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Highly divergent *Staphylococcus aureus* isolates from African non-human primates

F. Schaumburg,[1,3*] A. S. Alabi,[3,4] R. Köck,[2] A. Mellmann,[2] P. G. Kremsner,[3,4] C. Boesch,[5] K. Becker,[1] F. H. Leendertz[6] and G. Peters[1]

Institutes of [1]Medical Microbiology and [2]Hygiene, University Hospital Münster, Münster, Germany.

[3]Medical Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon.

[4]Institute of Tropical Medicine, University Hospital Tübingen, Tübingen, Germany.

[5]Department of Primatology, Max-Planck-Institute for Evolutionary Anthropology, Leipzig, Germany.

[6]Research Group Emerging Zoonoses, Robert-Koch-Institut, Berlin, Germany.

Summary

***Staphylococcus aureus* is a bacterium that colonizes and infects both humans and animals. As little is known about the phenotypic and molecular characteristics of *S. aureus* from wild animals in sub-Saharan Africa, the objective of the study was to characterize *S. aureus* isolates from wildlife and to analyse if they differed from those found among humans. The resistance to penicillin was low in *S. aureus* isolates from non-human primates (2.9%). Phylogenetic analysis based on the concatenated sequences from multilocus sequence typing revealed two highly divergent groups of isolates. One group was predominated by *S. aureus* that belonged to known human-related STs (ST1, ST9 and ST601) and mainly derived from great apes. A second clade comprised isolates with novel STs. These isolates were different from classical human *S. aureus* strains and mainly derived from monkeys. Our findings provide the basis for future studies addressing the inter- and intra-species transmission of *S. aureus* in Africa.**

Introduction

Staphylococcus aureus is one of the most frequent causes of superficial skin and deep-seated infections in humans. It has been demonstrated that many mammals, reptiles and birds can also be colonized and infected by *S. aureus* (Cuny *et al*., 2010). Most recently, the impact of *S. aureus* as a zoonotic pathogen has been highlighted. Exemplarily, in many western countries industrially raised livestock (pigs, veal calves, poultry) was found to represent a significant reservoir of methicillin-resistant *S. aureus* and a major source of human methicillin-resistant *S. aureus* colonization and infections in regions characterized by a high density of livestock farming (Köck *et al*., 2010). The transmission of *S. aureus* between humans and animals can be bidirectional as *S. aureus* isolates can also be transmitted from human to animals (i.e. poultry) (Lowder *et al*., 2009). Whereas 'host switching' of *S. aureus* has been intensively studied in industrialized countries, nothing is known about interspecies transmission and its public health impact in developing regions. Close contact of humans to wild animals occurs during butchering, trade and consumption of bushmeat and may facilitate a transmission of potential pathogens between humans and animals (Wolfe, 2005; Leendertz *et al*., 2006; Jones *et al*., 2008). Bushmeat is meat from wild animals living in the African rainforest or savannah (Barnes, 2002). To provide the basis for future investigations into inter- and intra-species transmission of *S. aureus* in Africa, we characterized the susceptibility to antibiotic agents, genotypes and virulence patterns of *S. aureus* isolates from African non-human primates, which are important targets for hunters and whose meat is frequently consumed in sub-Saharan regions.

Results and discussion

Animal population

Samples were derived from monkeys ($n = 64$) and great apes ($n = 31$, Table S1). One *Cercopithecus nictitans* from Gabon and two *Piliocolobus badius* from Côte d'Ivoire carried two genotypically different *S. aureus* isolates. Five samples from fruit wadges from chimpanzees in Côte d'Ivoire harboured two different *S. aureus* isolates and one fruit wadge carried three different isolates. Fruit wadges are the spit out remains of forest fruits, chewed by chimpanzees for several minutes to suck out the juice. All *S. aureus* isolates ($n = 58$) were coagulase-positive, showed a typical biochemical profile and were confirmed as *S. aureus* by 16S rRNA gene sequencing.

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Antimicrobial susceptibility

In total, 57 isolates (98.3%) were susceptible to penicillin. The beta-lactamase encoding gene *blaZ* was only detected in the penicillin-resistant isolate. This isolate derived from a chimpanzee from Côte d'Ivoire. All isolates were susceptible to methicillin (as confirmed by the absence of *mecA*), aminoglycosides, fluoroquinolones, macrolides, lincosamides (including inducible clindamycin resistance), nitrofurantoin, fosfomycin, rifampicin, tetracycline, cotrimoxazole and vancomycin.

Penicillin resistance is rare in *S. aureus* isolates from remote African regions, even in humans who are more likely to be exposed to antibiotics (Schaumburg *et al*., 2011a). The penicillin resistance could be due to a treatment of chimpanzees with respiratory tract infections in Taï National Parc, Côte d'Ivoire with benzathine benzylpenicillin, a slowly absorbed penicillin.

Molecular typing

Sequence based typing of the hypervariable region of *S. aureus* protein A (*spa*-typing) resulted in 32 different *spa* types (Table S2). Two *spa* types were found in two different species: t127 (*Cercopithecus polykomos*, *Pan troglodytes*, both from Côte d'Ivoire) and t6533 (*Cercopithecus cephus* and *C. nictitans*, both from Gabon) indicating a transmission between these species. Chimpanzees hunt and consume other monkeys such as *Colobus* sp. and *Cercopithecus* sp. (Boesch and Boesch, 1989). This might explain a transmission of *S. aureus* from prey to hunters as it has been already shown for various viruses (Leendertz *et al*., 2004; 2008).

To analyse the phylogenetic relatedness of the *S. aureus* isolates, we used the concatenated sequences of the seven multilocus sequence typing (MLST) housekeeping genes of each ST and constructed a Neighbor-Joining tree (Fig. 1). All sequences clustered into two groups and the mean distance between the two groups was 0.085 base substitutions per site. The split into two groups was supported by a bootstrap value of 100. The majority of *S. aureus* isolates from group 1 derived from great apes ($n = 24$, 72.7%) and clustered with ST8, ST15, ST36 and ST152, which are well known ST from human *S. aureus* isolates. This might mirror the co-divergence of *S. aureus* and its primate host and might reflect the close phylogenetic relation between chimpanzees and humans.

In contrast, isolates from group 2 mainly originated from monkeys ($n = 14$, 93.3%) and clustered with the ST of a divergent isolate recently found in a human *S. aureus* carrier from rural Gabon (ST1822) (Schaumburg *et al*., 2011b) and the known divergent ST1223 (Ruimy *et al*., 2009). Central African Pygmies, who frequently hunt and consume bushmeat, are not colonized with isolates belonging to the divergent clade, which argues against a frequent transmission of theses isolates from animals to humans. Therefore, targeted studies are needed to investigate the transmissibility of animal-adapted strains to humans.

Despite its divergent position, STs of group 2 are phylogenetically closer to group 1 than to *Staphylococcus simiae* the closest related coagulase-negative species of *S. aureus* (Fig. 1).

Virulence factors and capsular types

In total, 18 virulence factors were tested (Table S3). The median number (range) of virulence genes was 2 (1–5) per isolate, which was markedly lower than in isolates from remote Gabonese Pygmies (2 vs. 3.3) who share the same habitat with many animals species of this study (Schaumburg *et al*., 2011a).

Pyrogenic toxin superantigens were encoded by 26 (44.8%) isolates with a predominance of *sei* (29.3%), *seh* and *seg* (19.0% each) (Table S2). The Panton-Valentine leukocidin (PVL) encoding genes were found in isolates from *C. nictitans* (ST1855, Gabon) and *P. troglodytes* (ST 1928, Côte d'Ivoire, Table S3). Other virulence genes were not detected (*sea*, *sed*, *see*, *sej*, *tst*, *eta*, *edin-A*, *edin-C*). PVL is an important pore forming protein toxin that is frequently associated with abscesses. *S. aureus* harbouring PVL seem to be endemic in humans in Africa (Gillet *et al*., 2002; Breurec *et al*., 2011; Schaumburg *et al*., 2011a,b). In contrast, the low prevalence of PVL in non-human primates suggests a different selection pressure for PVL-positive isolates. Because monkey cells have a reduced susceptibility to PVL our finding argues against a selective advantage of PVL-positive isolates in terms of clonal spread, if the host is not susceptible to this toxin (Olsen *et al*., 2010).

Capsular polysaccharides (CP) are virulence determinants as they impede phagocytosis by monocytes and macrophages. The genes encoding CP5 ($n = 21$, 36.2%) and CP8 ($n = 20$, 34.5%) were equally distributed, 17 (24.6%) isolates were CP non-typeable.

Concluding remarks

African monkeys can be colonized by highly divergent *S. aureus* isolates, which are rarely found in humans while great apes mainly carry human-related *S. aureus* lineages. The characterization of these isolates is the basis for future investigations into inter- and intra-species transmission of *S. aureus* in sub-Saharan Africa.

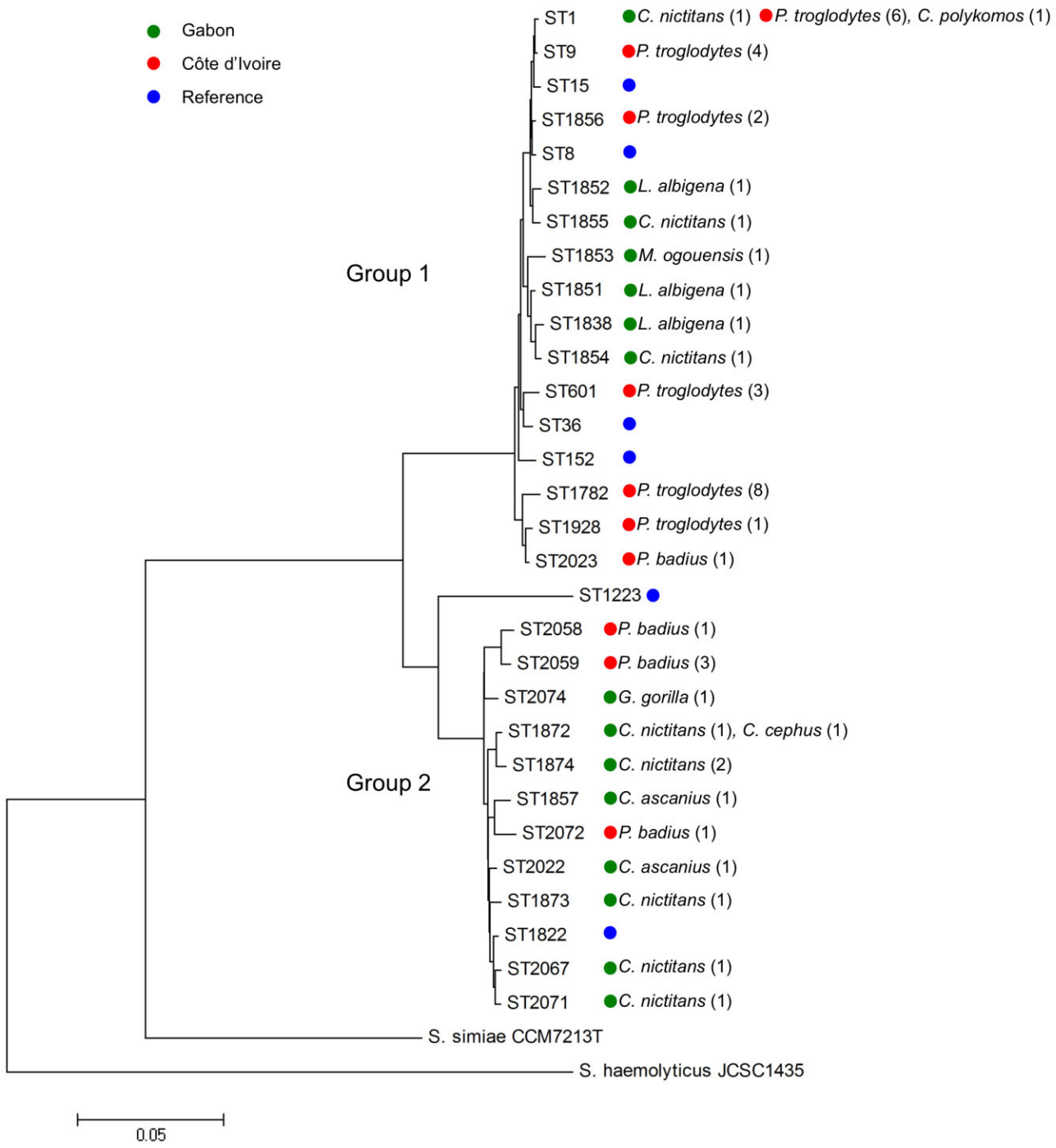

Fig. 1. Phylogenetic relation of *S. aureus* from African wildlife, *S. simiae* and *S. haemolyticus*. A Neighbor-Joining tree was constructed using the concatenated sequences of the seven *S. aureus* MLST housekeeping genes. Colours indicate the country of origin of the isolate; the name of the species from which the isolate derived is shown in italics; total numbers of isolates per ST are shown in brackets.

Experimental procedures

Sample collection

From Gabon, samples originated from 45 monkeys and one gorilla sold for consumption at a commercial market or on the roadside in the province 'Moyen Ogooué' (Table S1). Nasal swabs were taken within 6–12 h after death of the animals.

In Côte d'Ivoire, nasal swabs were taken from 10 red colobus monkeys (*P. badius*) and 10 black and white colobus monkeys (*C. polykomos*) anaesthetized in the course of a primate health study in the Taï National Park, Côte d'Ivoire

(Leendertz *et al*., 2008; 2010). Furthermore, fruit wadges were collected from 30 chimpanzees habituated to human presence in the same area of the Taï National Park (Boesch and Boesch, 2000). All animals in this study were considered to be wild and have never been touched by humans.

Identification and antimicrobial susceptibility testing

All samples were streaked on SAID and blood agar plates (bioMérieux, Marcy l'Etoile, France). Presumptive *S. aureus* colonies were identified by colony characteristics, catalase, coagulase (Staph-ase, bioMérieux), and latex agglutination test (Pastorex Staph-Plus, Bio-Rad Laboratories, Marnes-la-Coquette, France). Biochemical species confirmation and antimicrobial susceptibility testing were performed using Vitek2 automated systems (bioMérieux). All isolates were subjected to ribosomal 16S rRNA gene sequencing for genotypic species confirmation (Becker *et al*., 2004b). To confirm susceptibility to penicillin and methicillin, we performed a PCR targeting the *blaZ* and *mecA* genes respectively (Becker *et al*., 2006; Kaase *et al*., 2008).

Virulence factors

We used multiplex PCR approaches to detect genes encoding the pyrogenic toxin superantigens including toxic shock syndrome toxin (*tst*), enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*), the PVL (*lukS*-PV/*lukF*-PV), the exfoliative toxins (*eta*, *etb*, *etd*), and members of the epidermal cell differentiation inhibitor (*edin-A*, *edin-B*, *edin-C*) (Becker *et al*., 2004a; von Eiff *et al*., 2004).

Molecular typing

All isolates were *spa* typed based on the repeat pattern of the hypervariable region of the *S. aureus* protein A (*spa*) gene (Mellmann *et al*., 2006). Multilocus sequence typing was performed exemplarily for one isolate of each *spa* type (Enright *et al*., 2000). Alternative primers were used for *aroE* and *glpF* (Ng *et al*., 2009; Ruimy *et al*., 2009). Subtypes of the accessory gene regulator (*agrI–IV*) and CP types 5 and 8 were analysed by multiplex PCR (von Eiff *et al*., 2004; Goerke *et al*., 2005).

Phylogenetic analysis

All MLST STs of this study were assigned to known clonal complexes by the eBURST algorithm using the whole MLST dataset and the stringent group definition of six out of seven shared identical alleles (http://saureus.mlst.net). Related *spa* types were identified and clustered into *spa* clonal complexes (spa-CC) using the BURP-algorithm (Staph Type 2.1.1 software, Ridom GmbH, Münster, Germany) with preset parameters as published (Mellmann *et al*., 2007). Phylogenetic analyses were performed with MEGA4 (http://www.megasoftware.net) using the concatenated sequences of seven MLST genes and the Neighbor-Joining method. We did not include *arcC* in the concatenated sequences of *S. simiae* due to difficulty in amplifying this gene (Ruimy *et al*., 2009). As a reference for common STs found among carrier isolates from sub-Saharan Africa we selected ST8, ST15; ST36 and ST152 (Ruimy *et al*., 2008; Schaumburg *et al*., 2011b). To refer to known divergent isolates we chose ST1223 (Ruimy *et al*., 2009) and ST1822 (Schaumburg *et al*., 2011b). The evolutionary divergence of the concatenated sequences was calculated using the Maximum Composite Likelihood method.

Ethics

The non-invasive samples from animals were collected in accordance with international guidelines and under the permission of the national authorities (OIPR, Office Ivoirienne des Parcs et Reserves). Ethical approval for samples from bushmeat was not required as samples were taken from dead animals only. The investigators did not have any influence on the amount and the animal species that were hunted.

Statistics

Odds ratio and the 95% confidence interval were calculated to analyse the association between categorical variables. Significance of association was analysed using chi-squared test or Fisher's exact test. Statistical analysis was performed using the software 'R' (http://cran.r-project.org, Version: 2.10.1) and package 'epicalc'.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of African wildlife.
Table S2. Characteristics of *S. aureus* genotypes from bushmeat and wild-living African animals.

Table S3. Distribution of virulence factors among *S. aureus* isolates from African animals.

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