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Genomic Analysis of Companion Rabbit 
Staphylococcus aureus

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Abstract

In addition to being an important human pathogen, Staphylococcus aureus is able to cause a variety of infections in numerous other host species. While the S. aureus strains causing infection in several of these hosts have been well characterised, this is not the case for companion rabbits (Oryctolagus cuniculus), where little data are available on S. aureus strains from this host. To address this deficiency we have performed antimicrobial susceptibility testing and genome sequencing on a collection of S. aureus isolates from companion rabbits. The findings show a diverse S. aureus population is able to cause infection in this host, and while antimicrobial resistance was uncommon, the isolates possess a range of known and putative virulence factors consistent with a diverse clinical presentation in companion rabbits including severe abscesses. We additionally show that companion rabbit isolates carry polymorphisms within dltB as described as underlying host-adaption of S. aureus to farmed rabbits. The availability of S. aureus genome sequences from companion rabbits provides an important aid to understanding the pathogenesis of disease in this host and in the clinical management and surveillance of these infections.

Introduction

Staphylococcus aureus is a major human and veterinary pathogen, responsible for a wide range of diseases. Among economically important livestock animals these diseases include mastitis in dairy cows and small ruminants, lameness in commercial broiler chickens and virulent epidemics of skin abscesses, mastitis and septicaemia in farmed rabbits (Oryctolagus cuniculus). S. aureus colonisation and infection of animals is not only important from the perspective of animal wellbeing and economic impact but may also lead to zoonotic infection of humans [1, 2]. Although also recognised as a versatile and virulent pathogen among companion rabbits, in particular as a cause of severe abscesses [3], few data are available on the S. aureus strains causing disease in this host. This is despite the popularity of rabbits as a companion animal; for
instance the population of companion rabbits in the UK is estimated at ~ 1 million [4]. Furthermore S. aureus, including methicillin-resistant S. aureus (MRSA) is a well-documented pathogen among other companion animals such as cats, dogs and horses, [5–7]. Interest in the S. aureus population among companion animals is further heightened by the discovery of emergent mecC MRSA in a farmed rabbit [8], and sporadic reports of MRSA in companion rabbits [9], including livestock-associated clonal complex 398 MRSA [10] and Panton-Valentine Leucocidin-positive isolates [11]. Furthermore, rabbits are a frequently used experimental model for S. aureus infections and a better understanding of the natural bacterial-host interactions in this setting may facilitate improved model systems.

In order to address this paucity of data on the S. aureus population among companion rabbits we have genome sequenced a collection of companion and research unit S. aureus from this host species in the United Kingdom. This novel genome-level study provides insight into host-pathogen interactions, antimicrobial resistance and the phylogenetics of S. aureus among rabbits. These data will inform clinical management in rabbits and the future surveillance of this widespread and important pathogen.

### Materials and Methods

**Bacterial isolates and antimicrobial susceptibility**

A request for S. aureus isolates from companion rabbits was made to personal contacts and veterinary diagnostic laboratories in the UK. Isolates where collected by veterinary microbiology laboratories in the course of their routine diagnostic work, with the study approved by the Department of Veterinary Medicine, University of Cambridge Ethics and Welfare Committee (reference: CR76 Collection of S. aureus isolates from domestic and wild animals for genome sequencing). The resultant ten isolates that were collected and their associated details are shown in Table 1. Antimicrobial susceptibility testing was performed using the Staph AST-P620 card on the Vitek 2 system (bioMérieux, Basingstoke, UK) following the manufacturer’s instructions with S. aureus NCTC6571 and NCTC12493 as control strains.

**Genome sequencing and analysis**

Genomic DNA was extracted using the MasterPure™ Gram Positive DNA Purification Kit (Cambio, Dry Drayton, UK) from overnight cultures grown from single colonies in 5 ml of tryptic soy broth overnight at 37°C. Illumina library preparation was carried out as described previously [12], and genome sequencing using Hi-Seq 2000 performed following the manufacturer’s standard protocols (Illumina, Little Chesterfield, UK). Nucleotide sequences been deposited in the European Nucleotide Archive, accession numbers provided in Table 1. Genome assembly was performed de novo using Velvet [13] and antimicrobial resistance genes and virulence factors identified using BLAST and ResFinder [14]. Genome-derived multi-locus sequence types (MLST) were assigned as described previously [15]. The phylogenetic relationships among the isolates was assessed using core genome (cg)MLST using SeqSphere+ software (Ridom GmbH, Münster Germany) as described previously [16] and including twenty-eight reference genomes to place the rabbit isolates within the context of the wider S. aureus population. 1475 core genome loci found in all isolates were used. spa typing was performed using Sanger sequencing of PCR products using primers spa-1113f (5' - TAA AGA CGA TCC TTC GGT GAG C -3') and spa-1514r (5' - CAG CAG TAG TGC CGT TTG CTT -3') as per Ridom GmbH (Würzburg, Germany).
Table 1. Rabbit isolates included in this study.

| Isolate name | ERA Accession | Coverage | Biosample | Assembly Accessions | Geographical location | Site of isolation | Date of isolation | ST1 | CC2 | spa type(s) | Phenotypic resistance | Resistance genes/mutations | Additional notes |
|--------------|---------------|----------|-----------|---------------------|-----------------------|-------------------|-------------------|-----|-----|------------|-----------------------|---------------------------|---------------------|
| FP01         | ERR387096     | 163x     | SAMEA1929514 | FJNS01000001-FJNS01000044 | Manchester area, England | Not known | Apr-2013 | 30  | 30  | 1021       | benzylpenicillin         | blaZ, tet(38), norA       | same animal as FP02   |
| FP02         | ERR387097     | 156x     | SAMEA1929515 | FJNW01000001-FJNW01000043 | Manchester area, England | Not known | Apr-2013 | 30  | 30  | 1021       | benzylpenicillin         | blaZ, tet(38), norA       | same animal as FP01   |
| 557472       | ERR387166     | 113x     | SAMEA1929647 | FJNT01000001-FJNT01000024 | England | Ventral vulva abscess | Jun-2013 | 3126 | 291 | 11614      | benzylpenicillin         | blaZ, tet(38), norA       |                     |
| M197098/1    | ERR387195     | 160x     | SAMEA1929516 | FJNU01000001-FJNU01000024 | Scotland | Lesion | 1998   | 3120 | 425 | 113114     | susceptible              | tet(38), norA            |                     |
| M503044/99/1 | ERR387196     | 147x     | SAMEA1929517 | FJNP01000001-FJNP01000029 | Scotland | Sub-cutaneous abscess | 1999   | 121  | 121 | 1645       | susceptible              | tet(38), norA            |                     |
| 543471       | ERR387256     | 90x      | SAMEA1929646 | FJNO01000001-FJNO01000011 | England | Dentricystitis | Feb-2013 | 6    | 6   | 15413      | benzylpenicillin, fusidic acid | blaZ, tet(38), norA, H457Y in elongation factor G |                     |
| 559622       | ERR387257     | 101x     | SAMEA1929648 | FJNO01000001-FJNO01000024 | England | Skin infection | Jun-2013 | 15   | 15  | 12574      | benzylpenicillin         | blaZ, tet(38), norA       |                     |
| 61908        | ERR494744     | 134x     | SAMEA2298602 | FJNO01000001-FJNO01000014 | Stirlingshire, Scotland | Nasal sample at post-mortem | Sep-2009 | 3092 | 425 | 115410     | susceptible              | tet(38), norA            | feral rabbit         |
| 68850        | ERR494745     | 144x     | SAMEA2298603 | FJNV01000001-FJNV01000032 | Glasgow, Scotland | Oesophagus | Jun-2012 | 39   | 30  | 115409     | benzylpenicillin         | blaZ, tet(38), norA       | experimental research unit |
| 68901        | ERR494746     | 130x     | SAMEA2298604 | FJNR01000001-FJNR01000031 | Glasgow, Scotland | Lower jaw abscess | Jun-2012 | 2257 | 22  | 11977      | benzylpenicillin         | blaZ, tet(38), norA       | experimental research unit |

1 Multi-locus sequenced type (new multi-locus sequence types shown in bold)
2 MLST clonal complex assigned by e-Burst
3 New spa type(s) shown in bold
4 Tested against: benzylpenicillin, cefoxitin, oxacillin, ciprofloxacin, erythromycin, chloramphenicol, daptomycin, fusidic acid, gentamicin, linezolid, mupirocin, nitrofurantoin, rifampicin, teicoplanin, tetracycline, tigecycline, trimethoprim, vancomycin and clindamycin as well as inducible resistance to clindamycin.

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Study strains, multi-locus sequence types and spa types

Ten rabbit *S. aureus* isolates collected between 1998 and 2013 in the UK were included in this study, Table 1. Eight were from companion rabbits including two isolates from the same rabbit, with a further two isolates from research unit rabbits. MLST showed the ten isolates belonged to nine different sequence types, the only duplication of ST being the two isolates from the same rabbit which both belonged to ST30. Three new ST were identified in this study; ST3092 and ST3120 being single locus variants (SLV) of ST425 in *aroE* and *tpi* respectively, whilst ST3126 is a SLV of ST291 in *tpi*. These STs belonged to eight clonal complexes, Table 1. Similarly to MLST, nine different spa types were found among the ten isolates, the only duplication again being the two isolates from the same rabbit. Two new spa types, t15409 and t15410 were found.

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**Fig 1.** Clonal relationships among rabbit *S. aureus* and their context within the wider *S. aureus* population. A phylogenetic dendrogram (UPGMA) generated from the allelic profiles of 1475 cgMLST target genes, based on (16) and comprising the ten rabbit isolates from this study and twenty-eight reference *S. aureus* genomes from Genbank. Rabbit isolates are denoted by *, isolate name and Genbank accession provided for the reference genomes. The last figure in the text line indicates the multi-locus sequence type of each isolate, where available. The scale bar indicates the number of differing alleles comprising the calculated distance.

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Whole genome phylogenetic analysis

In agreement with the diversity indicated by MLST, whole genome analysis using cgMLST across 1475 loci showed a diverse population among rabbit isolates, Fig 1. The average pairwise difference in allele profile between rabbit isolates was 1271 alleles, representing 86% of the core genome loci assessed. The two isolates from the same rabbit, FP01 and FP02 differed in 51 alleles and the largest pairwise difference in profile was 1402 (95% of the 475 core loci analysed). Inclusion in the analysis of twenty-eight reference S. aureus genomes showed the rabbit isolates to be distributed across the wider S. aureus population, Fig 1.

Antimicrobial Resistance and resistance determinates

Resistance to benzylpenicillin occurred in seven of the isolates and correlated with the presence of \( \textit{blaZ} \), Table 1. Resistance to other antimicrobials was restricted to a single isolate, 543471, showing fusidic acid resistance, Table 1. This resistance correlated with a single amino acid substitution, H457Y in elongation factor G. Whilst no other phenotypic resistance was seen, all ten isolates were positive for the efflux pump genes \textit{tet(38)} and \textit{norA}.

Virulence factors and markers of host adaption

The sequenced rabbit isolates were assessed for the presence or absence of \textit{S. aureus} virulence factors, Table 2. Several genes, including those encoding \( \alpha \)-(hla), \( \beta \)-(hlb) and \( \gamma \)-haemolysins (hlgACB) were present in all the isolates with others present in a subset, Table 2. None of the ten isolates possessed the genes encoding for Panton-Valentine Leukocidin but isolates FP01 and FP02 were both positive for toxic shock syndrome toxin-1. Four of the strains were positive for the phage-encoded immune evasion genes, \textit{sak} and \textit{scn} which are taken as indicative of strains of human origin. Among clinical rabbit isolates from commercial rabbitries in mainland Europe, Viana \textit{et al.} have demonstrated a critical role in host adaption for polymorphisms in \textit{dltB}, encoding the D-alanine teichoic acid esterification protein [17]. We therefore compared
the DltB sequence in our isolates to that from human isolated S. aureus. Every rabbit isolate in our collection had at least one amino acid polymorphism in DltB, Table 2. These comprised both novel and previously described polymorphisms including the experimentally validated T113K dltB mutation [17].

Discussion

To gain insight into the molecular epidemiology and disease pathogenesis of rabbit staphylococcosis we have genome sequenced a collection of S. aureus isolates from companion rabbits. Strain typing by MLST and spa typing showed a diverse population of isolates with no duplication of ST or spa type between isolates from different animals and minimal overlap even at the level of MLST clonal complex. This finding of a diverse S. aureus population able to infect rabbits was further supported by the use of high-resolution whole genome analysis using cgMLST. Bacterial diversity extended to two isolates from the same individual rabbit which differed by 51 alleles and demonstrates that the within host diversity of S. aureus described previously in humans and dogs [18–21] extends to the rabbit host also. This diversity among the isolates indicates that a variety of S. aureus lineages are able to cause disease in companion rabbits, with no strong suggestion that any lineages are predominant, albeit based on a relatively small sample. However, the finding that this relatively small collection led to the identification of three novel STs and two novel spa types strongly indicates that the S. aureus population among this host has been poorly sampled to date and includes strains rare in humans and other animals.

With the exception of penicillin resistance, present in the majority of isolates and which correlated with the possession of blaZ, antimicrobial resistance was scarce. No MRSA isolates were found and the only additional phenotypic resistance was a single isolate resistant to fusidic acid. This isolate possessed a single amino acid substitution in elongation factor G which has previously been found associated with fusidic acid resistance in naturally occurring clinical isolates and in experimentally selected resistance mutants [22–25]. Furthermore, when introduced into a susceptible strain on a plasmid, this mutant fusA allele confers fusidic acid resistance [22]. All ten strains were positive for norA, a multidrug efflux pump which confers resistance to ciprofloxacin among a broad spectrum of agents [26] and tet(38), an efflux pump conferring tetracycline resistance [27]. In both cases however, phenotypic resistance is associated with mutations leading to over-expression which likely explains the absence of phenotypic resistance in these rabbit isolates [26, 27]. The presence of these genes in rabbit isolates, however, indicates the potential for such resistance to manifest in the future.

The importance of dltB polymorphisms in host adaption of S. aureus to rabbits has been demonstrated previously with a single amino acid substitution (T113K) sufficient to confer virulence in rabbits to a human ST121 isolate otherwise avirulent in that host [17]. Furthermore, while dltB is highly conserved in human isolates, thirty-nine rabbit isolates belonging to a range of STs and CCs all contained one or more non-synonymous SNPs in dltB thus suggesting convergent evolution among rabbit-adapted S. aureus [17]. All ten rabbit isolates in our collection carried at least one amino acid polymorphism in DltB with each strain encoding a different pattern of polymorphism(s) to each other. Two isolates belonging to ST121 and ST15 carried the experimentally validated T113K substitution. A second distinct dltB allele containing two SNPs and associated with the S. aureus ST96 rabbit clone was also shown experimentally to confer virulence in rabbits [17]. While one of those two SNPs, "405Q, was present in some of the isolates reported here, the second SNP, K402R, was not. We show therefore that host-adaption via dltB polymorphism occurs in companion rabbit isolates and provide further evidence for convergent evolution at this locus across diverse S. aureus lineages infecting...
rabbits. In addition to the DltB polymorphisms described previously we have identified six novel amino acid substitutions, Table 1. Using a predicted membrane topology model of the DltB protein, Viana et al. noted that the majority of mutations they described were predicted to be in the extracellular loops or proximal to the outer surface of the membrane [17]. Using that model, while none of the novel mutations described here appear to be located extracellularly, four are predicted to be proximal to the outer surface of the membrane (data not shown). Interestingly, the pattern of distinct polymorphisms between the isolates included the two related ST30 isolates, FP01 and FP02, cultured from the same animal. These two isolates shared one amino acid insertion but had a distinct second amino acid substitution. This suggests that the selective pressure exerted on dltB by the rabbit host may be acting to drive divergent evolution within clones within the same individual host. Although the independent acquisition of two related strains with divergent dltB alleles cannot be excluded.

To conclude, we have used antimicrobial susceptibility testing and whole genome sequencing to characterise S. aureus isolates from companion rabbit. Isolates came from a diverse bacterial population, including three new STs and two new spa types. While antimicrobial resistance was uncommon, except for penicillin resistance, isolates possessed a number of virulence factors consistent with the ability to cause severe abscesses in companion rabbits. The availability of these genome sequences will underpin improved understanding of disease pathogenesis, clinical management and pathogen surveillance in this popular companion animal.

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Author Contributions
Conceived and designed the experiments: GKP MAH. Performed the experiments: GKP EMH EAF. Analyzed the data: MAH GKP. Contributed reagents/materials/analysis tools: MAH JP EMG GF. Wrote the paper: GKP. Contributed to the drafting and approval of final manuscript: MAH EMH EAF EMG JP GF GKP.

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