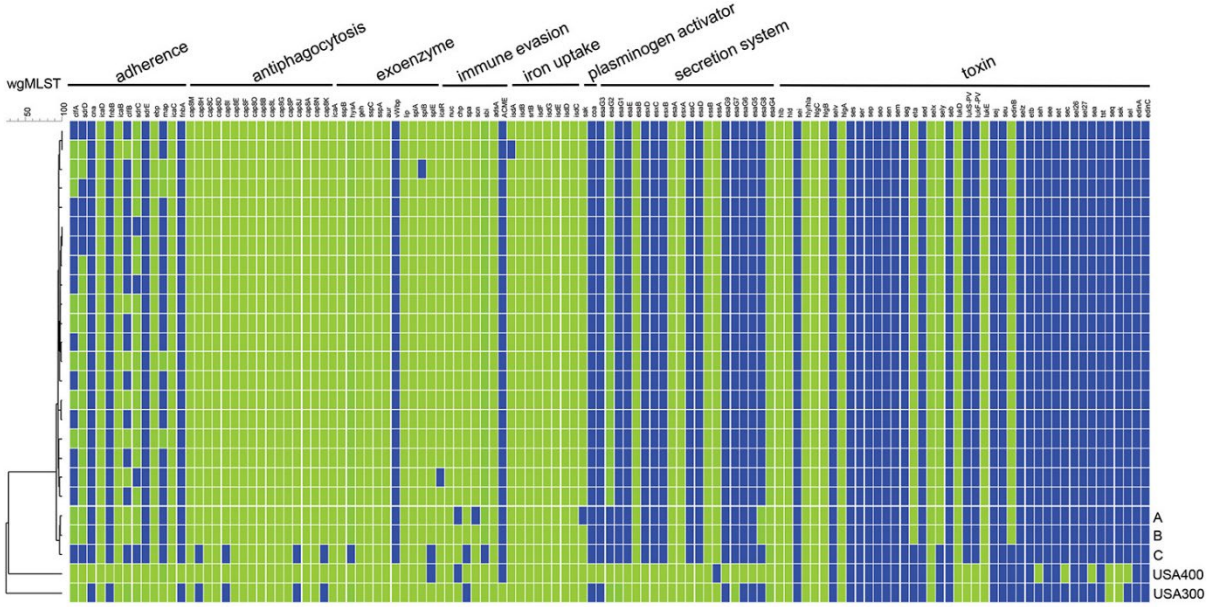
#	Ref lab	Sector	Sample Collection Date	Originator	Residence Place	AST Pattern	WGS	CHL Int	Cip Int	Clinda Int	Dtest 1 Int	Dtest 2 Int	Ery Int	Genta Int	Levo Int	Moxi Int
1	SA09749	Orthodox	7/27/2017	HMO	Modi'in Illit	A	Orthodox_R to Chloramphenicol only	**R**	S	S	S	S	S	S	S	S
2	SA10528	Arab	11/17/2017	Hospital	Unknown	A	Arab_R to Chloramphenicol only	**R**	S	S	S	S	S	S	S	S
3	SA08727	Orthodox	2/28/2017	Hospital	Modi'in Illit	B	Jewish_R only to Erythromycin	S	S	S	**R**	**R**	**R**	S	S	S
4	SA11866	Orthodox	5/23/2018	HMO	Elazar	B	Orthodox_R only to Erythromycin	S	S	S	**R**	**R**	**R**	S	S	S
5	SA13166	Arab	1/16/2019	Hospital	Rahat	B	Arab_R only to Erythromycin	S	S	S	**R**	**R**	**R**	S	S	S
6	SA08765	Arab	3/5/2017	Hospital	Jerusalem	C	Arab_R to Chloramphenicol and Gentamicin	**R**	S	S	S	S	S	**R**	S	S
7	SA10010	Orthodox	8/27/2017	HMO	Beit Shemesh	C	Orthodox_R to Chloramphenicol and Gentamicin	**R**	S	S	S	S	S	**R**	S	S
8	SA09965	Arab	8/17/2017	Hospital	Huzail tribe	D	Arab_First strain R to Chloramphenicol and Erythromycin	**R**	S	S	**R**	**R**	**R**	S	S	S
9	SA12099	Arab	7/25/2018	Hospital	Rahat	D	Arab_Last strain R to Chloramphenicol and Erythromycin	**R**	S	S	**R**	**R**	**R**	S	S	S
10	SA08479	Orthodox	12/21/2016	HMO	Modi'in Illit	E	Orthodox_First strain S to all antibioticss tested	S	S	S	S	S	S	S	S	S
11	SA08650	Arab	2/16/2017	Hospital	Modi'in Illit	E	Arab_First strain S to all antibioticss tested	S	S	S	S	S	S	S	S	S
12	SA09589	Jewish	7/11/2017	Hospital	Jerusalem	E	Jewishl_First strain S to all antibiotics tested	S	S	S	S	S	S	S	S	S
13	SA13843	Orthodox	4/29/2019	HMO	Jerusalem	E	Orthodox_Last strain S to all antibioticss tested	S	S	S	S	S	S	S	S	S
14	SA14456	Arab	8/14/2019	Hospital	Umm Batin	E	Arab_Last strain S to all antibioticss tested	S	S	S	S	S	S	S	S	S
15	SA14675	Jewish	9/12/2019	Hospital	Shlomit	E	Jewishl_Last strain S to all antibiotics tested	S	S	S	S	S	S	S	S	S
16	SA11087	Orthodox	1/17/2018	HMO	Bnei Brak	F	Middle strain R to Gentamicin	S	S	S	S	S	S	**R**	S	S
17	SA12245	Orthodox	7/19/2018	HMO	Tel Sheva	F	Last strain R to Gentamicin	S	S	S	S	S	S	**R**	S	S
18	SA07499	Orthodox	8/21/2016	HMO	Beitar Illit	H	First strain from HMO	S	**R**	S	S	S	**R**	S	**R**	**R**
19	SA09727	Orthodox	2/5/2017	HMO	Jerusalem	I	R to Ciprofloxacin only	S	R	S	S	S	S	S	S	S
20	SA06277	Jewish	3/13/2016	Hospital	Beersheba	L	First strain from Hospital	S	R	S	S	S	S	S	R	S



Appendix Figure 1. Prevalence comparison of spa types t991, t002, t008 and all other spa types among clinical MRSA isolates isolated from SSTIs between 2012–2020.



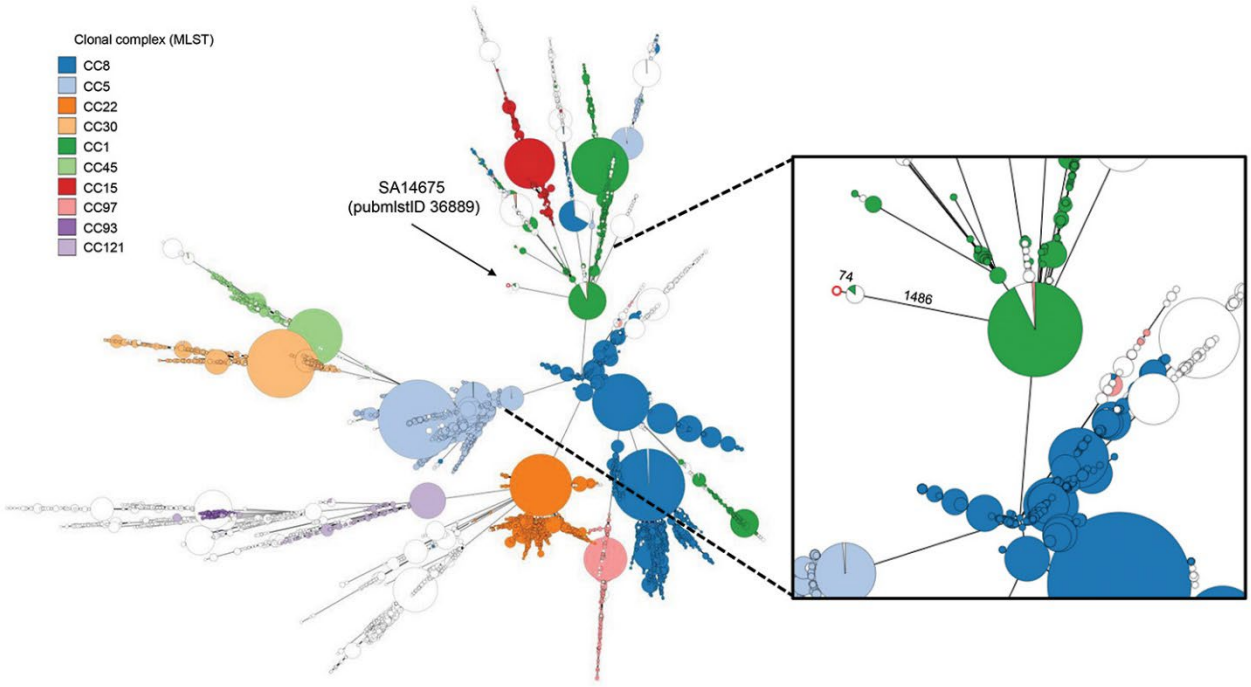
Appendix Figure 2. AMR pattern of t991 isolates, isolated from patients who live in Jewish and Arab localities.



Appendix Figure 3. Comparison of virulence factors profile of t991 MRSA isolates, isolated in Israel and Germany alongside USA300 and USA400 strains. Virulence factors analysis was performed using BioNumerics *S. aureus* functional genotyping tool. Presence or absence of gene is represented by green or blue tile respectively. The dendrogram on the left is based on wgMLST data. A – strain 012 isolated from Syrian refugee, B – strain 073 isolated from non-refugee patient, C – this strain was isolated in Germany from refugee.

	Virulence Pattern	icaB	icaC	icaD	ebp	sdrC	sdrD	clfA	clfB	map	sdrE	cna	fnbB	fnbA
SA08479	A	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->	<->	<->
t991 isolated from refugee in Germany	A	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->	<->	<->
SA14456	B	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->	<->
SA08650	B	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->	<->
SA09749	B	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->	<->
SA09965	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA07499	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA11866	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA13843	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA10010	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA12245	C	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->	<->
SA10528	D	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->
SA12099	D	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->
SA08727	D	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->
Strain 012 from Syrian refugee	D	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->
Strain 073 from non-refugee patient	D	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->
SA14675	E	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->
SA08765	E	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->
SA09589	F	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<+>	<->	<->	<->	<->
SA06277	F	<+>	<+>	<+>	<+>	<+>	<->	<+>	<->	<+>	<->	<->	<->	<->
SA09727	F	<+>	<+>	<+>	<+>	<->	<+>	<->	<->	<->	<->	<->	<->	<->
SA13166	F	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->	<->	<->	<->
SA11087	F	<+>	<+>	<+>	<+>	<->	<+>	<->	<+>	<->	<->	<->	<->	<->
USA300 (t991 comparison)	USA300	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<->	<->	<->
USA400 (t991 comparison)	USA400	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>	<+>

Appendix Figure 4. Comparison of virulence pattern as predicted by BioNumerics.



Appendix Figure 5. Genomic relationships of t991 isolates and 37,883 *S. aureus* strains isolated worldwide. The graph illustrates the genetic distance between the genomes included in the analysis. Grape tree was created on pubMLST site based on wgMLST data. Each node corresponds to a single CC. Node is proportional to genomes number. Strain SA14675 is marked by red circle and black arrow.