Recombination-mediated host-adaptation by avian Staphylococcus aureus

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**Running title:** Poultry-associated genetic elements in *S. aureus*
ABSTRACT

*Staphylococcus aureus* are globally disseminated among farmed chickens causing skeletal muscle infections, dermatitis and septicaemia. The emergence of poultry-associated lineages has involved zoonotic transmission from humans to chickens but questions remain about the specific adaptations that promote proliferation of chicken pathogens. We characterised genetic variation in a population of genome-sequenced *S. aureus* isolates of poultry and human origin. Genealogical analysis identified a dominant poultry-associated sequence cluster within the CC5 clonal complex. Poultry and human CC5 isolates were significantly distinct from each other and more recombination events were detected in the poultry isolates. We identified forty-four recombination events in 33 genes along the branch extending to the poultry-specific CC5 cluster, and 47 genes were found more often in CC5 poultry isolates compared to those from humans. Many of these gene sequences were common in chicken isolates from other clonal complexes suggesting horizontal gene transfer among poultry associated lineages. Consistent with functional predictions for putative poultry-associated genes, poultry isolates showed enhanced growth at 42°C and greater erythrocyte lysis on chicken blood agar in comparison with human isolates. By combining phenotype information with evolutionary analysis of staphylococcal genomes, we provide evidence of adaptation, following a human-to-poultry host transition. This has important implications for the emergence and dissemination of new pathogenic clones associated with modern agriculture.
INTRODUCTION

The expansion of the global poultry industry and rapid increase in the number of chickens presents opportunities for increased incidence of zoonotic diseases. Staphylococci are among the leading agents of bacterial infection in chickens (Peton and Le Loir 2014) and Staphylococcus aureus causes a wide range of chicken diseases, including septic arthritis, subdermal abscesses and gangrenous dermatitis (Bystron, et al. 2010). Molecular epidemiology studies have described highly structured S. aureus populations with clusters of related isolates, grouped into clonal complexes (CCs) that share 5 or more alleles at 7 MLST loci (Enright and Spratt 1999; Urwin and Maiden 2003). Clonal complexes vary in the range of sources from which they have been isolated. For example, isolates belonging to the CC385 have not been previously identified among human and mammalian species but have been principally found in birds including poultry (Lowder, et al. 2009). Isolates from other lineages, such as CC5 and CC398, have been frequently isolated from chickens, humans and other hosts (Abdelbary, et al. 2014; Monecke, et al. 2013; Price, et al. 2012). CC5 is of particular concern in food production as it is the most frequent disease-causing lineage in chickens (Bystron, et al. 2010; Hasman, et al. 2010; Lowder, et al. 2009).

Host association has previously been described in staphylococci (Shepheard, et al. 2013). Global dissemination and the high level of genetic diversity observed among CC5 isolates from humans suggests long-term association with the human host (Nubel, et al. 2008). The emergence of CC5 as a common lineage in poultry (Monecke, et al. 2013) is thought to be the result of a single recent human-to-poultry host jump approximately 38 years ago (Lowder, et al. 2009). In common with bovine-adapted S. aureus lineages (Herron-Olson, et al. 2007), host transition of poultry CC5 isolates has been accompanied by genetic changes, including the loss
of several genes involved in human disease pathogenesis and acquisition of novel mobile
genetic elements from an avian-specific accessory gene pool (Lowder, et al. 2009). Host-
associated genetic variation of this kind is influenced by various factors. These include: (i)
genetic bottlenecks leading to reduced genetic diversity in the founder population; (ii)
diversification in allopatry following isolation from the ancestral population; (iii) adaptation
involving the acquisition of genomic elements that provide a competitive advantage in the new
niche.

There are several examples of host adaptation following zoonotic transmission of S. aureus
al. 2012), including acquisition of genes that contribute to transmission, colonization and
virulence (Wiedenbeck and Cohan 2011; Yan, et al. 2016). For example, it is believed that
methicillin-susceptible S. aureus CC398 acquired the methicillin resistance cassette in livestock
host adaptations becomes particularly important when they promote the spread of disease. This
is the case with plasmids that have contributed to strain virulence and conferred antibiotic
resistance (pT181, pT127, pC194, pC221, pC223 and pUB112) (Ehrlich 1977) among S. aureus
isolates causing a wide range of chicken diseases that are particularly robust and difficult to
treat (Bystron, et al. 2010).

Comparison of individual S. aureus isolates from human and chicken has been instructive in
identifying host-associated genetic elements (Lowder, et al. 2009). However, it remains
difficult to differentiate genetic changes associated with bottlenecking and drift from those that
confer an advantage in the poultry niche. Evidence for a role in adaptation is provided if specific
genomic changes occur in divergent lineages that are not present in their common ancestor (homoplasy). In this study, we build on the work by Lowder, et al. (2009) which identified poultry-associated plasmid and phage elements, and conduct population genomic analysis of the emergence of disease-associated *S. aureus* in chickens. We: (i) conduct a survey of disease causing *S. aureus* in chickens; (ii) characterize the population structure of disease-associated strains and investigate the abundance of CC5; (iii) compare chicken-disease-associated isolate genomes with clonally related bacteria from human hosts; (iv) identify the genetic elements associated with the emergence of disease; (v) quantify homologous and accessory genome recombination in populations of *S. aureus* from different sources and genetic backgrounds. By characterising the evolutionary events in CC5 isolates that accompanied the colonization of chickens from humans, and comparison with phylogenetically divergent *S. aureus* lineages, we provide evidence for the genetic basis of poultry adaptation. Relating adaptive hot-spots to gene function, and using laboratory phenotyping assays, we describe an evolutionary history of rapid avian host adaptation of a globally disseminated animal pathogen.
METHODS

Isolate collection

In total, 191 isolates were sampled from diseased chickens from various breeder farms predominantly in the UK (n=161), and also the USA (n=25) and the Netherlands (n=5) (Table S1). Samples were collected from chickens suffering from infections of the leg (153), liver (8), footpad (7), bone marrow (4), yolk sac (4), peritoneum (3), brain (3), kidney (1), pericardium (1), spleen (1), lung (1), from a day old chick and four samples from the surrounding environment. Samples were collected between 2008 and 2013. S. aureus were identified, sub-cultured and transported on slopes of Dorset’s egg medium before storing at minus 80 °C. All isolates were sequenced and 165 assembled genomes were analysed.

Genome sequencing

Staphylococcus isolates were cultured on Columbia blood agar plates at 37 °C for 24h. Single-colony cultures were harvested and re-suspended in 3ml of peptone yeast extract medium to minimize clumping and incubated at 37 °C with overnight shaking. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, UK), using manufacturer’s instructions with the addition of 1.5 μg/μL lysostaphin (Ambi Products, USA) to facilitate cell lysis. The DNA of 224 isolates was sequenced at the Wellcome Trust Centre for Human Genetics (Oxford, UK) using a HiSeq 2500 (Illumina, USA). The 100 bp short read paired-end data was assembled using the de novo assembly algorithm, Velvet (Zerbino and Birney 2008)(version 1.2.08). The VelvetOptimiser script (version 2.2.4) was run for all odd k-mer values from 21 to 99. The minimum output contig size set to 200 bp with the scaffolding option switched off; all other program settings were left unchanged. For comparison, we also sequenced several human isolates from wound infections (n=13), prosthetic joint infections (n=31), nasal swabs (n=9)
and the SH1000 lab strain. Overall, the average number of contiguous sequences (contigs) for all 224 genomes sequenced in this study was 77 which gave rise to an average total assembled genome size of 2,893,678 bp and an average N50 of 337 Kbp. Short reads are available from the NCBI short read archive (SRA) associated with BioProject: PRJNA312437.

Our collection of avian isolates was augmented with published genomes from NCBI (n=189) and further isolates were sequenced to compliment our collection. Twenty-four additional avian isolates were sequenced by the Edinburgh genomics facility at the Roslin Institute using an Illumina MiSeq (USA), including 8 isolates from CC385 - a clonal complex that has not previously been found outside of avian hosts (ENA project accession: PRJEB18782; average number of contigs: 382, average genome size: 2,967,259, average N50: 52 Kbp). In total, 432 *Staphylococcus aureus* genomes (198 from poultry, 228 from humans and 6 from ruminants) were used to investigate the association of genomic elements with human or poultry hosts (individual accession numbers can be found in Table S1).

**Genealogical studies**

A reference pan-genome approach (Meric, et al. 2014) with gene-by-gene alignment (Maiden, et al. 2013; Sheppard, et al. 2012) was implemented using the open source Bacterial Isolate Genome Sequence Database: BIGSdb (Jolley and Maiden 2010), which included functionality to call MLST profiles defined by the pubMLST database (https://pubmlst.org/saureus). The publicly accessible genomes used in this study are archived in the Sheppard Lab Staphylococcal database and can be accessed at: http://www.sheppardlab.com/resources. The annotated genome of ED98 (accession NC_013450.1) and 3 plasmids (pAVY, pAXY, pT181) were combined and used as a reference genome (Meric, et al. 2014). The BLAST algorithm was used to scan all genomes for gene orthologs at each locus in the reference genome. An ortholog was
defined as a reciprocal best hit of the sequence with >70% nucleotide identity over 50% of the alignment length. MAFFT software was used to align gene orthologs on a gene-by-gene basis, and these data concatenated into contiguous sequence for each isolate genome, including gaps (Sheppard, et al. 2013). Alignment based on the ED98 reference genome and poultry plasmids was constructed (2,780 genes) and a heuristic maximum-likelihood tree generated using FastTree2 (version 2.1.0)(Price, et al. 2010) with the generalised time reversible substitution model enabling reconstruction of branch lengths greater than 0.0000005 (1,000 times higher than the default FastTree parameters). In addition, gene-by-gene alignments were extracted from the BIGSdb for individual genes present in CC5 poultry isolates and completely absent from CC5 human isolates. The RAST automated annotation server was used to predict putative gene function (Aziz, et al. 2008).

Core genome recombination

Isolate genomes (n=432) were aligned using MAFFT (Katoh, et al. 2002). Mutations found in single isolates were excluded, as they carry no information about the shared ancestry between lineages and a total of 279,646 SNPs were recorded in the isolates. Isolates were grouped into clusters through hierBAPS analysis (version 6.0)(Cheng, et al. 2013) of the resulting core genome alignment using 5 replicate runs with the upper bound for the number of clusters varying between 50 and 100. All estimation runs converged to the same estimate of the posterior mode clustering, indicating strongly peaked posterior distribution. CC5 was identified as one of the hierBAPS clusters and subsequently, BratNextGen (BNG) software (Marttinen, et al. 2012) was used to estimate the amount of homologous recombination in the core genome for the 68 isolates assigned to that cluster. Within BNG, 20 iterations of hidden Markov model parameter estimation were performed and 30 groups of isolates were identified. Statistically significant (p-value < 0.05) recombination in the core genome was determined with 100 parallel
permutation runs. We report the ratio at which changes in nucleotide sequence are introduced by recombination relative to mutation (r/m) as in McNally, et al. (2013).

Inference of recombination regions

ClonalFrame infers the clonal relationship of bacteria and the chromosomal position of homologous recombination events that disrupt a clonal pattern of inheritance (Didelot and Falush 2007). Gene-by-gene alignment (XMFA) files of all ED98 and plasmid genes present in each of the *S. aureus* strains were generated. A genealogy for these alignments was estimated using ClonalFrame (version 1.2) on concatenated sequences of 46 CC5 genomes with 100,000 iterations, half of which were discarded as burn-in. Substitution mutation and recombination regions were categorized from the output of ClonalFrame. The posterior probability of recombination and substitution at each site is calculated by ClonalFrame and recombination events were defined with a probability of recombination more than 75%, reaching 95% at any one site.

Phenotype assays

**Growth at human and avian body temperature:** Twelve *S. aureus* CC5 poultry isolates were selected for comparison with four representative *S. aureus* human isolates (from clades containing poultry isolates: 2 CC5 and 2 CC1) to assess their growth under human (37°C) and avian body temperatures (42°C). Isolates were cultured on CBA plates at 37°C overnight prior to inoculation of 3 ml Mueller-Hinton broth with shaking at 37°C overnight until stationary phase (OD$_{600}$ between 1.0 and 1.5). Aliquots were diluted 1:100 in fresh Mueller-Hinton medium and 195 µl of each sample loaded into a 96-well plate with absorbance read every hour using the FLUOstar Omega microplate reader (BMG Labtech, Germany) at 600 nm. Experiments were performed in triplicate and the average of three readings for each run was
calculated. We performed an extra sum-of-squares F-test (p value threshold of 0.05) to test the null hypothesis that a single sigmoid regression curve fits the distribution of both human and poultry samples at human and avian body temperature.

**Erythrocyte lysis assays:** The same isolates used in growth experiments were also used to assay for their ability to digest chicken and human erythrocytes. Isolates were cultured on CBA plates at 37°C overnight prior to inoculation of 3 ml Mueller-Hinton broth with shaking at 37°C for up to 3 h until late exponential phase (OD600 ~0.6). Each sample was diluted 1:10 using fresh Mueller-Hinton broth and 2 µl added to wells of a six-well plate containing 3 ml of defibrinated chicken blood (Rockland antibiotics and assays, USA #R102-0100) or pooled whole human blood (collected using haematology tubes with EDTA from volunteer scheme at Swansea University). Plates were incubated at 37°C for 48 h and positive results recorded for erythrocyte lysis if a halo (>1mm) was measured around the colony (Table 1). Unpaired t-tests were used to evaluate the significance of differences between human and chicken isolates, in addition to isolates with and without the Staphostatin B gene.
RESULTS

Emergence and rapid clonal expansion of CC5 in chicken

The majority of avian isolates collected belonged to CC5 (85%). Isolates collected from the UK (91%) and the USA (76%) were predominantly of CC5, but no CC5 isolates were collected from the Netherlands, although these five isolates represent only a small proportion of our collection (Figure 1a). Our isolates are predominantly from 2012 and 2013 and are dominated by isolates from CC5 (Figure 1b). Samples from all disease types studied contained CC5 isolates (Figure 1c) and the only other CC detected in our collection was the livestock-associated CC398. As in other poultry *S. aureus* studies (Lowder, et al. 2009), CC5 is the dominant lineage within our collection. Further long-term surveillance is necessary to establish the timescale of emergence of this lineage.

A maximum-likelihood phylogenetic tree of all 432 isolates was constructed using a reference pan-genome combining the ED98 isolate genome and three plasmids previously identified from poultry: pAVX (17,256 bp); pAVY (1,442 bp); pT181 (4,439 bp) (Lowder, et al. 2009; Meric, et al. 2014) (Figure 2A). Four distinct lineages contained isolates from poultry (Price, et al. 2010) and within the CC5 clade a clear poultry sub-lineage was evident (there was also a single poultry isolate in CC30). Hierarchical Bayesian estimations of the genetic population structure (hierBAPS) also grouped poultry and human isolates within the CC5 cluster.

Increased recombination in CC5 poultry isolate core genomes

The number of ancestral populations was estimated by grouping isolates into genetically divergent clusters using BratNextGen (Marttinen, et al. 2012). The algorithm inferred the positions and sizes of DNA sequence segments with evidence of homologous recombination
and grouped isolates according to recombination pools (Figure S1). At the highest level of BAPS clustering there were 30 *S. aureus* sequence clusters composed of 2 or more isolates (Figure 2a). Recombination was unevenly detected across *S. aureus* sequence clusters (Figure S1). The rate at which recombination introduced nucleotide changes, relative to mutation (*r/m*), in the CC5 complex was 1.08. Disproportionally more recombination events were identified in the chicken CC5 isolates ($\bar{x} = 34.6$ per isolate) compared to the human CC5 isolates ($\bar{x} = 18.5$). In order to assess the statistical significance of this we assumed independence of the isolates and estimated the probability of finding at least one recombination event in an isolate from each population. In human isolates there were 40/59 isolates with at least one recombination event and in poultry there were 9/9. The z-score test statistic was 2.005 and a p-value for a one-sided test was 0.022, which was significant at 5% level.

**Poultry-associated core-genome recombination**

A subset of CC5 isolates (human and poultry) were selected for further analysis with ClonalFrame (Didelot and Maiden 2010), which infers the clonal relationship of bacteria and the chromosomal position of homologous recombination events. Inferred regions of recombination were removed (and exported) prior to phylogenetic reconstruction of the clonal frame (Figure 2b). Execution of ClonalFrame with 46 *S. aureus* strains (20 CC5 poultry and 26 CC5 human) gave rise to a genealogy that also demonstrated complete separation of the poultry and human isolates within the CC5 complex. In comparison to the isolate phylogeny prior to the removal of recombination, there is tighter clustering of the poultry CC5 isolates, which supports our observation that CC5 poultry isolates have been subject to a greater level of recombination.
ClonalFrame inferred 196 substitution sites and 44 recombination regions mapping to 33 genes (Table S2). BLAST was used to locate exact matches (100% identity) of the recombination regions (between 6 and 913 bp in length) in poultry isolates from other clonal complexes (Table S2). The shortest recombination region detected by ClonalFrame was 6 bp in length located within SAAV_2007 (hilb, β-haemolysin) and upstream of the phage-associated virulence factor SAAV_2008 (ornithine cyclodeaminase) (Lowder, et al. 2009; Price, et al. 2012) and was similar to a ribosome-binding site (TTATAA). This recombination region was too short to be detected using the BLAST algorithm, requiring a manual search of all isolates to identify the region in all CC1, CC5, CC385 and CC398 poultry isolates, but was completely absent from human isolates.

Quantification of the poultry associated recombination regions in poultry and human isolates was carried out for each clonal complex (Table S2). In all four lineages studied here (CC1, CC5, CC385 and CC398), the putative recombination regions were detected more often in isolates obtained from chicken compared to humans. All recombination regions (43/44 by BLAST plus the short 6 bp recombination region) were found in CC5 poultry isolates (n=177), but only 31 of those were present in the human CC5 isolates (n=53) (Table S2). In CC1, thirty five recombination regions were found in chicken isolates (n=3) compared to 24 in the isolates from humans (n=11). Many of the inferred recombination regions were found in isolates from both chicken and human sources in the livestock-associated CC398 clonal complex, 38 in chicken isolates (n=9) and 28 in the human CC398 isolates (n=8). Forty-three recombination regions were found in chicken isolates (n=8) from the poultry ancestral clonal complex CC385 (no human isolates). In total, 31 recombination regions were found more often in chicken isolates in all four of the investigated clonal complexes, consistent with ongoing poultry
adaptation. A summary of the relative abundance of all poultry-associated genes and recombination regions found by analysis of CC5 is presented in Table 2.

**Poultry associated accessory genes**

Comparison of gene content revealed that 2,716 genes from the poultry-associated reference pan-genome were shared between CC5 human and CC5 poultry isolates. No genes were found exclusively in CC5 human isolates and 47 genes were found predominantly (≥95%) in CC5 poultry but absent in human isolates (Table S2). Thirty-eight of the 47 genes were also present in CC1 poultry isolates with variable frequency from 33% to 100% (Table S2). Forty-one genes were present in 22% to 89% of CC398 poultry isolates (Table S2). All 47 poultry-associated genes were present in CC385 poultry isolates to some degree (13% to 100%) (Table S2). Only one poultry-associated gene, SAAV_0809, which belongs to the *S. aureus* pathogenicity island (SaPI), was present in 82% of CC1 human isolates. Thirteen genes were found in 13-25% of CC398 human isolates (Table S2). In total, 36 of the genes associated with poultry in CC5 were present more often in poultry isolates from all four investigated clonal complexes.

**Co-localisation of poultry-associated genes and recombination regions in three recombination hotspots**

Thirty-three genes containing homologous recombination regions and forty-seven poultry-associated accessory genes were mapped to the ED98 reference genome and to three plasmids (pAVX, pAVY and pT181). Where sequence mapped to regions of unknown function, for example genes encoding hypothetical proteins, the locus ID from ED98 was recorded and the putative gene function was investigated using RAST and BLAST comparison with the NCBI database (Table S2). Three genes (*SAAV_A2*, *SAAV_B1* and *SAAV_C17*) containing recombination regions were mapped to locations on the three poultry-associated plasmids,
pAVY, pT181, and pAVX (Lowder, et al. 2009). Nine poultry associated genes were mapped to pAVX (SAAV_C01, SAAV_C03, SAAV_C04, aurT, scpB, SAAV_C12, SAAV_C15, SAAV_C18 and the pemK-like SAAV_20). Many of the eighty genes located on the reference genome clustered into three distinct genomic regions (Figure 3), including genes with putative roles in heat shock response, haemolysis, adhesion, mobile elements and transposons (Table S2).

A total of 58 poultry-associated genes and genetic elements are predicted to be involved in the transfer of mobile genetic elements, phage proteins, and hypothetical proteins (Figure 4a, Table S2). Putative gene function was inferred using the RAST annotation server (Aziz, et al. 2008) and BLAST sequence comparisons made against the NCBI database. The genes included in region 1 map to a similar location on the ED98 reference genomes and may form a transposon comprised of 16 genes with predicted function including hypothetical proteins, lipoproteins a transposase, a plasmid-related conjugal transfer protein (TraG, SAAV_0051) and an FtsK/SpoIIIIE family protein (SAAV_0054)(Figure 4b). Regions 2 and 3 are phage-related. Genes encoding hypothetical proteins, phage proteins, the fibronectin-binding protein (Fnb, SAAV_2566) and a putative β-haemolysin (SAAV_2007) were found in region 3. The β-toxin gene is common among S. aureus isolates, but often truncated by insertion of a beta-converting phage in most human infection isolates (Salgado-Pabon, et al. 2014). The three poultry-associated plasmids were composed of several hypothetical proteins, a replication associated protein (SAAV_C12), the staphylococcal virulence factor Staphostatin A (ScpB, SAAV_C09) and the plasmid-retention PemK-like protein (SAAV_C20).

Poultry isolates show enhanced growth and erythrocyte lysis at avian body temperature on chicken blood agar
A subset of CC5 poultry (12) and human (4) isolates were grown at mammalian (37°C) and poultry body temperatures (42°C) overnight. The poultry isolates reached a peak OD$_{600}$ of 1.8, while the human isolates grew to a similar OD$_{600}$ of 1.7 when grown at 37°C. However, when grown at 42°C the poultry isolates grew to a similar density (OD$_{600}$ of 1.8) but the human isolates did not (OD$_{600}$ of 1.4; Figure 5). Using an extra sum-of-squares F-test (p-value threshold of 0.05) the distribution of human isolates at both temperatures could be fitted to a single sigmoid regression curve. This null hypothesis was rejected when comparing poultry isolates, which showed significant difference when grown at avian and human body temperatures.

The ability to lyse chicken erythrocytes was tested on a subset of isolates. Most of the isolates from chicken (11/12) lysed chicken erythrocytes compared to only one (out of four) of the isolates from humans. Conversely, most of the human isolates (3/4), but only around half (7/12) of the poultry isolates were able to lyse erythrocytes on blood agar plates with human blood (Table 1). The differences observed between human and poultry isolates on chicken blood agar were statistically significant (p-value = 0.0048, unpaired t-test), but the results using human blood agar were not (p-value > 0.05). Several poultry-associated genes have been implicated in increased pathogenicity in chicken (Abdalrahman, et al. 2015; Lowder and Fitzgerald 2010), including scpB (Staphostatin A) which encodes a putative cysteine protease (Lowder, et al. 2009; Takeuchi, et al. 1999) and is located on the pAVX plasmid (Takeuchi, et al. 2002) previously identified in studies of poultry infection. All eight isolates containing this gene lysed chicken blood in Columbia Blood Agar (Cheung, et al. 2012). Half of the isolates (1/4 human and 3/4 poultry) not carrying the gene lysed chicken blood (Table 1). Only the differences observed on chicken blood agar were statistically significant (p-value = 0.0192, unpaired t-test).
The poultry ancestral lineage CC385 exhibits more genetic diversity than CC5

All of the 47 poultry-associated genes and alleles identified in CC5 were also found in at least one other clonal complex. Individual gene phylogenies were constructed for all 47 genes from all 197 poultry isolates, including isolates from CC1, CC5, CC385 and CC398 (Figure S2). The average branch length per isolate was estimated for each clonal complex (Table S3). CC385 is considered an avian-associated lineage, only isolated from wild bird species. Longer association with the host results in increased genetic diversity through mutation and genetic drift. We therefore tested if average branch length is shorter in CC5 isolates, which are believed to have colonised more recently. In total, the number of polymorphic sites per isolate was much lower for CC5 (867) than for isolates from CC385 (2012) and CC398 (2505). The total number of unique/shared alleles for the 47 poultry-associated genes was similar across all clonal complexes where there was more than one isolate in this study (Table S3).
DISCUSSION

Genealogical analysis of the isolates in CC5 identified a sub-lineage of poultry-associated isolates. This is consistent with a scenario where chicken colonisation by CC5 resulted from a host transition event from a human ancestral population (Lowder, et al. 2009). The majority of poultry disease isolates in this study belonged to CC5 and the widespread distribution of this clonal complex among poultry is consistent with the emergence of an important disease-causing lineage following the human-to-poultry host jump (Figure 1) (Bystron, et al. 2010; Hasman, et al. 2010; Lowder, et al. 2009).

Reconstruction of the clonal frame following removal of inferred recombination regions, further supports this hypothesis demonstrating complete segregation of the poultry and human CC5 isolates in this study and the emergence of a single lineage from an ancestral genetically diverse human CC5 population (Figure 2). This pattern of genetic bottle-necking and the emergence of epidemic clones from a founder population are well established in *S. aureus*, for example in the spread of globally disseminated healthcare-associated methicillin-resistant *S. aureus* (MRSA) clones (Hsu, et al. 2015). While the impact of clinical practice and antibiotic usage have been well studied in relation to the evolution of nosocomial pathogen populations (Enright, et al. 2000; Gill, et al. 2005; Hiramatsu, et al. 2001; Mwangi, et al. 2007; Wertheim, et al. 2005), less is known about the emergence of disease-associated staphylococci in agriculture. Rapid dissemination of clones is likely facilitated by global food production networks, but to identify the specific genomic changes related to the emergence of pathogenic lineages, it is instructive to use a comparative functional genomics approach.
Adaptation is mediated by recombination in *S. aureus* and our estimate of the rate at which recombination is introducing nucleotide changes, relative to mutation in CC5 (*r/m*=1.08), is consistent with previous estimates based on core genomes in ST-239 (*r/m*=1.13 Castillo-Ramirez, et al. 2012) and several lineages (STs 38, 36, 22 and 12) by MLST (*r/m*=1.1 by Feil, et al. 2003). Different recombination rates have been observed between *S. aureus* lineages with admixture occurring between some, but not all clonal complexes (Castillo-Ramirez, et al. 2012; Meric, et al. 2015). There is also variation in recombination estimates within CC5 human isolates (Duchêne, et al. 2016; Everitt, et al. 2014; Meric, et al. 2015). In this study the amount of realised recombination in CC5 poultry isolates is almost double that inferred in isolates from humans. This is consistent with a scenario of adaptation mediated by recombination following colonisation of a new niche.

Characterisation of the genetic changes associated with the divergence of CC5 poultry isolates from human CC5 isolates identified genomic variations that may have been involved in adaptation to poultry. Broadly, these can be considered as differences in the core and accessory genome compared to CC5 isolates from humans. Whole genome comparison of CC5 isolates identified 47 genes found in >95% of poultry isolates but absent from human isolate genomes (Table S2). The predicted function of these genes included staphylococcal virulence factors, such as toxin production, adhesion, stress response, plasmid maintenance and antibiotic resistance, as well as mobile genetic elements. For accessory genome characterisation it was necessary to compare CC5 genomes with the chicken *S. aureus* reference strain ED98. While this is less well annotated than some human reference strains, comparison allowed genes with orthologues of known function to be associated with the CC5 poultry accessory genome. These included: the *Hlb* toxin gene which contributes to tissue damage and can influence disease severity (Bramley, et al. 1989; Guinane, et al. 2008); the gene encoding fibronectin-binding
protein $FnbB$, which facilitates colonisation and attachment (Foster and Hook 1998; Peacock, et al. 2002), and mobile genetic elements involved in DNA transfer (Everitt, et al. 2014; Malachowa and DeLeo 2010). Several other accessory genes ($SAAV\_0806$, $SAAV\_0807$, $SAAV\_0808$, and $SAAV\_0809$) have been identified as components of $S.\ aureus$ pathogenicity islands (SaPI) (Viana, et al. 2010). In addition to variation in accessory genes, recombination in homologous sequence can be associated with host adaptation (Didelot and Falush 2007; Sheppard, et al. 2013). Inferred recombination regions ($n=44$) in the poultry-associated CC5 isolates were mapped to 33 genes in the reference pan-genome. A total of 9% (3/33) mapped to plasmid genes, with the remaining recombinant sequences mapping to 30 chromosomal genes. The function of these genes varied including those associated with hypothetical proteins, transposition and DNA regulation.

The absence, or relative scarcity, of the 80 poultry-associated accessory genes ($n=47$) and genes containing poultry-associated recombination regions ($n=33$) in the ancestral human CC5 population suggests that acquisition of these elements was associated with chicken colonisation. However, this alone is not confirmation of adaptation. Evidence for significance of these genes in poultry adaptation is supported by their differential presence in poultry and human isolates from other clonal complexes. The majority of CC5 poultry-associated genes / alleles were more commonly present in poultry isolates from CC398 and CC1 with only 15% being more common among isolates from humans (Table S2). All of the CC5 poultry-associated accessory genes, and 96% of associated homologous recombination segments, were present in the analysed CC385 isolates that have only been isolated from birds. This is consistent with an evolutionary scenario where horizontal gene transfer (HGT) occurred, introducing genetic material that has evolved in a different genetic background to $S.\ aureus$ strains colonising chickens. CC385 has not been previously associated with humans or mammals but has been isolated from various
wild and reared birds suggesting that the CC385 lineage has had long-term avian host restriction (Lowder, et al. 2009). Consistent with this, most of the genetic variation in poultry-associated genes and alleles, identified in CC5, is present within isolate genomes from other complexes (Table 2). Furthermore, in these genes there were more than twice as many polymorphisms per isolate in CC385 compared to CC5 (Table S3). It is possible that each CC could have acquired the genes from a human-associated lineage and then transferred to each CC at different times. However, the absence of CC structuring on individual genes phylogenies (Figure S2), where avian alleles cluster together, implies that poultry isolates from different CCs have acquired the genes necessary for adaption to the poultry niche from pre-existing avian-associated lineages.

Some evidence of the functional significance of poultry-associated genes was investigated in laboratory phenotyping assays. Three genes located in the three poultry-associated CC5 hotspots had putative functions related to chicken colonisation (beta-haemolysin, SAAV2007 and SAAV_C21). Among the most obvious environmental challenges for S. aureus colonising chickens is the higher host body temperature of 42°C in chickens compared to 37°C in mammals. Two poultry-associated genes in CC5 isolates (SAAV0062 and SAAV0064) had >85% nucleotide identity to genes involved in temperature-dependent growth, including dnaK and dnaE which have been shown to be important for growth at 42°C in S. aureus poultry strains (Inoue, et al. 2001; Singh, et al. 2007). In laboratory assays chicken CC5 isolates containing these thermo-tolerance genes demonstrated enhanced growth at avian body temperature compared to growth at 37°C (Figure 5).

A number of the chicken associated genes potentially had a role in pathogenicity including beta-haemolysin, SAAV2007 and SAAV_C21 putatively involved in haemolysis contributing to S. aureus pathogenicity (Kuroda, et al. 2007). In laboratory assays, CC5 isolates of human origin
showed very little haemolytic activity when grown on agar plates containing chicken blood. However, eleven of twelve poultry strains lysed chicken blood in agar under the same conditions (Table 1). The pAVX plasmid has previously been implicated in lysis of avian erythrocytes and contains a putative thiol protease, scpA (SAAV_C10), which - when expressed - contributes to S. aureus virulence (Bonar, et al. 2016). Other protease genes are also likely to contribute to lysis of erythrocytes as S. aureus has several orthologous haemolytic genes (Jusko, et al. 2014).

Sustainable food production in intensive agricultural systems is threatened by the spread of zoonotic pathogens. The recent host transition of CC5 S. aureus from humans to poultry has resulted in the emergence of a major pathogen that causes various diseases in chickens in agricultural systems. Comparative genomics of commensal and pathogenic staphylococci offers considerable opportunities to improve understanding of the epidemiology and evolution of these organisms (Yahara, et al. 2016). Here, by identifying the evolutionary events associated with chicken colonisation, we provide evidence for the role of lateral gene transfer and homologous recombination in the emergence of CC5 as a major poultry pathogen. Many of the genes involved have putative functions that could be related to adaptation to chicken, but all were present in other S. aureus clonal complexes isolated from chickens. This is consistent with adaptation though HGT within the resident poultry staphylococcal community, potentially leading to convergent evolution. The importance of HGT in pathogen emergence is well documented in staphylococci, for example in the emergence of MRSA in hospitals. While it may be difficult or impossible to prevent adaptation through HGT in recombining bacteria, characterising the genes associated with adaptation can provide important information about the genetic basis of pathogen emergence. A clearer understanding of this may provide
opportunities for improving agricultural practices and targeting interventions to reduce animal disease.
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REFERENCES


1471-2105-11-595 [pii]


PONE-D-13-53614 [pii]


genes3020261 [pii]


PONE-D-14-06502 [pii]


DATA ACCESSIBILITY

Accession numbers can be found in Table S1 for all genomes used in this study. Short reads of all isolates sequenced have been uploaded to the short read archive, which can be accessed via the BioProject PRJNA312437 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA312437). Additional genomes sequenced at the Edinburgh Genomics facility at the Roslin Institute, Edinburgh are available on the European Nucleotide Archive via project accession PRJEB18782 (http://www.ebi.ac.uk/ena/data/view/PRJEB18782). Assembled genomes can also be accessed via the resource section of our group website (https://sheppardlab.com/resources/) in the public Sheppard Staphylococcal BIGS database server.

Supplementary figures and tables have been deposited on figshare:

https://figshare.com/articles/Recombination-mediated_host-adaptation_by_avian_Staphylococcus_aureus/3863736

FIGURES AND TABLES

**Figure 1:** Samples were collected from infected breeder chickens across the UK (n=161), USA (n=25) and the Netherlands (n=5). Grouped bar charts show the relative proportion of isolates belonging to CC5 by country (1a), year of collection (1b) and disease site (1c). MLST clonal complexes were assigned based on shared sequence at 5 or more MLST house-keeping loci.

**Figure 2:** Genetic relatedness of *S. aureus isolates* from different hosts. (a) Host origin of all *S. aureus* isolates from chicken (blue), human (red), and other species (yellow). Clonal complex (CC) designations are based on shared MLST housekeeping loci. Chicken isolates were found
in five sequence clusters, corresponding to CCs 1, 5, 30, 358 and 398 which are highlighted. The tree was constructed from a core genome alignment (2,789,909 bp) of 1,700 genes using an approximation of the maximum likelihood algorithm. (b) Reconstruction of the clonal frame with putative recombination regions removed of CC5, including 20 chicken (blue) and 26 human (red) isolates (2,302,773 bp alignment in ClonalFrame).

**Figure 3:** Genes and recombination regions identified as poultry-associated in ClonalFrame analysis mapped to the ED98 reference genome and three plasmids (pAVX, pAVY and pT181). The frequency of these genes (red circles) and recombination regions (black crosses) in chicken and human isolate genomes is shown for CC5, CC398, CC1 and CC385 (chicken only). The relative abundance of these genes / recombination regions was calculated as presence in chicken minus presence in human isolates. 0 score denotes equal presence in poultry and human isolates. The majority of poultry-association in the core and accessory genome is co-localized in three chromosomal regions, which are labelled.

**Figure 4:** Genome positions of co-localised poultry-associated genes and recombination regions. (a) Poultry-associated genes (blue) and recombination regions (red) mapped to the ED98 reference chromosome. Hot spots of co-localisation of poultry-associated elements are numbered. (b) Schematic diagrams of each hot spot showing gene content, including poultry-associated genes and genes containing recombination regions. Genes with no poultry-association in the same regions are also shown (grey). Poultry-associated genes and genes containing recombination regions are labelled, including *S. aureus* pathogenicity island genes (1), transposon-related genes (2), hypothetical proteins (3) and phage-related genes (4). More details of putative gene function can be found in supplementary table S2.
**Figure 5:** Growth of *S. aureus* isolates from poultry (red) and human (black) in TSB medium. Curves represent growth levels in vitro (OD$_{600}$) over a period of 20 h at 37°C (dashed lines) and 42°C (solid lines) in medium. Mean growth levels and standard deviation (dotted lines) was calculated for 12 poultry and 4 human clinical samples.

**Table 1:** Lysis of chicken and human erythrocytes on Columbia Blood Agar (Cheung, et al. 2012) plates with *S. aureus* isolates from humans (n=4) and poultry (n=12). A positive result was recorded if a halo of lysis was observed around the colony after 24 h, experiments were performed in triplicate.

**Table 2:** Prevalence of chicken-associated genes and recombination regions as defined by Clonal Frame analysis of CC5 isolates.

**Supplementary material**

**Figure S1:** Genetic relatedness of *S. aureus* isolates from different hosts alongside visualisation of recombination events identified by BRAT NG analysis.

**Figure S2:** Individual neighbour joining phylogenetic trees of all 47 chicken-associated accessory genes and 33 genes that contain recombination regions. Isolates from CC5, CC1, CC398 and CC385 are coloured blue, turquoise, red and pink, respectively.

**Table S1:** List of isolates used in this study.

**Table S2:** List of all chicken-associated genes (47) and genes containing recombination regions (33). Function of the genes was predicted using RAST and NCBI BLAST.

**Table S3:** The number of polymorphic sites found in each chicken-associated allele from CC5, 398, 1 and 385.
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*Genes and recombination regions found to be associated with CC5 poultry isolates.*