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Staphylococcus aureus Sequences from Osteomyelitic Specimens of a Pathological Bone Collection from Pre-Antibiotic Times

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Abstract: *Staphylococcus aureus* is a major pathogen causing osteomyelitis, amongst other diseases, and its methicillin-resistant form (MRSA) in particular poses a huge threat to public health. To increase our knowledge of the origin and evolution of *S. aureus*, genetic studies of historical microorganisms may be beneficial. Thus, the aim of this study was to investigate whether osteomyelitic skeletal material (autopsy specimens collected from the mid 19th century until the 1920s) is suitable for detecting historical *S. aureus* DNA sequences. We established a PCR-based analysis system targeting two specific genes of *S. aureus* (*nuc* and *fib*). We successfully amplified the historical S. *aureus nuc* and *fib* sequences for six and seven pre-antibiotic, osteomyelitic bone specimens, respectively. These results encourage further investigations of historical *S. aureus* genomes that may increase our understanding of pathogen evolution in relation to anthropogenically introduced antibiotics.

Keywords: *Staphylococcus aureus*; osteomyelitis; ancient DNA; pathological bone collection; skeletal material; historical pathogens; infectious diseases; PCR; pre-antibiotic era

1. Introduction

Staphylococcus aureus is a major pathogen for both humans and animals, causing a variety of diseases ranging from minor skin and soft tissue infection to severe, life-threatening medical conditions like bacteraemia, endocarditis and toxic shock syndrome. Of all diseases caused by *S. aureus*, only osteomyelitis enters the archaeological record. The clinical picture is characterized by an increased irregular osteolytic destruction of the bone surface, the production of pus, and the formation of woven bone, which is deposited on the periosteal surface, thus enlarging the affected skeletal element. Although osteomyelitis is an unspecific clinical picture that can be caused by several agents, *S. aureus* is the most frequently isolated causative organism in diagnosed osteomyelitis cases [1]. *S. aureus* is the causative organism in 90% of the cases of modern diagnosed osteomyelitis infection (e.g., [2]); historical references indicate that this pathogen played a major role in historical osteomyelitis infections as well (e.g., [3]). Roberts and Manchester [4] assumed that primarily initiated infection of the throat, ear, or the chest resulted in bacteremia and that the pyogenic bacteria reached organs and bones via the bloodstream. Skinner and Keefer [5] estimated that, in pre-antibiotic times, the mortality rate due to bacteremia initiated by *S. aureus* exceeded 80%. The discovery of penicillin in 1928 by Sir Alexander Fleming [6] and its introduction into medical use in 1938 [7] laid the primary foundation for improving health care. However, only a few years later, the first penicillin resistant *S. aureus* strains were recognized [8]. These isolated strains acquired *blaZ* horizontally

encoding ß-lactamase, which made them resistant to all β-lactamase susceptible penicillins. After the introduction of β-lactamase unsusceptible agent methicillin into medical use, approximately 20 years later, strains which were resistant against all known β-lactam agents (methicillin-resistant *S. aureus* MRSA) were described (e.g., [9–11]) and subsequently spread through hospitals and care facilities (reviewed in [12]). Since *S. aureus* became an increasing threat to human health, in the nineties, national (Paul-Ehrlich-Gesellschaft für Chemotherapie e.V. [13]) and international (European Antimicrobial Resistance Surveillance Network [14]) organisations started to monitor MRSA rates. Evaluating data from these organisations, after constantly increasing MRSA rates up until the beginning of the twenty-first century, the improvement in hygienic standards led to a decreasing trend over the last decade in Germany. Nevertheless, *S. aureus* still poses a huge threat to public health, as there are large differences in the frequency of MRSA-rates both between various German hospitals and across European countries.

To increase our knowledge about the origin and evolution of MRSA, Deurenberg and Stobberingh [15] recommended focusing on historical *S. aureus* strain collections. Several studies have shown that genes encoding resistances to multiple modern antibiotics are not only a result of the treatment with clinical antibiotics but can be found in genomes of ancient bacteria [16,17]. Those bacteria were found in different remote places never before in contact with human activities (e.g., caves and permafrost). Their genomic make-up is interpreted to prove the existence of a natural resistome [18,19] beyond modern human pharmaceutical influence [16,17]. If such a characteristic were to be proven in historical *S. aureus* this may be seen in the context of the emergence and persistence of MRSA-strains. Further, studies of microorganisms from ancient remains can also help us understand host–pathogen interactions in the context of the emergence of infectious diseases [20]. Generally, comparing the genetic profiles of modern bacterial isolates with those of historical strains increases our understanding of the transmission and spread of infectious diseases and the corresponding pathogens [21].

The invention of the polymerase chain reaction (PCR) three decades ago enabled ancient DNA (aDNA) studies. Only a short time later, the first paleomicrobiological applications were presented by Salo et al. [22], who reported the successful extraction and amplification of specific *Mycobacterium tuberculosis* sequences from the soft tissue of a 1000-year-old Peruvian mummy. Spigelman and Lemma [23] first extracted and amplified *M. tuberculosis* DNA from skeletal material using the PCR technique, which was later successfully applied to pathological and archaeological skeletal material (e.g., [24–26]). Furthermore, evidence of other infectious diseases and their causative pathogens, like *Mycobacterium leprae* (e.g., [27–29]), *Yersina pestis* (e.g., [30,31]), and *Bartonella quintana* (e.g., [32,33]) from archaeological material was provided.

To the best of our knowledge, there is no report of a successful DNA extraction and amplification of historical *S. aureus* DNA sequences from human remains. Thus, the aim of this study was to investigate whether the osteomyelitic skeletal material assembled in the pathological collection of the Department of Historical Anthropology and Human Ecology is suitable for detecting the presence of analysable *S. aureus* DNA sequences. Positive results would encourage further investigation of historical, pre-antibiotic *S. aureus* sequences to improve our understanding of the molecular events that led to the origin and rise of resistance to anthropogenically introduced antibiotics.

2. Materials and Methods

2.1. Materials

2.1.1. Osteomyelitic Bone Specimens

Bone specimens from seven different adult individuals (represented by few bones each) with apparent signs of an osteomyelitis infestation were chosen from the pathological collection of the Department of Historical Anthropology and Human Ecology (Figure 1). This large collection was assembled from autopsy specimens from the mid 19th century until the 1920s. The bones used in

this study were permanently stored in the collections' magazine, and have thus not been handled intensively over the last decades. Each bone was sampled at the diaphysis and four of the specimens were sampled additionally at a position close to the metaphysis that revealed morphologically detectable signs of osteomyelitis (Figure 1 arrows). Hence, eleven samples were investigated to prove the presence of *S. aureus* DNA sequences. Unlike archaeological skeletal material, the bone specimens of the pathological collection have never been in contact with soil-inhabiting bacteria, which can colonize skeletal material [34]. Nevertheless, the PCR system should be designed in a way that it is applicable to archaeological material, which has usually been buried. To test the PCR system in the presence of DNA from soil-inhabiting bacteria as well as PCR inhibitors like humic acids, which are present in every type of soil [35], several spiking experiments were performed (Supplementary Text S1).

Figure 1. Bone specimens from seven different individuals with apparent signs of an osteomyelitis infestation. From left to right: 13:K:40:4 (femur, distal), 13:K:40:7 (femur, proximal), 13:K:40:9 (femur, distal), 13:K:42:3 (tibia and fibula, distal), 13:K:42:12 (tibia, distal), 13:K:42:14 (tibia, proximal), 13:K:43:1 (tibia, distal). Sampling sites: diaphysis all specimens, four specimens additionally close to the metaphysis (red arrows).

2.1.2. *Staphylococcus aureus* Genomic DNA

To adjust the newly designed PCR-based analysis system to the requirements of degraded DNA samples, various control experiments were carried out (Supplementary Text S1). The genomic DNA of the *S. aureus* strain NCTC 8325 (accession CP000253.1 [36]) was used for initial spiking experiments and also served as a positive control for the PCR-amplification of the historical samples.

2.2. Methods

2.2.1. DNA Extraction

In order to remove possible cellular contamination from the sample surfaces, they were exposed to 6% sodium hypochlorite solution (Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany) (e.g., [37,38]). Lysis and DNA extraction was performed according to a protocol as described by Frischalowski et al. [39]. For decalcification and lyses, 0.25 mg bone powder was incubated with 3900 µL EDTA (0.5 M, pH 8.0, Invitrogen™, Carlsbad, CA, USA) and 100 µL proteinase K solution in Tris/HCl (pH 7.5, 0.01 mol/L, 600 mAnson-U/mL, Merck, Darmstadt, Germany) for 18 h at 37 °C under constant rotation. An additional 50 µL proteinase K was added and a second lyses step, again under constant rotation, was performed for another 2 h at 56 °C. Afterwards, a 10 min incubation with 50 µL sodium dodecyl sulphate (10 mg/mL, Sigma-Aldrich®, St. Louis, MO, USA) was performed at 65 °C. After a 3 min centrifugation at 3300 rcf (Type 5430R, Eppendorf, Hamburg; Germany), the entire lysate of approximately 4100 µL was used for phenol-chloroform extraction. For this purpose, the entire

lysate was transferred to 3 mL phenol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and manually inverted for 6 min at room temperature. For phase separation, the extracts were incubated at 56 °C for 10 min. As much as possible of the aqueous phase was transferred to 4.5 mL chloroform (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Again, the samples were manually inverted for 6 min at room temperature and afterwards incubated at 56 °C for 10 min. The aqueous phase was then transferred to a mixture of 16 mL PB-Buffer (Qiagen, Hilden, Germany) and 100 µL sodium acetate buffer (pH = 5.2, 3 M Sigma-Aldrich®, St. Louis, MO, USA). The mixture was transferred to MinElute™ spin columns (Qiagen, Hilden, Germany) and DNA purification was performed via the QIAvac-System (Qiagen, Hilden, Germany) following the manufacturer's protocol. The elution of the DNA from the MinElute™ spin columns (Qiagen, Hilden, Germany) was performed by using 20 µL RNAse free water (Qiagen, Hilden, Germany). After 5 min of incubation, centrifugation was performed at 13,000 rpm for 1 min. Elution was repeated twice to a total elution volume of 60 µL. The DNA extracts were stored frozen at −20 °C. For samples of the diaphysis at least two independent DNA extracts were prepared, for the samples close to the metaphysis one DNA extract each (Supplementary Text Table S1).

2.2.2. Primer Design

Ancient DNA analyses are highly dependent on optimal analysis conditions. This holds particularly true for the historical bone specimens investigated in this study, since they are expected to contain only traces of pathogenic DNA along with comparatively large quantities of human and microbial DNA, in case of the pathological collection due to maceration and taphonomic processes. Amplification sensitivity and specificity are mainly determined by primer characteristics [40]. Hence, the prerequisites for optimized primers—appropriate energy profile, avoidance of hairpins and primer dimer formation, and matching melting temperatures—were met. For the primer design for the amplification of specific sequences of the genes *nuc* [41] and *fib* [42] of *S. aureus*, the software PrimerSelect™ (DNASTAR, Madison, WI, USA) and the reference sequence of strain NCTC8325 (accession CP000253.1, cf. material-Staphylococcus aureus genomic DNA) was used. Primer specificity to the *S. aureus* complex was subsequently confirmed by BLASTN 2.2.32+ analysis [43,44] (Table 1). A cross reaction particularly with other Staphylococcus species (e.g., *Staphylococcus epidermidis*, common on the skin of humans) can be excluded.

Table 1. Primer sequences for the *S. aureus* loci [1].

Position	Name	Primer Length	Sequence	Position in *S. aureus* Reference Sequence (NCTC 8325, Accession CP000253.1)	Product Length
Forward	S.aur nuc up [2]	26-mer	5′-GGCAATTGTTTCAATATTACTTATAG-3′	800069 … 800094	122 bp
Reverse	S.aur nuc low	28-mer	5′-TTGAAACTACAACTAAAGTTAACACTAA-3′	800163 … 800190	
Forward	S.aur fib up	20-mer	5′-GAAGGATACGGTCCAAGAGA-3′	1073163 … 1073128	111 bp
Reverse	S.aur fib low	23-mer	5′-AGGTGTTGAGTTAAATTTTGGTC-3′	1073251 … 1073273	

[1] Using the amplification parameters described in this paper for both the *nuc* and the *fib* system, as little as 10 genomes/targets can be amplified and analysed by gel electrophoresis (data not shown). [2] The "T" at the sequence position seven of S.aur nuc up indicates a polymorphism ("C" instead of a "T") at the primer binding site of *S. aureus* strain NCTC 8325 (positive control).

2.2.3. Amplification of *S. aureus* DNA

The amplification reactions each consisted of a total volume of 25 µL with one part AmpliTaq Gold®360 Master Mix (Applied Biosystems®, Waltham, MA, USA), 0.4 µM of each primer (forward and reverse), and a DNA inset varying between 0.5 and 6 µL (the DNA amount in the extracts varied because of the differential state of sample preservation). PCR conditions were as follows: initial phase at 94 °C for 7 min, 40 cycling steps consisting of 94 °C for 1 min, 46 °C for 1 min (*nuc*) or 50 °C (*fib*), and 72 °C for 1 min, and soak for 10 min at 10 °C.

Amplification of the X region of the protein A gene (*spa* typing) (e.g., [45,46]) and sequence analysis was done by the Robert Koch Institute as described by Strommenger et al. [47] (Supplementary Text S6).

The amplified PCR products were separated by gel electrophoresis. Run time was approximately 30 min at 120 V in a 2.5% gel in 1xTBE buffer.

2.2.4. Sequence Analysis of PCR Products

Sequencing of the purified amplification products (Supplementary Text S3) was carried out in a direct *taq* cycle sequencing approach with a total reaction volume of 20 µL. Each reaction mix consisted of one part BigDyev1.1 5X Sequencing Buffer (Applied Biosystems®, Waltham, MA, USA), one part BigDye®Terminator v1.1 Ready Reaction Mix (Applied Biosystems®, Waltham, MA, USA), 0.24 µM primer (forward or reverse), and a PCR product inset varying between 0.5 and 5 µL. The PCR cycling conditions were as follows: initial phase at 96 °C for 10 min, 25 cycling steps consisting of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, and a final soak at 10 °C. Analysis of the purified sequencing products (Supplementary Text S3) was conducted via 3500 Genetic Analyzer (Applied Biosystem®, Waltham, MA, USA) and data processing was applied by using SeqA (Applied Biosystem®, Waltham, MA, USA) and the DNASTAR Lasergene 10 Core Suite software package (DNASTAR, Madison, WI, USA). The specificity of all amplified sequences was confirmed by BLASTN 2.2.32+ analysis [43,44].

2.2.5. Contamination Prevention

Concerning contamination, all aDNA analyses were conducted following a rigorous cleaning protocol and established laboratory standards that minimalize the contamination risk (e.g., [40,48]. All laboratory surfaces, instruments and bone samples underwent a strict cleaning protocol with 6% sodium hypochlorite solution (Aug. Hedinger GmbH & Co. KG, Stuttgart, Germany).

In order to easily detect possible external contamination with either human or even pathogenic DNA, there were three general strategies: (i) All DNA extracts of the bone specimens were checked for foreign human DNA via short tandem repeat typing (STR typing). Since STR typing only identifies human DNA and does not exclude a possible contamination with pathogenic DNA through a processor. (ii) The processors were screened for *S. aureus* colonization via culture of nasal swabs (Supplementary Text S4). In the case that none of the processors was a carrier of *S. aureus,* a late introduction of a modern contamination through a processor (e.g., contamination of a single tube during the PCR-setup) could be excluded. In the case that one of the processors had been a carrier of *S. aureus*, sequence analysis of the cultures would have been carried out allowing a differentiation e.g., through single nucleotide polymorphisms or *spa* typing. (iii) Several control samples and extraction blank controls were amplified with the *S. aureus* primer sets and the corresponding amplification parameters (Supplementary Text S1) to screen for possible *S. aureus* contaminants in extraction and PCR reagents.

The positive control sample of *S. aureus* was stored and handled separately from the historical DNA extracts during all steps in a working station (UV-bench DNA/RNA UV-Cleaner, UVT-S-AR) in a separate room and was always prepared last. Those precautions were taken to avoid a cross-contamination between the positive control sample (*S. aureus* strain NCTC8325) and the DNA extracts of the historical bone specimens.

2.2.6. STR Typing

STR typing was conducted with a miniSTR system [49] which was designed particularly for aDNA applications. Every DNA extract was tested twice, before and after amplifications were carried out for *S. aureus* analysis. The allele determination was performed by capillary electrophoresis via 3500 Series Genetic Analyzer (Applied Biosystems®, Waltham, MA, USA) and data processing was applied with the GeneMapper5 (Applied Biosystems®, Waltham, MA, USA) software (Supplementary Text S5).

3. Results

3.1. Amplification of Staphylococcus aureus Sequences from Osteomyelitic Bone Specimens

This study successfully conducted analyses of historical *S. aureus* sequences. The extraction revealed variable amounts of both human and pathogenic DNA from the different samples as judged by the differential brightness of amplification product in the respective gel pictures. The 122 bp fragments of the *nuc* gene was reproducibly amplified for eight out of eleven samples (Figure 2, Supplementary Figure S1, Table 2). The band intensity of most of the amplification products compared to that of the positive control indicates that there were less than 100 targets in the respective PCR. In contrast, the gel bands of 13:K:40:7 are much brighter than that of the positive control, indicating by far more than 100 targets in the reaction. The 111 bp *fib* fragment was amplified for ten out of eleven samples (Supplementary Figure S2, Table 2).

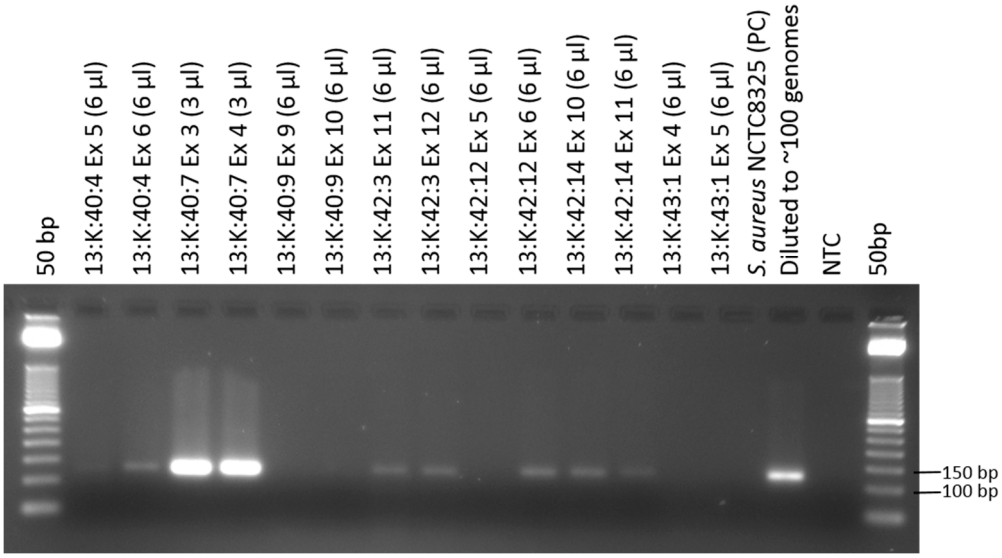

Figure 2. Results of the PCR-based detection of *S. aureus* specific 122 bp *nuc* sequences after agarose gel electrophoresis (DNA inset bracketed). Gel bands are located between the 100 bp and 150 bp fragments of the DNA ladder, indicating that the sought amplification product of 122 bp was generated. 9/14 amplifications resulted in visible bands; the negative template control (NTC) shows no evidence of pathogenic DNA presence in the PCR reaction mix. As a positive control, DNA of the strain NCTC8325 (approximately 100 genomes) was used. Electrophoresis parameters: 8 µL PCR product with 2 µL loading dye, 4 µL size standard: 50 bp DNA ladder (Invitrogen™, Carlsbad, CA, USA), 2.5% gel, 120 V, exposure time: 1.00 s. For detailed description of the extract nomenclature see Supplementary Text Table S1.

Concerning *spa* typing, only the sample 13:K:40:7 revealed an amplification product with a fragment length between 350 and 500 bp (Supplementary Text S6) visualized by gel electrophoresis. The *spa* type of this sample was determined as "t017" (Ridom StaphType, Ridom GmbH, [50]). Clustering analysis via the *Ba*sed *U*pon *R*epeat *P*attern algorithm [47] revealed an affiliation to the clonal complex "CC30".

Table 2. Overall amplification success of *S. aureus* sequences visualized by gel electrophoresis (Figure 2, Supplementary Figures S1 and S2).

Bone Specimen	Sampling Position (Figure 1)	*nuc*	*fib*
13:K:40:4	diaphysis	+	+
	metaphysis	+	+
13:K:40:7	diaphysis	+	+
13:K:40:9	diaphysis	+	+
13:K:42:3	diaphysis	+	+
	metaphysis	−	+
13:K:42:12	diaphysis	+	+
	metaphysis	+	+
13:K:42:14	diaphysis	+	+
13:K:43:1	diaphysis	−	−
	metaphysis	−	+

+ = specific amplification product; − = no amplification product.

3.2. Sequencing of Staphylococcus aureus Sequences from Osteomyelitic Bone Specimens

For each bone sample revealing a positive signal in one of the PCR attempts for either the *nuc* (eight samples, Supplementary Figure S3) or *fib* locus (ten samples, Supplementary Figure S4), forward and reverse sequencing was performed. A BLASTN 2.2.32+ search [43,44] of the amplified historical sequences revealed sequence similarities to the reference sequences of the positive control sample NCTC 8325 between 97% and 99% for *nuc* and between 99% and 100% for *fib*, respectively. The alignment of the amplified historical *nuc* sequences and the genome of strain NCTC 8325 showed several polymorphisms (Figure 3). All amplified historical sequences reveal three single-base polymorphisms (C42T, A113T, and T129C) deviating from the reference sequences of the positive control sample NCTC 8325. Positions 89 to 90 revealed a polymorphic region where either double adenine or double guanine is present. For two amplified sequences (13:K:42:12_Ex3 and 13:K:42:14_Ex7), a double purine base (R = A or G) was determined. The alignment of the amplified *fib* sequences revealed only three base deviations in two historical samples (Supplementary Figure S5).

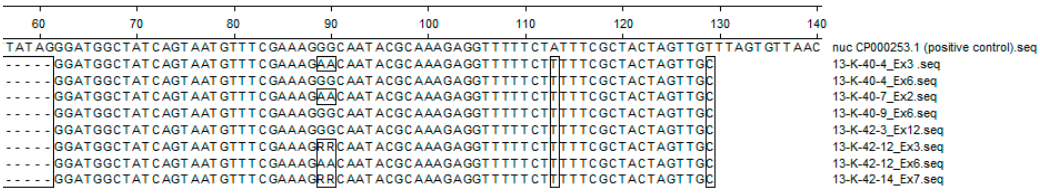

Figure 3. Alignment of the consensus *nuc* sequence of the historical bone specimens (primer binding sequence was removed) and the *nuc* gene of the *S. aureus* reference strain NCTC 8325 (CP000253.1, positive control sample). Bases differing from the reference *nuc* sequence are framed. Software: *MegAlign*™ (DNASTAR, Madison, WI, USA). For the alignment of all amplified forward (fwd.) and reverse (rev.) sequences and the corresponding electropherograms see Supplementary Figures S3 and S6.

With the *nuc* sequencing results a phylogenetic analysis was performed. A comparison of the two *nuc* sequence variants obtained from the historical samples (13:K:40:4 Ex6 and 13:K:40:7 Ex2) with publically available sequences of *S. aureus* belonging to different clonal complexes was conducted. Furthermore, the sequences of *Staphylococcus schweitzeri* [51] and *Staphylococcus argenteus* [52,53] were used as an outgroup, as they both also belong to the *S. aureus* complex. Multiple alignments were

performed using MegAlign™ (DNASTAR, Madison, WI, USA) and the phylogenetic relationship was visualized (Figure 4). As can be deduced from the phylogenetic tree, the 68 bp sequence variant as realised in 13:K:40:7_Ex2 clusters with the *S. aureus* strain MRSA 252 [54] and CA-347 [55]. The respective sequence characteristics of the 13:K:40:4_Ex6 cluster with the *S. aureus* isolates Mu3 [56], MW2 [57], RF122 [58], 08-02300 [59], JH9 [60], HO 5096 0412 [61], 08-02119 [59], N315 [62], Mu 50 [62], and MSSA476 [54].

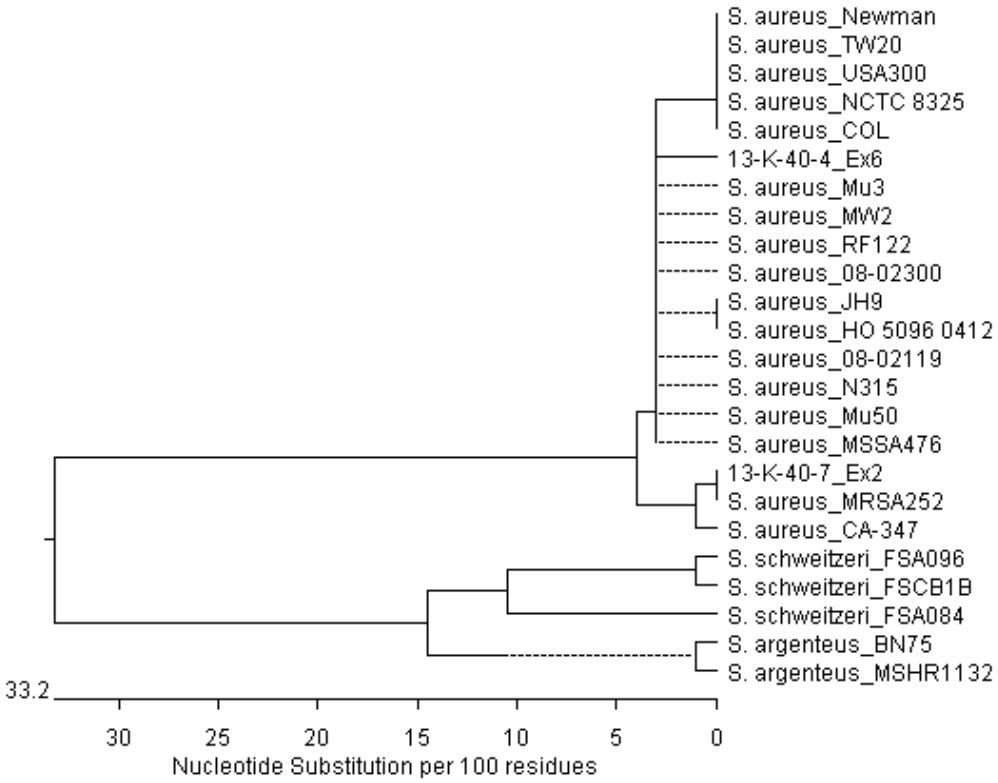

Figure 4. Phylogenetic relations based on the alignment (Figure S7 in supplementary data) of two historical *nuc* sequence variants of 13:K:40:4 Ex6 and 13:K:40:7 Ex2 and *nuc* sequences of seventeen contemporary *S. aureus* strains. *S. schweitzeri* and *S. argenteus* were used as the out group. Software: MegAlign™ (DNASTAR, Madison, WI, USA), Method: Jotun Hein.

3.3. STR Typing

Human STR typing of the historical DNA samples indicated that there were considerable differences in DNA preservation amongst the investigated bone specimens. When working with aDNA STR typing those differences may include variability in amplification success, allelic drop-out, preferential amplification of smaller STR systems (at the expense of the larger systems), or artefacts as a consequence of overamplified stutter products (e.g., [40,63,64]). The specimens 13:K:40:4, 13:K:40:7, and 13:K:42:3 revealed excellent DNA preservation resulting in full genetic fingerprints, whereas the remaining bone specimens (13:K:40:9, 13:K:42:12, 13:K:42:14, and 13:K:43:1) showed a poorer state of DNA preservation resulting in only a limited number of alleles (Supplementary Text Tables S4–S10).

4. Discussion

This study succeeded in finding specific DNA of *S. aureus* in seven investigated specimens from a pathological bone collection from historical times. Therefore, it is most likely that *S. aureus* was the causative organism of the morphologically manifested osteomyelitis infection. We succeeded in amplifying *S. aureus*-specific sequences of the two genes *nuc* and *fib*.

The investigation demonstrated that, as expected for aDNA analyses, different amounts of both pathogenic and human DNA were extracted from the pathological bone specimens. Due to the nature of DNA degradation, e.g., DNA fragmentation, human and pathogenic DNA were not quantified. We rather conducted initial spiking experiments with different amounts of *S. aureus* reference DNA demonstrating that approximately 10 to 50 genome equivalents are sufficient for a light band to be visible in the agarose gel picture (data not shown). Similar band intensities could be observed during the investigation of the DNA extracts of the pathological bone specimens, indicating that at least approximately 10–50 intact targets ought to be present in most of the historical extracts. Extracts of the bone specimen 13:K:40:7 were always amplified with a decreased DNA inset and yet revealed a brighter amplification product in gel electrophoresis (Figure 2, Supplementary Figures S1 and S2). The comparison of the band intensities of the spiking experiments with those of 13:K:40:7 revealed that by far higher amounts of pathogenic DNA was extracted and amplified from this sample (data not shown).

The evaluation of all amplified historical *nuc* sequences showed various polymorphisms differing from the *nuc* sequence of the strain NCTC 8325 (Figure 3). Hence, signals for historical *S. aureus* sequences are not a result of a contamination from the positive control. When analysing aDNA, contaminations should always be considered as a possible cause of positive signals during investigations. In this context, a processor in contact with the samples or the positive control is usually suspected of being the origin of a contamination. However, the spatial separation of the positive control from the historical samples makes a cross-contamination between them very unlikely. Furthermore, concerning *S. aureus* contamination through one of the processors (Flux, A.L. and Mazanec, J.), screening for *S. aureus* via a cultivation of swabs from the nasal mucosa on blood agar plates (Institute for Medical Microbiology, University Medical Center Goettingen) detected no colonization of the processors by the bacteria. In case of a contamination with DNA from one of the processors or from another bone specimen the detection of multiple allels in the STR typing results would have been expected. However, the comparison of the subsequent fingerprints showed that a contamination could be excluded (Supplementary Text Tables S4–S11). Therefore, it can be assumed that the results are authentic and not a result of a modern contamination.

Sequence variations of the *nuc* gene of 13:K:42:12, 13:K:42:14, and 13:K:40:4 were observed, within different samples (metaphysis and diaphysis) of the corresponding bone specimen, possibly indicating the presence of different *S. aureus* strains. Historically, *S. aureus* carriage as well as infection has been assumed to be monoclonal [65]. However, recent research data has indicated that polyclonal colonization is more common than previously anticipated [66,67]. During the investigation of the pathological samples sequencing of two different DNA extracts from one specimen (13:K:40:4, Figure 3) revealed different sequence characteristics, both being reproduced by forward and reverse sequencing reactions. This may be an indication that two different *S. aureus* strains colonized the individual in its lifetime. Specimen 13:K:42:12 also revealed two different sequence characteristics in two investigated extracts. It should be noted, however, that the sample 13:K:42:12 Ex3 revealed different characteristics in the forward and reverse amplified sequence that may be explained as a PCR artefact through e.g., base misincorporation. This assumption is strongly supported by the unusually high number of amplification cycles of the sample (in total, 70 cycling steps), because it was necessary to reamplify the PCR product before the sequencing reaction (Supplementary Text S3). The sequence of the sample 13:K:42:14 Ex7 revealed sequence characteristics similar to the sequences of sample 13:K:42:12 Ex3, but it was not reamplified before direct sequencing. The differences between the forward and reverse amplified sequences occurred repeatedly, making it difficult to evaluate whether the sequence characteristics can also be explained as a PCR artefact or as two sequences of different *S. aureus* strains being amplified.

Concerning *spa* typing, the amplification failure of the *spa* locus for the entire historical samples, except for 13:K.40:7, was not surprising since the *spa* amplicon size (approx. > 200 base pairs) is comparatively long with respect to aDNA analysis. Additionally, the primers for the *spa* locus

were designed for the amplification of contemporarily isolated *S. aureus* DNA and do not meet aDNA requirements (Section 2.2.2). However, the *spa* type of 13:K.40:7 was determined as "t017" clustering to the clonal complex "CC30" (Section 3.1). Clonal complex 30 (CC30) had been involved in different pandemic "waves" and was also part of the toxic shock syndrome epidemic. It includes the historic phage-type 80/81 strains and contemporary community, as well as hospital associated MRSA (CA-/HA-MRSA). Related methicillin-susceptible *S. aureus* (MSSA) are common human nasal colonizers worldwide [68].

Phylogenetic analysis shows different clustering for both sequence variants obtained from the historical samples (13:K:40:4 Ex6 and 13:K:40:7 Ex2) to the reference sequences of the *S. aureus* complex. Sequence 13:K:40:7 Ex2 clusters with MRSA252, which is in concordance with the determined *spa* type and clonal complex (CC30). The other historical sequence (13:K:40:4 Ex6) clusters in a group with *S. aureus* sequences belonging to various clonal complexes. This is not surprising, since there were no sequence variations within the amplified *nuc* sequences. Since the analysis was carried out with only 68 bp, further genome-wide analysis will be necessary to assess a robust phylogenetic relation between the historical sequences and those of contemporary *S. aureus* strains.

To the best of our knowledge, recent investigations focussing on analysing the genome of historical pathogens with PCR- or NGS-based methods were limited to archaeological skeletal material (e.g., [69–77]) or non-archaeological preserved soft tissues (e.g., [78–80]). The first successful investigation on non-archaeological, macerated bones assembled in the pathological collection was performed by Baron et al. [24]; we have now demonstrated yet again that these bone samples are suitable for extracting analysable amounts of both human and pathogenic DNA. In addition to archaeological material, these pathological samples are especially beneficial for the investigation of pathogens causing diseases limited to only single individuals in a skeletal series and not those leading to epidemics and mass burials in the archaeological context. Hence, these findings encourage further investigation of sample material from pathological collections and might establish a new approach to historical genomes of not only *S. aureus* but other pathogens causing the clinical picture of osteomyelitis, as well as other diseases. In the case of *S. aureus*, genome analysis of strains colonizing patients before the introduction of penicillin into medical use may increase our understanding of host–pathogen interactions and pathogen evolution in relation to anthropogenically introduced antibiotics.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/4/43/s1. Flux et al. 2017 Supplementary Text Figure S1. Results of the PCR-based detection of the *S. aureus* specific nuc sequence; Figure S2. Results of the PCR-based detection of the *S. aureus* specific fib sequence; Figure S3. Electropherograms of all amplified historical nuc sequences; Figure S4. Electropherograms of all amplified historical fib sequences, Figure S5. Alignment of forward and reverse fib sequences of the historical bone specimens; Figure S6. Alignment of forward and reverse nuc sequences of the historical bone specimens; Figure S7. Alignment of two historical nuc sequences and reference sequences.

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