Multilocus Sequence Typing Analysis of *Staphylococcus lugdunensis* Implies a Clonal Population Structure

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Staphylococcus lugdunensis is recognized as one of the major pathogenic species within the genus *Staphylococcus*, even though it belongs to the coagulase-negative group. A multilocus sequence typing (MLST) scheme was developed to study the genetic relationships and population structure of 87 *S. lugdunensis* isolates from various clinical and geographic sources by DNA sequence analysis of seven housekeeping genes (*aroE*, *dat*, *ddl*, *gmk*, *ldh*, *recA*, and *yqiL*). The number of alleles ranged from four (*gmk* and *ldh*) to nine (*yqiL*). Allelic profiles allowed the definition of 20 different sequence types (STs) and five clonal complexes. The 20 STs lacked correlation with geographic source. Isolates recovered from hematogenic infections (blood or osteoarticular isolates) or from skin and soft tissue infections did not cluster in separate lineages. Penicillin-resistant isolates clustered mainly in one clonal complex, unlike glycopeptide-tolerant isolates, which did not constitute a distinct subpopulation within *S. lugdunensis*. Phylogenies from the sequences of the seven individual housekeeping genes were congruent, indicating a predominantly mutational evolution of these genes. Quantitative analysis of the linkages between alleles from the seven loci revealed a significant linkage disequilibrium, thus confirming a clonal population structure for *S. lugdunensis*. This first MLST scheme for *S. lugdunensis* provides a new tool for investigating the macroepidemiology and phylogeny of this unusually virulent coagulase-negative *Staphylococcus*.

Since its first description in 1988, *Staphylococcus lugdunensis* (15) has rapidly been recognized as one of the major pathogenic species within the genus *Staphylococcus*, despite belonging to the coagulase-negative group. *S. lugdunensis* is recovered from the normal skin flora (1), and numerous infections have been related to inguinal-area carriage (37). Cutaneous infections account for more than 50% of *S. lugdunensis* infections (38), but tissue infections with various abscess localizations are reported, with tissue damage or poor clinical outcome (13). Bacteremia is frequently associated with endocarditis (36), which develops more frequently on native valve than on prosthetic material (28) and has a clinical course resembling *Staphylococcus aureus* infection (36). Hematogenic infections, such as prosthetic joint infections or osteomyelitis, are also considered as aggressive as with *S. aureus* (30). The pathogenic potential of *S. lugdunensis*, which is thus closer to that of *S. aureus* than those of other coagulase-negative staphylococci, may be related to virulence factors such as a fibrinogen-binding protein (25), a von Willebrand factor-binding protein (26), or synergistic hemolysins (7). In addition, the genome of *S. lugdunensis* N920143 contains an *isd* locus (19), which encodes proteins involved in iron acquisition in *S. lugdunensis* (18) or in cutaneous colonization in *S. aureus* (6).

Unlike *S. aureus* or numerous coagulase-negative staphylococci, *S. lugdunensis* remains largely susceptible to antistaphylococcal antibiotics. Penicillinase production is observed for less than 50% of isolates (14), and methicillin resistance is still rare (30, 38). However, a particular feature of *S. lugdunensis* is vancomycin and/or teicoplanin tolerance (4, 14). We found, by analyzing seven coagulase-negative staphylococcal species, that this glycopeptide tolerance phenomenon was restricted to *S. lugdunensis*. Indeed, all the *S. lugdunensis* isolates investigated displayed strict glycopeptide tolerance or at least decreased and slower susceptibility to glycopeptide bactericidal activity (4).

Phylogenetic analyses within pathogenic species provide pre-

cious knowledge about their genetic population structure and their relation to patterns of virulence and/or antimicrobial susceptibility. Multilocus sequence typing (MLST), which characterizes bacterial multilocus genotypes by using intragenic sequences of a set of housekeeping genes, was initially proposed for population genetics analysis of *Neisseria meningitidis* (12). It allowed the characterization of recombinant population structures for *N. meningitidis* or *Streptococcus pneumoniae* (10, 12) and, conversely, clonal population structure for *S. aureus* (8) or *Clostridium difficile* (21), whose mutational evolution generates deeper recognizable phylogenetic lineages. MLST has proved essential for analyzing the evolution of methicillin-resistant *S. aureus* clones and their filiation from ancestral methicillin-susceptible clones (9). MLST also offers the possibility to open shared sequence databases on the World Wide Web that provide an extensive and continuously updated view about the long-term epidemiology and evolution of a given species (22).

Although MLST analyses have been promoted for *S. aureus* (8) and *Staphylococcus epidermidis* (24), there is still a need for an MLST scheme devoted to *S. lugdunensis*, whose phylogenetic structure remains unknown. The aim of the present work was to develop an MLST scheme for *S. lugdunensis* and to analyze the mode of evolution of this pathogenic *Staphylococcus* species in relation to antimicrobial susceptibility.

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MATERIALS AND METHODS

Bacterial isolates. A total of 87 *S. lugdunensis* isolates were studied: *S. lugdunensis* ATCC 49576, *S. lugdunensis* ATCC 43809, *S. lugdunensis* ATCC 700328, and 84 human clinical isolates (skin or soft tissue isolates, osteoarticular isolates, blood isolates, and material device isolates) collected from 1991 to 2011 from 8 different geographic sources in France, Belgium, and Slovenia. Isolates were identified as *S. lugdunensis* by Gram stain, colony morphology, biochemical profile from the Phoenix automated microbiology system (Becton Dickinson), and detection of pyrolydonyl-arylamidase (Oxoid biochemical identification system [O.B.I.S.]; Oxoid). For some isolates, the species identification was confirmed by real-time PCR amplification of an internal fragment of the *fbl* gene (5) using *fbl forward* (5'-GTAAATAGCGAGGCACAAGC-3') and *fbl reverse* (5'-GGTAAATCGTATCTGCCGCT-3') primers. β -Lactamine susceptibility was determined by chromogenic detection of penicillinase production (Cefinase; bioMérieux) and by the agar disk diffusion method according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (2) for oxacillin and moxalactam (detection of methicillin resistance). The bactericidal activities of vancomycin and teicoplanin were determined previously for 13 strains (4).

PFGE. Pulsed-field gel electrophoresis (PFGE) typing of *SmaI* (New England BioLabs)-digested DNA was performed as previously described (23). Briefly, genomic DNA incorporated in agarose plugs was digested at 25°C overnight, and large restriction fragments were separated in a 1% agarose gel at 14°C for 16 h by using the Gene Path system (Bio-Rad). The patterns were digitized with Molecular Analyst Fingerprinting software (Bio-Rad), and PFGE patterns were interpreted as recommended by Tenover et al. (33).

MLST. Seven housekeeping loci were selected for the characterization of *S. lugdunensis* isolates by MLST (Table 1): *aroE* (shikimate dehydrogenase), *dat* (D-amino acid aminotransferase), *ddl* (D-alanine:D-alanine ligase), *gmk* (guanylate kinase), *ldh* (L-lactate dehydrogenase), *recA* (recombinase), and *yqiL* (acetyl-coenzyme A acetyltransferase). The choice of these housekeeping genes was based on their use in MLST schemes of *S. aureus* (8), *S. epidermidis* (34), *Listeria monocytogenes* (29), or *C. difficile* (16, 21) and on the availability of sequence data from *S. lugdunensis* HKU09-01 (35). The seven loci were present in single copies and physically scattered on the *S. lugdunensis* HKU09-01 genome.

Each DNA sample for PCR amplification was extracted from a single bacterial colony from a tryptic soy agar culture using InstaGene DNA (Bio-Rad) according to the manufacturer's recommendations. PCRs were performed on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) in a final volume of 25 μ l containing 0.50 μ M each primer, 12.5 μ l of Reddymix (Thermo Fisher Scientific), and 5 μ l of extracted DNA. The PCR mixtures were heated for 4 min at 95°C, and then a touch-down procedure was performed, consisting of 30 s at 95°C, annealing for 30 s at temperatures decreasing from 60°C to 50°C during the first 11 cycles (with 1°C decremental steps in cycles 1 to 11), and ending with an extension step at 72°C for 30 s. A total of 50 cycles were performed.

PCR products were then purified with a Nucleospin extract II kit (Macherey-Nagel) and sequenced with the same primers as for PCR by using the DTCS sequencing kit (Beckman) on a GenomeLab GeXP analyzer (Beckman Coulter). Different sequences of a given locus were given allele numbers, and each unique combination of alleles (multilocus allelic profile) was assigned a sequence type (ST). Single-point polymorphisms were assessed by sequencing both DNA strands from two separate PCR experiments.

Computer analysis of MLST data. Clustering of the 87 isolates (and sequence data from *S. lugdunensis* HKU09-01) was performed using BioEdit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) for alignment of the nucleotide sequences, and gene trees were constructed from sequence data using the neighbor-joining method and bootstrapping algorithms contained in MEGA 4.0 software (32). The average numbers of nucleotide differences between alleles and the ratios of nonsynonymous to synonymous substitutions (*dN/dS*) were calculated

TABLE 1 Genetic polymorphism of the seven housekeeping genes analyzed by MLST

Gene	PCR and sequencing primers (5'→3')	Size (bp) of analyzed fragments	No. of alleles	No. of polymorphic sites	% of polymorphic sites	No. of nucleotide differences between alleles	<i>dN/dS</i> ^a
<i>aroE</i>	Forward, ATCGGAGATCGGATTTCACATTC; reverse, GGCGTTGTATTATAATATATC	460	6	5	1.1	1–3	0.0628
<i>dat</i>	Forward, TCGTGGTTATGTTTGGTGACGGT; reverse, CTATGAGAAAGTAAAGCCAGGAAT	412	8	7	1.7	1–4	0.0836
<i>ddl</i>	Forward, ATGCGGAGCAGCAGGTTTCA; reverse, ACACCTTGATCCCAAAATCGCGGT	420	7	6	1.4	1–4	0.0000
<i>gmk</i>	Forward, ATAGTTCTTCCGGACCATC; reverse, TCATTGACTACAGTAATCATAT	431	4	5	1.2	1–5	0.0000
<i>ldh</i>	Forward, ACTTGACGGTCCACGTCGA; reverse, GCTACGCAATTCGAATGGTAACGCA	414	4	5	1.2	1–4	0.0000
<i>recA</i>	Forward, GCACGGCCACCAAGGTGTGT; reverse, AGGCCGTCCGGTATCTAGTGT	463	5	4	0.9	1–3	0.0000
<i>yqiL</i>	Forward, GTGCTAAACGCACACCAATGGA; reverse, CTTCAGCATCGATTAGAGGCAC	450	9	8	1.8	1–5	0.2412

^a *dN/dS*, ratio of nonsynonymous to synonymous substitution.

TABLE 2 Characteristics of the 20 allelic profiles (STs) and clonal complexes

ST ^a	Allelic profile							Clonal complex	No. of isolates	Geographic origin(s) ^b
	<i>aroE</i>	<i>dat</i>	<i>ddl</i>	<i>gmk</i>	<i>ldh</i>	<i>recA</i>	<i>yqiL</i>			
1	1	1	1	1	1	1	1	CC1	11	A, B, C, D, F
2	3	3	2	2	1	2	3	CC2 ^c	15	A, B, D, E, G
3	4	3	4	2	3	4	6	CC3 ^c	18	A, B, C, D, E, F
4	6	4	3	1	1	3	3	CC4	3	A, C
5	5	6	5	3	4	4	7	CC5	4	A, B, C
6	1	1	1	1	1	1	2	CC1 ^c	8	A, B
7	1	1	1	1	1	1	9	CC1	1	D
8	2	3	2	2	1	2	3	CC2	1	A
9	1	4	3	1	1	3	4	CC3	1	A
10	3	5	2	2	2	3	5		8	A, B, C, F
11	1	1	1	1	1	6	1	CC1	1	— ^d
12	1	1	7	1	1	1	2	CC1	4	A, C
13	3	3	6	4	1	5	3		3	A, G
14	3	3	2	2	1	2	5	CC2	1	B
15	1	2	1	1	1	1	2	CC1	4	A, D, E
16	5	3	4	2	3	1	6	CC4	1	A
17	6	4	6	1	1	3	3	CC3	1	D
18	5	6	5	3	4	4	8	CC5	1	D
19	3	7	2	2	1	2	3	CC2	1	C
20	4	8	4	2	3	4	6	CC4	1	C

^a ST, sequence type.^b Geographic origins are as follows: A, Rouen, France; B, Nantes, France; C, Nancy, France; D, Bordeaux, France; E, Montpellier, France; F, Versailles, France; G, Louvain, Belgium; H, Maribor, Slovenia.^c Ancestral genotype within the clonal complex.^d *S. lugdunensis* HKU09-01, unknown geographic source (35).

to test the degree of selection operating on a locus using the START program (<http://www.mlst.net>).

The index of association (I_a) between alleles (31) was used to test for linkage disequilibrium between alleles of the seven loci analyzed (<http://www.mlst.net>). The observed variance in the distribution of allelic mismatches in all pairwise comparisons of the allelic profiles was compared to that expected in a freely recombining population (linkage equilibrium). The significance of the difference in the observed and expected variances was evaluated by computing the maximum variance in the distribution of allelic mismatches obtained using 100 randomizations of the data set.

The BURST program (<http://www.mlst.net>) was used to define clonal complexes (CCs; groups in which every isolate shares at least five identical alleles with at least one other isolate) and to characterize ancestral genotypes and their corresponding single-locus variants (SLVs, isolates that differ at only one of the seven loci) within these clonal complexes.

RESULTS

Allelic variation in *S. lugdunensis*. Data reporting the allelic variation of the seven housekeeping genes are summarized in Table 1. The number of individual alleles for each of the seven housekeeping genes ranged from 4 for *gmk* and *ldh* to 9 for *yqiL*. The number of polymorphic sites on a given locus varied from 4 (for *recA*) to 8 (for *yqiL*), and the maximum number of nucleotide differences between alleles of a given locus ranged from 3 for *aroE* and *recA* to 5 for *yqiL* and *gmk*. The variations in the sequences extended over the whole stretch of each of the seven genes investigated (<http://www.pasteur.fr/mlst>). Most polymorphisms resulted in synonymous substitutions, with the ratios of nonsynonymous to synonymous substitutions (dN/dS) varying from 0 (for *ddl*, *gmk*, *ldh*, and *recA*) to 0.2412 (for *yqiL*). These low ratios indicate a lack or a very limited contribution of environmental selection to the sequence variation in the seven housekeeping genes analyzed, which are thus assumed to be suitable for a population genetic study.

MLST analysis of *S. lugdunensis* isolates. MLST analysis of the 87 *S. lugdunensis* isolates generated a total of 20 different STs (Table 2). For each ST shared by at least two isolates, PFGE analysis displayed identical or closely related profiles, further arguing for probable clonal relationships between these isolates. An MLST database specific for this species and containing allelic and ST data has been made available on the website of the Pasteur Institute of Paris (<http://www.pasteur.fr/mlst>).

The results of clustering from concatenated sequence data are shown in Fig. 1. Half of the STs were represented by a single isolate. Among STs shared by several isolates, the most frequently encountered were ST3 (18 isolates), ST2 (15 isolates), and ST1 (11 isolates). Five clonal complexes (CCs) could be characterized by using the method and the criteria described by Feil et al. (11) (Table 2). The ancestral genotypes and their single-locus variants (probable derived genotypes) could be identified for CC1, CC2, and CC3 (Fig. 2). Of note, the sl96 strain, corresponding to the first published complete genome of *S. lugdunensis*, belongs to CC1 but harbors a unique ST (ST11).

Dendrograms based on sequence data of each housekeeping gene. Dendrograms from the sequences of the seven separate housekeeping genes were found to be congruent overall, indicating a predominantly mutational evolution of these genes. No recombination event could be detected by comparing monolocus trees (data not shown).

Estimation of the relative contributions of recombination and mutation to genomic evolution of *S. lugdunensis*. Since the congruence of dendrograms based on allelic variation of each housekeeping gene suggested a low frequency of recombination, a quantitative analysis of the linkage between alleles from the seven loci was performed by calculating the index of association (I_a)

FIG 2 Clonal complexes with ancestral genotypes identified. (A) Clonal complex 1, including ST6 (ancestral genotype), ST1, ST7, ST12, and ST15 (descended from single-locus variant genotypes), and ST11 (descended from double-locus variant genotype). (B) Clonal complex 2, including ST2 (ancestral genotype) and ST8, ST14, and ST19 (descended from single-locus variant genotypes). (C) CC3 including ST3 (ancestral genotype) and ST16 and ST20 (descended from single-locus variant genotypes).

(31). When all the isolates were included in the analysis, a significant linkage disequilibrium was detected (I_a of 2.91). At the level of STs (one isolate from each ST, to avoid bias due to a possible epidemic population structure), I_a was calculated at 1.88, confirming the significant linkage disequilibrium between alleles and, thus, a clonal structure of the population studied.

Clustering of *S. lugdunensis* isolates. No correlation was found between genotype and geographic origin; for example, ST3, the ancestral genotype of CC3, was shared by isolates from 6 geographic origins. Similarly, ST1 and ST2 were each shared by isolates from 5 different geographic origins. Isolates recovered from hematogenic infections (blood or osteoarticular isolates) or from skin and soft tissue infections did not cluster in separate lineages.

Twenty-seven *S. lugdunensis* isolates (31% of isolates) were found to be resistant to penicillin G by penicillinase production; no isolate exhibited methicillin resistance. Three STs belonging to CC1 (ST6, the ancestral genotype, and ST12 and ST15) contained 13 of the 27 penicillin-resistant isolates and contained only 4 penicillin-susceptible isolates, indicating that penicillin-resistant isolates are associated with this phylogenetic lineage. Conversely, ST1 and ST7, which were also inferred to derive from the ancestral genotype ST6 in CC1, contain only penicillin-susceptible isolates, indicating the possible loss of the penicillinase-encoding plasmid. Alternatively, they may represent ancestral genotypes predating the clonal expansion of ST6 after the acquisition of resistance.

The six isolates that we previously characterized as exhibiting glycopeptide (vancomycin and/or teicoplanin) tolerance (4) belonged to five different STs (ST6, ST15, ST8, ST9, and ST10) and to three clonal complexes (CC1, CC2, and CC4) and, thus, did not define a specific lineage. In addition, two STs (ST6 and ST10) included both nontolerant isolates and glycopeptide-tolerant isolates, and ST5 included a nontolerant isolate together with a vancomycin-tolerant isolate. Overall, these data suggest that glycopeptide-tolerant isolates do not constitute a distinct subpopulation within *S. lugdunensis*, implying multiple indepen-

dent evolution of the genetic elements involved in this phenotype by isolates from different genetic backgrounds.

DISCUSSION

The aim of the present study was to design an MLST scheme, based on the allelic polymorphism of seven housekeeping genes, which should be suitable for population genetics and intraspecific phylogeny analysis of *S. lugdunensis*. Only macrorestriction PFGE (37) had been used previously for molecular typing of this organism, but this approach would not, henceforward, constitute the reference method for these purposes.

We analyzed 87 *S. lugdunensis* isolates from various clinical infections and geographic sources. The allelic polymorphism (number of distinct STs among the isolates and number of polymorphic sites per locus) appears lower than that of *S. aureus* or *S. epidermidis*, which themselves display a moderate genetic variability, with few well-conserved phylogenetic lineages (17, 24). The low apparent rate of recombination results in a clonal population structure for *S. lugdunensis*, as illustrated by the index of association between alleles of the different loci, which reveals linkage disequilibrium (31), the congruence of dendrograms of individual housekeeping genes, and the characterization of clonal complexes within the population studied. Thus, if homologous recombination does exist, it rarely contributes to the evolution of *S. lugdunensis*. Sequence data analysis revealed that only synonymous substitutions were detected in genes *ddl*, *gmk*, *ldh*, and *recA*, suggesting that most nonsilent mutations are eliminated through purifying selection.

PFGE typing allowed us to check for the lack of an epidemiological link between isolates sharing the same ST. Since PFGE was unable to further resolve these MLST clusters, this method did not demonstrate a clear improvement in the discrimination of isolates compared to MLST; thus, MLST should be proposed as an efficient alternative approach for molecular typing of *S. lugdunensis*, with the additional advantage of providing unambiguous and portable sequence data suited for large-scale epidemiology.

Although *S. lugdunensis* is recognized as one of the major pathogenic species within the genus *Staphylococcus*, little is known about the virulence determinants implicated in invasive infections. In the present study, isolates from skin and soft tissue infections or from hematogenic infections (blood or osteoarticular isolates) were found to be randomly distributed in the main lineages depicted from the MLST dendrogram. It should be informative to further analyze allelic polymorphism of genes encoding fibrinogen-binding protein (25), von Willebrand factor-binding protein (26), or synergistic hemolysins (7), which have been proposed as virulence determinants of *S. lugdunensis*, and to investigate how this variation is related to MLST data. This could contribute to the description of putative hypervirulent lineages within the species.

S. lugdunensis remains largely susceptible to antibiotics and, surprisingly, displays a low frequency of methicillin resistance, unlike other coagulase-negative staphylococci or *S. aureus*. The

FIG 1 Dendrogram showing genetic relationships of the 87 *S. lugdunensis* isolates (and sequence data from *S. lugdunensis* HKU09-01) based on the composite sequence of the seven housekeeping genes (*aroE*, *dat*, *ddl*, *gmk*, *ldh*, *recA*, and *yqiL*). Clonal complexes (CC) are represented with brackets. Rooting of the dendrogram was established using homologous sequence data from *Staphylococcus haemolyticus* JCSC1435. Origin: A, Rouen, France; B, Nantes, France; C, Nancy, France; D, Bordeaux, France; E, Montpellier, France; F, Versailles, France; G, Louvain, Belgium; and H, Maribor, Slovenia. Clinical source: OA, osteoarticular; SST, skin and soft tissue; M, material device; B, blood. Penicillin: R, resistant; S, susceptible. *, ref1 (reference sequence 1), *S. lugdunensis* ATCC 700328; ref2, *S. lugdunensis* ATCC 43809; ref3, *S. lugdunensis* ATCC 49576; **, sl96 is *S. lugdunensis* HKU09-01.

present collection of clinical isolates is concordant with these data, with 31% of isolates producing penicillinase and a lack of methicillin-resistant isolates. Half of the penicillinase-producing isolates belong to a single clonal complex (CC1), reflecting vertical transmission within this lineage. Of note, ST1 and ST7, which are single-locus variants of the ST6 ancestral genotype of CC1, contain penicillin-susceptible isolates, suggesting a probable loss of the penicillinase-encoding plasmid or transposon (19, 20). Besides this clonal complex, penicillinase-producing isolates are widely distributed, either as a result of horizontal transmission or multiple independent acquisitions. Concerning methicillin resistance, which is still uncommon in *S. lugdunensis*, collecting MLST data from methicillin-resistant isolates could allow the study of their filiation from ancestral methicillin-susceptible clones, as was successfully deduced from MLST data for *S. aureus* (9, 27).

Vancomycin and/or teicoplanin tolerance (lack of bactericidal activity of these presumed bactericidal antibiotics) appears as a particular feature of *S. lugdunensis* (4, 14). We previously investigated a total of 13 isolates for glycopeptide tolerance and characterized six isolates tolerant to vancomycin and/or teicoplanin (4). These six tolerant isolates are distributed in different MLST lineages in the present study, and some STs (ST6, ST10, and ST5) include both nontolerant and glycopeptide-tolerant isolates. Thus, although glycopeptide tolerance was not investigated in the whole population analyzed by MLST, it may be assumed that this phenomenon is not restricted to a distinct subpopulation within *S. lugdunensis*. Further studies are currently in progress to investigate the implication of the major peptidoglycan hydrolase AtlL (3) in this impaired bactericidal activity of vancomycin and/or teicoplanin.

This work provides the first phylogenetic analysis of *S. lugdunensis* and gives strong arguments for a clonal population structure and mutational evolution of this pathogen. MLST combines the advantages of (i) a sequence-based typing method, which makes the data unambiguous and readily comparable between different laboratories, and (ii) a phylogenetic approach to genetic diversity, since it is based on sequence polymorphism of housekeeping genes apart from selective pressure. The *S. lugdunensis* MLST database, which is hosted at the website of the Institut Pasteur Paris (<http://www.pasteur.fr/mlst>), should be enriched from sequence data from worldwide and clinically diverse isolates, to follow the global epidemiology and evolution within the species. In addition, MLST data could be used as typing markers, possibly in combination with virulence gene data, to increase the discriminatory power and to investigate the short-term epidemiology of *S. lugdunensis*, which remains until now poorly investigated.

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