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Evaluation of an 'all-in-one' seven-day whole-genome sequencing solution in the investigation of a *Staphylococcus aureus* outbreak in a neonatal intensive care unit

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SUMMARY

Background: Meticillin-susceptible and -resistant *Staphylococcus aureus* (MSSA and MRSA) are responsible for outbreaks in intensive care units. MSSA infections have the same morbidity and mortality rate as MRSA infections but are studied less often. Whole-genome sequencing (WGS) is used increasingly for outbreak monitoring, but still requires specific installation and trained personnel to obtain and analyse the data.
Aim: To evaluate the workflow and benefits of EpiSeq solution (bioMérieux, Marcy l'Etoile, France) in exploring the increased incidence of *S. aureus* bloodstream infections in a neonatal intensive care unit (NICU).
Methods: Four *S. aureus* bacteraemia isolates and 27 colonization isolates obtained between January and July 2016 were submitted to the 'all in one solution' EpiSeq [WGS, quality data assessment, multi-locus sequence typing (MLST), spa typing, virulome and resistome characterization, and phylogenetic tree construction]. More in-depth analyses were performed (whole-genome MLST and whole-genome single nucleotide polymorphism (wgSNP)] with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).
Findings: Nine different sequence types and 13 different spa types were found among the 31 isolates studied. Among those isolates, 11 (seven patients) were ST146 spa type t002, five (four patients) were ST30 and four (four patients) were ST398. The 11 ST146 isolates had a maximum of seven pairwise SNP differences.
Conclusion: Use of EpiSeq solution allowed fast demonstration of the polyclonal profile of the MSSA population in neonates, and enabled the suspicion of a global outbreak to be ruled out. However, wgSNP analysis showed the transmission and persistence of one

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sequence type for over six months in the NICU, and enabled the infection control team to adapt its response.

Introduction

Staphylococcus aureus is the second most common cause of late-onset sepsis (LOS) after coagulase-negative staphylococci within the neonatal intensive care unit (NICU) population [1—4]. Although the incidence of LOS has decreased, 34% of very-low-birth weight children (VLBW) will experience at least one episode [1]. LOS has been associated with a mortality rate of 12%, while increased neurodevelopmental impairment is observed in the surviving infants [1]. *S. aureus* colonization may be acquired by neonates from healthcare workers, parents and visitors, or from inanimate objects in the environment [5]. Meticillin-susceptible *S. aureus* (MSSA) infections have equivalent morbidity and mortality in preterm infants, and are more common than meticillin-resistant *S. aureus* (MRSA) infections [6,7]. However, due to their antibiotic resistance, MRSA outbreaks are reported more frequently in NICUs. Recently, studies have used whole-genome sequencing (WGS) for MRSA outbreak investigation in adults and neonates [8,9]. Indeed, the discriminatory power of WGS in analysing isolate relatedness, as well as the possibility of predicting resistance and virulence phenotypes, is extremely advantageous. As a result, routine use of this technology is being implemented worldwide. However, acquisition of WGS data is not easy for all hospital laboratories, and the standardization of WGS remains challenging. In response to this, EpiSeq solution (bioMérieux, Marcy l'Etoile, France), dedicated to the epidemiological monitoring and control of nosocomial infections by WGS, has been introduced. This solution combines a service for WGS (subculture, DNA extraction, library preparation and sequencing run) with secure cloud-based software for data analysis [reads assembly, multi-locus sequence typing (MLST) and spa typing, resistome and virulome characterization, and core-genome single nucleotide polymorphism (SNP)-based phylogeny]. Analysis involves several knowledge bases, including a proprietary and curated collection of well-characterized epidemic *S. aureus* isolates associating genomic and epidemiological data. All of these data are provided directly to the client via intuitive software. Moreover, WGS data can be used for whole-genome MLST (wgMLST) analysis, which could also represent an answer to the problem of standardization. The US Centers for Disease Control and Prevention has recently validated the use of wgMLST and the *Escherichia coli* genotyping plug-in tool employing the commercial software platform BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). This tool has the advantage of combining quality assessment, data analysis and databasing of metadata in a single platform [10]. An *S. aureus* wgMLST scheme has also been developed for BioNumerics and could be used in outbreak investigations, as shown recently [8].

The aim of this study was to evaluate the relevance and workflow of EpiSeq solution and the BioNumerics software in order to confirm or refute the epidemiological link between different MSSA strains.

Methods

Case description

The Antoine Béclère University Medical Centre includes an academic, tertiary referral unit with 28 neonatal intensive care cots. All infants admitted to the NICU are screened for MSSA and MRSA carriage on anterior nares swabs on admission and then weekly thereafter until discharge. During the first quarter of 2016 (January, February and March), an increase in MSSA bacteraemia was detected in the NICU [incidence of 3.8% (5/130)] compared with the same period in 2015 [incidence of 0.9% (2/213)]. This increase triggered a genomic investigation of colonization and bloodstream isolates for outbreak determination. Four bacteraemia isolates and 27 *S. aureus* colonization isolates from 25 patients obtained between January 2016 and July 2016 were studied (Figure 1). Isolates are labelled with the patient's number and a 'B' if the isolate came from a bacteraemia or a 'C' if the isolate came from a nasal colonization. To explore the possible relationship between bacteraemia isolates, the three MSSA isolates in blood cultures from 2015 were also sequenced (2015B1, 2015B2 and 2015B3).

S. aureus isolation, identification and drug susceptibility testing

S. aureus was detected in blood cultures and nasal swabs used to screen for *S. aureus* colonization on blood agar plates (bioMérieux) after incubation for 24—48h at 37°C in an ambient atmosphere. Bacterial identification was confirmed by mass spectrometry (MALDI-TOF) (Brucker, Leipzig, Germany). Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Hercules, CA, USA) as recommended [11].

Whole-genome sequencing and bioinformatics analysis

EpiSeq solution included the different steps listed in the Introduction. This full service costs 300€ per sample, with a guaranteed turnaround time of seven days starting from receipt of the isolate at the sequencing facility. Isolates should be sent on a Copan swab after 24 h of incubation on blood agar. DNA libraries are prepared using a Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). Sequencing is performed on a MiSeq (Illumina) instrument to generate 200 bp paired end reads. Molecular resistance and virulence determinants, MLST and *spa* typing are inferred from WGS data through the EpiSeq knowledge base and data analysis workflow (https://biomerieux-episeq.com). Initial phylogenetic analysis is also conducted directly from WGS data. All results are made available to the customer through a user-friendly web platform.

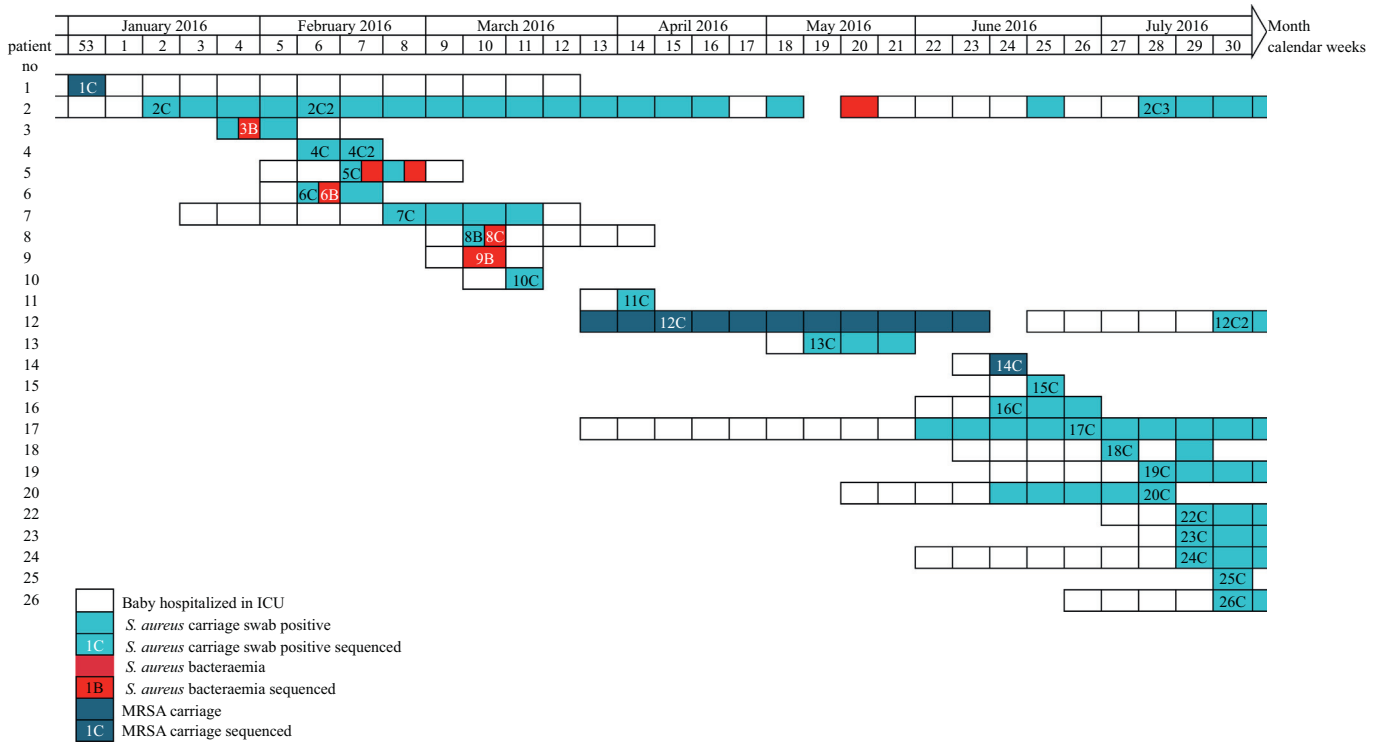

Figure 1. Timeline of *Staphylococcus aureus* isolation from carriage swabs and blood cultures during the 7-month study period. Isolates are labelled with the patient's number and a 'B' if the isolate came from a bacteraemia or a 'C' if the isolate came from a nasal colonization. ICU, intensive care unit; MRSA, meticillin-resistant *S. aureus*.

Sequenced genomes of the study isolates were also processed for more phylogenetic analysis in this study using BioNumerics v7.6 (Applied Maths). BioNumerics is a unique software platform for integrated analysis of bioinformatics, which has the ability to combine information from various genomic and phenotypic sources into a global database and to conduct conclusive analysis. A de-novo assembly was performed on a cloud-based calculation engine using SPAdes Assembler Version 3.7.1 [12]. Gene prediction and annotation of the contigs were carried out using BioNumerics. wgMLST was performed on 3897 loci (core and accessory genes) on the cloud-based calculation engine of BioNumerics Version 7.6 [12]. To further determine variation among isolates clustered in the same sequence type (ST), the strain first isolated in 2016 from ST146 and ST30 (Isolates 2C and 3B for the ST146 and ST30 lineages, respectively) was selected as the reference. The reads from each isolate in the group were mapped to this reference, and focused on single nucleotide polymorphisms (SNPs) in these mapped sequences (wgSNPs). Clustering of the wgMLST and wgSNP results was performed by an unweighted pair group method with an arithmetic-means-based tree. A minimum spanning tree based on pairwise wgSNP differences was also constructed using BioNumerics.

Results

Antibiotic resistance profiles of S. aureus isolates

All *S. aureus* isolates responsible for bacteraemia were meticillin susceptible, and had the same antibiotic susceptibility profile with resistance to penicillin G alone (Figure 2). Colonizing *S. aureus* isolates were also mainly MSSA. However, some strains had additional resistance mechanisms to macrolides.

Genomic investigation

Overall, nine different STs and 13 different *spa* types were found among the 31 isolates from the study period, with three predominant STs (Figure 2). Among these, 11 isolates from seven patients belonged to ST146 *spa* type t002. Five isolates from four patients belonged to ST30 and four isolates from four patients belonged to ST398. Assessment of clonal relationships with the wgMLST approach confirmed clustering among ST146 and ST30 isolates. One additional isolate (20C), with a new ST, clustered with the four ST398 isolates in the wgMLST-based dendrogram (Figure 2). The phylogenetic relationship was not further investigated for ST398 given that the allelic pairwise difference in wgMLST analysis was between 20 and 209. Regarding isolates from bacteraemia alone, they belonged to three different STs for 2016 isolates and two additional STs for 2015 isolates (Figure 2).

A wgSNP analysis was performed to further explore the relationship between isolates that belong to ST30 (Figure 1S, see online supplementary material). The three strains (8B, 2015B2 and 3B) isolated from blood cultures had a high number of SNPs with 402–435 pairwise differences (Figure 1S, see online supplementary material). In contrast, strains isolated from blood culture (8B) and a carriage swab (8C) from the same patient had a difference of just one SNP.

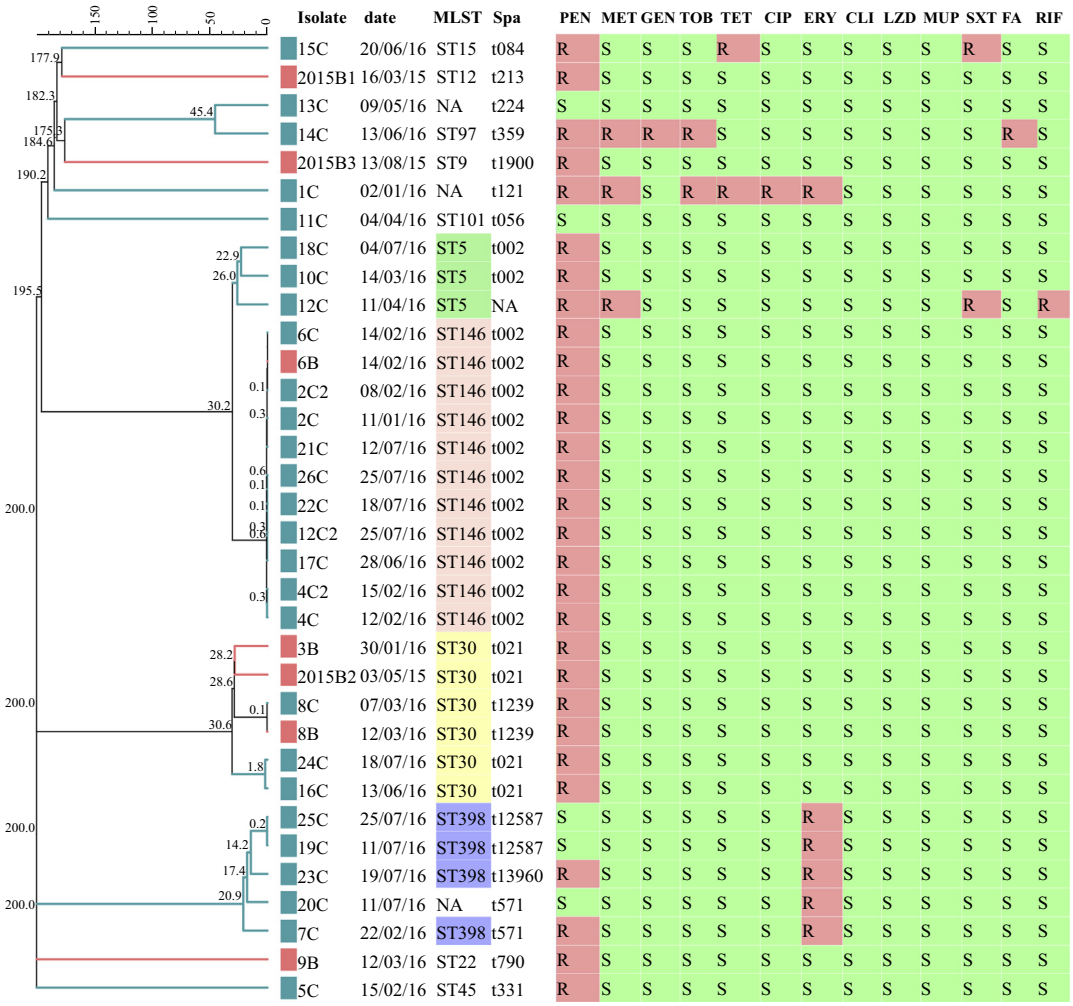

Figure 2. Whole-genome multi-locus sequence typing (wgMLST)-based dendrogram combined with collection date, molecular typing and antimicrobial susceptibility results. Branches corresponding to blood culture isolates and colonization isolates are shown in red and blue, respectively, using an unweighted pair group method with an arithmetic-means-based tree, with results clipped at 200-locus differences. The scale at the top and numbers at nodal positions refer to numbers of wgMLST allelic differences. PEN, penicillin; MET, meticillin; GEN, gentamicin; TOB, tobramycin; TET, tetracyclin; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; LZD, linezolid; MUP, mupirocin; SXT, trimethoprim-sulfamethoxazole; FA, fusidic acid; RIF, rifampicin.

The 11 isolates that clustered in ST146 had a maximum pairwise difference of seven SNPs (Figure 3B,C). The ST146 isolate from a blood culture (6B) was identical (zero SNPs) to the colonization strain (6C) isolated on the same day from Patient 6. Three of the ST146 isolates were recovered from Patient 2 (2C, 2C1, 2C2) who was hospitalized in the NICU from December 2015 to August 2016 (Figure 1). *S. aureus* carriage was detected throughout almost the entire length of this patient's stay. The sequenced isolates did not differ (zero SNPs) through January and February, and only differed by three SNPs in July. Patient 4 had two identical colonization strains (zero SNPs) recovered during hospitalization.

Resistome and virulome analysis

Analysis of antimicrobial resistance determinants (ARD) was concordant with the phenotypic antibiotic resistance profile (Figures 1 and 2S, see online supplementary material). The *bla*Z gene coding resistance to penicillin G was the only ARD present in the majority of isolates. However, different alleles of this gene could be identified and were associated with isolates that belonged to the three main groups (ST146, ST30 and ST398). The *erm*T gene was present in each of the five isolates of the ST398 group predicting resistance to erythromycin which was phenotypically associated with an inducible MLS_B phenotype. Other ARDs were present in the three MRSA isolates included in this study with the *mec*A gene for meticillin resistance, aph(6) and *aac*(6′)-*aph*(2″) for aminoglycoside resistance, *msr*A for erythromycin resistance, *fus*C for fusidic acid resistance, *dfr*G for trimethoprim resistance and *tet*K for tetracycline resistance. Resistance to fluoroquinolones and rifampicin was associated with known point mutations in *par*C and *rpo*B, respectively (Figure 2S, see online supplementary material). Of note, all ST30 isolates harboured the mutation Y737F in the *rpo*B gene, which has been described in vancomycin-intermediate *S. aureus* (VISA) strains [13].

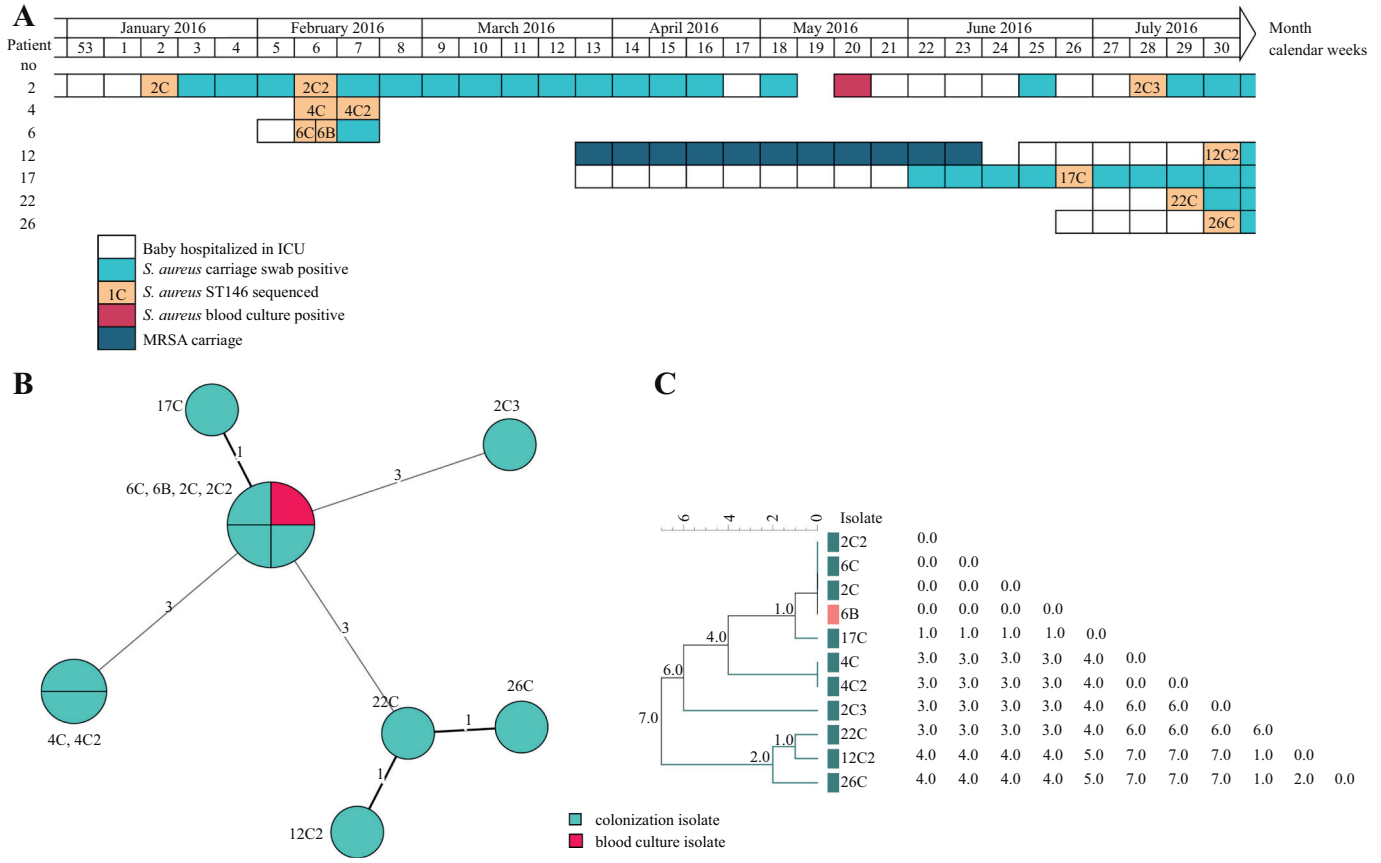

Figure 3. Dissection of ST146 isolates based on whole-genome single nucleotide polymorphism (wgSNP) analysis on an ST146-specific reference (B and C) and isolation timeline of the isolates of this group (A). (B) Circles corresponding to blood culture isolates and colonization isolates are shown in red and blue, respectively, in a minimum spanning tree. The numbers on branches indicate wgSNP distances between isolates. (C) Branches corresponding to blood cultures and colonization isolates are shown in red and blue, respectively, using an unweighted pair group method with an arithmetic-means-based tree. Numbers in the body of the table represent the number of pairwise wgSNPs between isolates.

Likewise, isolate 5C presented a D320N in *rpo*B, also associated with VISA [14]. Finally, no mupirocin resistance was phenotypically detected nor predicted from WGS data.

Among the virulence determinants, no particular toxin gene and no identical toxin profiles were found in isolates from blood cultures compared with colonization isolates. The *tst* gene was present in only two bacteraemia isolates and the enterotoxin *sea* gene, which has been associated with sepsis, was found in four isolates (Figure 2S, see online supplementary material). As with antimicrobial resistance, the global profile of toxin genes was associated with the different ST groups (Figure 2S, see online supplementary material). Only one carriage MRSA isolate (1C) encoded Panton-Valentin leucocidin.

Discussion

This study focused on investigation of an increased number of MSSA bacteraemias in an NICU using EpiSeq solution and the BioNumerics software. The use of EpiSeq solution is automated and does not require configuration by the user. It is therefore user-friendly. Although BioNumerics allows more in-depth analysis and annotation of contigs, it requires settings that entail user expertise and training. Use of the EpiSeq pipeline enabled the suspicion of a global outbreak in the NICU to be ruled out after seven days. Indeed, this solution allowed MLST, *spa* typing, resistome and virulome characterization, and phylogenetic analysis to be obtained from WGS data in one week for numerous isolates. This represents an effective time- and cost-saving strategy compared with the time and cost needed to obtain the same results by conventional methods [15].

This study determined the presence of a polyclonal MSSA population. The presence of various *S. aureus* clones in the NICU over a long period of time has been described previously for MSSA [16–19]. Using WGS tools, no relationship was detected between the four blood culture isolates of 2016, or between those from 2015. These findings demonstrate the absence of patient-to-patient transmission. Consequently, additional contact precautions were taken for patients colonized with MSSA. In addition, a review of hand hygiene, clothing and environmental biocleaning was performed. As a result, a programme of educational training (hand hygiene, standard precautions and contact precautions) was set up for the NICU staff by the infection control team. EpiSeq results of the polyclonal population of *S. aureus* in the NICU support the initial decision not to resort to staff and environmental screening.

Furthermore, an in-depth analysis with BioNumerics enabled identification of the transmission of one particular MSSA strain among seven patients. The clone-specific reference-based wgSNP analysis was useful in determining putative transmission events. Indeed, among the three major clones, different pairwise wgSNP numbers were observed. Previous studies have determined *S. aureus* mutation rates of five to 10 SNPs per year [20—22]. Moreover, in two studies on *S. aureus* transmission, cut-offs of 30 and 40 SNP differences, respectively, were used to indicate putative transmission. More precisely, the majority of acquisitions had fewer than 20 SNPs and most had fewer than four SNPs [23,24]. Accordingly, although an outbreak did not occur, wgSNP analysis demonstrated that transmission events occurred for strains of the three major clones. Among the ST146 clone, all isolates from different patients had a maximum of seven pairwise SNP differences. Among ST30, two isolates had 23 pairwise SNP differences. wgSNP analysis was not performed for ST398, but wgMLST showed that two isolates (19C and 25C) had very few allele differences and could also result from a transmission event. Transmission events of *S. aureus* have previously been linked to patient-to-patient transmission, transmission from healthcare workers or transmission from the environment. The exclusion of healthcare worker sampling in this study, although healthcare workers have been identified as an *S. aureus* reservoir in previous studies, could be considered a weak point of the present study [25,26]. However, Price *et al.* showed that when standard infection control measures were enforced in an intensive care unit, healthcare workers were not frequent sources of transmission [24]. The present epidemiological study showed that transmission events occurred during two distinct periods of time: February for ST146, and July for ST146, ST30 and ST398. One patient (Patient 2) carrying the ST146 isolate was present throughout the study period and may have been a reservoir.

WGS contributed to analysis of the virulome and resistome, which was concordant with the population structure observed in wgMLST and wgSNP analysis. Isolates responsible for bacteraemia did not have specific toxin genes. Some, but not all, have *tst* and/or *sea* genes, which have been correlated with sepsis [27]. Few antimicrobial-resistant determinants were detected among the majority of MSSA isolates that correlate with the phenotypic resistance profile and cannot help to discriminate isolates.

wgSNP analysis also showed that blood culture isolates were identical (zero or one SNP) to colonization isolates recovered from nasal carriage swabs in the same patient, and thus confirmed that infection was caused by their endogenous strains, as described previously [28]. Bacteraemia occurred in all patients except one immediately after acquisition of the strain, with colonization identified on the same day or a few days before infection.

To date, there is no consensus regarding routine screening for MSSA in neonates. However, given the association between carriage and subsequent infection among this population, screening is likely to be particularly beneficial in times of increased incidence, accompanied by the implementation of strategies to prevent the spread of *S. aureus* and to limit further colonization [5,16,17]. Recently, Wisgrill *et al.* demonstrated that active screening and decolonization of neonates lead to a decrease in MSSA infections in VLBW children [28]. Decolonization has not been performed in the study NICU because the MSSA infection outbreak was brought to a halt after infection control measures.

Finally, EpiSeq and BioNumerics could be very useful for the creation of a broad epidemiological database to allow comparison between new and older isolates.

For epidemiological purposes, WGS has become the most powerful tool for determining genetic relatedness among strains, helping to combat outbreaks effectively. Moreover, this technology allows the acquisition of genotyping information and characterization of the strains (including resistome and virulome characterization). With the availability of benchtop sequencers (such as Illumina iSeq 100), an improved turnaround time for outbreak detection is now feasible. In conclusion, the EpiSeq strategy is time-saving, user-friendly and potentially cost-saving.

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Conflict of interest statement
None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2019.01.029.

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