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Systematic surveillance detects multiple silent introductions and household transmission of methicillin-resistant *Staphylococcus aureus* USA300 in the east of England

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ABSTRACT

Background: The spread of USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) across the United States (US) resulted in an epidemic of infections. In Europe, only sporadic cases or small clusters of USA300 infections are described and its prevalence in England is unknown. We conducted prospective surveillance for USA300 in the east of England.

Methods: We undertook a 12-month prospective observational cohort study of all individuals with MRSA isolated from community and hospital samples submitted to a microbiology laboratory. At least one MRSA isolate from each individual was whole-genome sequenced. USA300 was identified based on sequence analysis, and phylogenetic comparisons were made between these and USA300 genomes from the US.

Results: Between April 2012-April 2013, we sequenced 2,283 MRSA isolates (carriage screens and clinical samples) from 1,465 individuals. USA300 was isolated from 24 (1.6%) cases. Ten cases (42%) had skin and soft tissue infection and two cases had invasive disease. Phylogenetic analyses identified multiple introductions and household transmission of USA300.

Conclusions: Use of a diagnostic laboratory as a sentinel for surveillance has identified repeated introductions of USA300 into the east of England in 2012-2013, with evidence for limited transmission. Our results show how systematic surveillance could provide an early-warning of strain emergence and dissemination.

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Identification of the community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) pulsotype USA300 in the United States (US) in 1999 was followed by widespread dissemination and an epidemic of MRSA infection in otherwise healthy people.[1, 2] By 2004, this lineage was responsible for up to 97% of skin and soft tissue infection (SSTI) presenting to US emergency departments (ED).[3] USA300 also causes invasive disease, such as pneumonia and osteomyelitis, and has become endemic in US hospitals where it causes hospital-associated infections including bacteraemia.[4, 5] USA300 is readily transmitted within households, which act as long-term reservoirs associated with repeated episodes of infection and onward transmission.[6, 7] Antimicrobial resistance to macrolides and fluoroquinolones is common.[3] An epidemic of CA-MRSA infection caused by a clone that is closely related to USA300 but which arose independently (USA300 Latin-American variant, USA300-LV) has also been identified in South America.[8]

International travel is an important contributor to the inter-continental spread of infectious diseases,[9] and the spread of USA300 and USA300-LV have been documented globally.[1, 10] In the United Kingdom (UK), the vast majority of MRSA infections continue to be caused by the dominant hospital-associated lineage EMRSA-15 (ST22).[11] USA300 is considered of low prevalence in continental Europe, presenting primarily as sporadic cases and discrete, small outbreaks.[12-18] Similarly, sporadic cases and a single hospital outbreak caused by USA300 have been described in the UK,[19-22] but the prevalence of USA300 carriage and infection is unknown. In the absence of systematic molecular surveillance of high-risk lineages, we hypothesised that USA300 would only become apparent in England if a high prevalence of SSTI presenting to emergency departments (ED) triggered concern. Here, we report the findings of

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systematic surveillance for USA300 in a major diagnostic microbiology laboratory serving part of the East of England.

METHODS

Study design

A prospective study was conducted over 12 months between April 2012 and April 2013 at the Public Health England Clinical Microbiology and Public Health Laboratory, Cambridge University Hospitals NHS Foundation Trust in Cambridge, UK. This laboratory processes samples submitted by four Cambridgeshire hospitals (Addenbrooke's hospital, the Rosie hospital, Papworth hospital and Hinchingbrooke hospital) and providers of community healthcare in the same geographic region. We identified all cases with MRSA isolated at least once from screening swabs and/or clinical specimens, and collected demographic and clinical data on MRSA-positive cases using electronic and paper medical records. Throughout the study period, universal MRSA screening was performed at the four hospitals, in accordance with national policy (multi-site MRSA screen of all patients on hospital admission, and weekly MRSA screens of patients in critical care units). The study protocol was approved by the National Research Ethics Service (ref: 11/EE/0499), the National Information Governance Board Ethics and Confidentiality Committee (ref: ECC 8-05(h)/2011), and the Cambridge University Hospitals NHS Foundation Trust Research and Development Department (ref: A092428).

Microbiology, DNA sequencing and analysis

MRSA was isolated from screening samples by plating swabs onto Brilliance MRSA chromogenic medium (Oxoid, Basingstoke, UK) and from all other samples by plating onto Columbia Blood Agar (Oxoid, Basingstoke, UK). *S. aureus* was identified using a commercial latex agglutination kit

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(Pastorex Staph Plus, Bio Rad Laboratories, Hemel Hempstead, UK). Antimicrobial susceptibility was determined to commonly used antibiotic agents (Fig. 1) using the VITEK 2 instrument (bioMerieux, Marcy l'Etoile, France). Inducible clindamycin resistance was detected using the D-test disk diffusion method. Antimicrobial susceptibility results were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria.

DNA was extracted, libraries prepared, and 150-bp paired end sequences determined on an Illumina HiSeq2000 as previously described.[23] MLST sequence types (STs) were identified from the sequence data using an in-house script and the MLST database (http://saureus.mlst.net/), and assigned to clonal complexes (CC). CC8 isolates were identified, and formed the basis for the remaining genetic analyses. CC8 were mapped using SMALT to the *S. aureus* USA300 genome FPR3757 (Genbank accession number CP000255.1). Mobile genetic elements, indels and regions of high-density single nucleotide polymorphisms (SNPs) were excluded. SNPs in this core genome were used to create maximum likelihood phylogenies using RAxML with 100 bootstraps.[24] Genome sequence data for 348 MRSA isolates reported previously by Uhlemann *et al* were sourced from the European Nucleotide Archive, ENA. Trees were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and iTOL.[25] Sequence data for the USA300 isolates from the East of England have been submitted to the ENA under the accession numbers listed in Table S1. *In-silico* PCR was used to determine the staphylococcal cassette chromosome *mec* (*SCCmec*) subtype.[26] To detect the presence of Panton-Valentine leukocidin (PVL) genes (*lukF-PV* and *lukS-PV*), the arginine catabolic mobile element (ACME), and the staphylococcal pathogenicity island 5 (SaPI5) corresponding sequences were extracted from the mapping alignment. Mutations in the *cap5* locus were retrieved from the assemblies by comparison to the *cap5* locus of the CP5+ reference strain Newman and USA300 reference strains TCH1516

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and FPR3757 as described.[27] The presence or absence of acquired genes and SNPs conferring resistance against quinolones (*grlA*, *grlB*, *gyrA*, *gyrB* genes) were determined as described previously.[23] For SNPs causing resistance in chromosomal genes the standard mapping and SNP calling approach was used as described earlier.

RESULTS

Description of cases positive for USA300

We identified 1,465 individuals over a 12-month period, with at least one sample submitted to the microbiology laboratory at CUH that was culture-positive for MRSA, and sequenced 2,283 isolates cultured from multisite screens or clinical samples from these cases. CC8 isolates were identified, and further evaluated to identify USA300 (SCC*mec* type IVa, presence of PVL genes, and phylogenetic clustering with USA300 reference genome FPR3757 (Fig. 1A)). This identified 56 USA300 isolates from 24 cases (Fig. 1B), giving a case prevalence among MRSA carriers of 1.6% (24/1489). The majority of USA300-positive cases were young (median 32 years, range 3 to 84 years, interquartile range 25 to 57 years), and males predominated (16/24, 67%). Over half of first positive samples were submitted from first-opinion services (ED (8/24, 33%) and general practices (5/24, 21%) rather than from hospital in-patient stays. Eleven cases had MRSA USA300 identified from carriage screens alone, 11 cases had samples taken from SSTI with or without associated carriage, and two cases had invasive infection with bloodstream infection and osteomyelitis, respectively (Fig. 1B). Mapping the residential address of the 24 cases demonstrated that 23 cases were scattered across the East of England (Fig. S1), while the remaining case was a US resident. Dates of collection of the positive samples were distributed throughout the 12-month study period, with median time between the first and last sequenced

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isolate 21 days (range 0 to 332 days) (Table S1). The finding that MRSA USA300 positive cases were distributed in time and space is suggestive of numerous independent introduction events.

Genomic investigation

The phylogenetic tree revealed three groups of closely related isolates containing isolates from more than one individual (termed pairs/clusters A, B and C, Fig. 1). Pair A contained one isolate each from two cases (person (P) P11 and P12) that differed by 6 SNPs. Samples were submitted three months apart from a GP (ear swab) and a hospital (multi-site MRSA screen) and cases shared the same registered address, suggesting household transmission. Pair B contained six isolates from two cases (P17 and P18), which differed by a median of 59 SNPs (range 57 to 60), but from other isolates by a minimum of 81 SNPs. These were isolated from samples collected within two weeks of each other and submitted by a general practitioner (multi-site screen) and hospital (blood culture), respectively. The two cases shared a surname but not address, and given the larger number of SNPs, potentially suggests spread between family contacts rather than household transmission. However, this must be interpreted with caution. Cluster C contained 17 isolates from three cases (P22, P23 and P24) with a median difference of six SNPs (range 0 to 9) between isolates from different individuals. P22 and P24 had the same registered residential address and had a total of 16 MRSA isolates sequenced, the most closely related of which were genetically identical. The single isolate from P23 was also highly related (the closest genetic distance to isolates from P22 and P24 was 1 and 7 SNPs apart, respectively), but no direct or indirect epidemiological link between P22/P24 and P23 could be identified. Overall, we found a maximum genetic distance of 6, 59 and 9 SNPs among epidemiologically linked cases. None of the cases had a recent history of hospitalisation in the same ward simultaneously (Fig. S2). The 56 USA300 genomes from this study were combined with the genomes of 348 MRSA

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USA300 isolates from New York that have been reported previously,[6] to provide genetic context to the UK isolates. A tree containing all isolates showed that the UK study genomes were interspersed throughout the tree (Fig. 2), suggesting repeated introductions of USA300 and ruling out a single importation and subsequent propagation of a single clone. This picture of multiple introductions is similar to recent USA300 studies from France and Switzerland.[18, 28]

Variability in the USA300 genome

We investigated the presence of mobile genetic elements proposed previously to be associated with USA300 fitness and epidemic spread (Fig. 1). Enterotoxins K and Q are thought to enhance pathogenesis through T-cell stimulation and are encoded by genes *sek*2 and *seq*2 within a pathogenicity island, SAPI5.[29, 30] SAPI5 was present in 25 of the 56 isolates. The ACME locus is a genomic island associated with USA300 that is composed of at least 33 putative genes and two operons.[31] The *arc* operon encodes genes involved in arginine catabolism, which are important for survival of USA300 in acidic environments.[32] The ACME *speG* gene, which encodes a spermidine acetyltransferase, confers the ability to survive levels of the polyamines spermidine and spermine that are lethal for other strains of *S. aureus*.[33] As described previously,[6] ACME was variably present in the USA300 isolates. Eight isolates from three cases were missing this gene cassette. The dispersed position of ACME-negative isolates in the phylogeny suggested multiple losses of the pre-existing island. ACME-negative isolates did not carry the copper and mercury resistance element (COMER), presence of which would be characteristic of South American strains of USA300-LV.[8] Boyle-Vavra *et al* recently reported that USA300 failed to produce capsular polysaccharide, which was associated with the presence of four conserved mutations associated with the *cap5* locus when compared with strain Newman.[27] We confirmed that these four mutations were present in all of our 56 USA300

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isolates. These *cap5* mutations together with SCC*mec* IVa and PVL therefore form a consistent marker of USA300 in our collection, whereas ACME and SAPI5 are variably present.

Antimicrobial resistance

The oral antibiotics used to treat MRSA SSTI in the UK and US in single or combination regimens are clindamycin, doxycycline and trimethoprim-sulphamethoxazole (TMP-SMX), rifampicin, trimethoprim and fusidic acid.[34-36] All 56 isolates were phenotypically susceptible to trimethoprim and clindamycin (constitutive), and only one isolate tested resistant to tetracycline. Of the 51/56 erythromycin resistant isolates, none tested positive for inducible clindamycin resistance. More than half of isolates (36/56) were phenotypically resistant to ciprofloxacin, and contained known mutations in both *grlA* and *gyrA* (*gyrA* 84L and *grlA* 80Y (n=33), and *grl*A 80F alone (n=3)). Previous studies have reported that USA300 isolates from the US segregated into two clades based on fluoroquinolone resistance genotypes (with or without *gyrA* 84L/A and/or *grlA* 80Y/F mutations).[6, 7] When the study isolates were considered in the context of the US isolates (Fig. 2), this was replicated for isolates from 22 of the cases, the two exceptions being isolates from P07 and P13. P07 carried the 84L/80Y mutations and tested phenotypically resistant but resided within the 'susceptible' clade. P13 resided at the base of the resistant clade and tested phenotypically resistant but with an 80F mutation within *grl*A.

DISCUSSION

This study represents, to our knowledge, the first prospective surveillance study for USA300 in the UK. It provides clear evidence of how systematic whole genome sequencing (WGS) might assist in monitoring the distribution of a potentially high-risk clone. Using WGS, we detected multiple introductions of USA300 into the East of England. In terms of epidemiological

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characteristics, most cases were young, and presented with SSTI to first-opinion services. This is reminiscent of the early disease epidemiology of USA300 in the USA, where this was largely associated with SSTI in the community before becoming introduced and established through US hospitals.[37] Genomic studies have revealed repeated introductions of USA300 into American cities. The source of the UK isolates is unknown, but it is likely that international travel has played an important role; one USA300 positive individual was normally resident in the USA. Intercontinental transmission is supported by the similar rates of resistance to oral antibiotics that are commonly used for SSTI caused by USA300, together with variability in the genome content reflecting the circulating strains in the US. We identified two likely independent acquisitions of ciprofloxacin resistance in addition to those strains which represented the ciprofloxacin resistant clade which is seen in the US (Fig. 1).[6, 7] We found no evidence for the introduction of USA300-LV from South America.

USA300 is known to have spread readily through communities in the USA, but we found limited evidence for such transmission in our study. We identified one two-member pair and one cluster of genetically closely related USA300. We found epidemiological links between all but one individual within these groups. Household transmission within pair A and cluster C is supported by the findings of previous US studies in which a median of three (range 0-772), six (range 0 to 199) and five (range 0 to 102) SNPs were identified per household in New York, Los Angeles and Chicago, respectively.[6, 7, 38]

Our prospective, systematic surveillance study found a prevalence rate of USA300 of 1.6%, which is more than three times higher than the prevalence of MRSA isolates positive for *mecC,* a *mecA* homolog associated with livestock.[39] Serial systematic prevalence studies for USA300

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are lacking across Europe, but single hospital studies from Switzerland have shown multiple importations and increasing rates,[28] with one study showing an increase in isolation rates from 0% to 9.2% between 2002 and 2012.[12] Like continental European studies, UK studies are sparse and strongly limited by methodology.[10] The potential for under-ascertainment within referral laboratory based infection surveillance studies is apparent when comparing ours to another UK report; molecular testing in a reference laboratory study identified 40 likely USA300 isolates (CC8 SCC*mec* IVa, *spa* t008, *agr* group 1, PVL positive) from 300 CA-MRSA across England and Wales over a two-year period (2004-05),[40] yet we have identified 60% of this total in one rather than two years and from a single region of England.

The reasons underlying the rapid and widespread dissemination of USA300 in the USA remain unknown, despite hypothesised roles for genetic elements such as ACME and more recently, the *copB* locus in raising fitness.[8, 32, 33] Phylogenetic analysis has indicated a process of repeated introduction throughout the Americas prior to the epidemic.[6, 7] Our data builds upon other European studies that suggest a similar pattern in Europe, but without systematic surveillance it is difficult to define the trajectory of USA300 in the UK. In contrast to when USA300 rapidly disseminated in the USA over 10 years ago, it is now feasible to implement comprehensive, genomic surveillance strategies to monitor lineage distribution and guide intervention. Had this been possible during the initial stages of the USA epidemic, alternative strategies such as an aggressive targeted search and destroy policy,[41] may have been implemented. Currently in Europe the majority of countries adopt a reactive rather than active management strategy.

The current methods for surveillance of invasive staphylococcal infections in the UK do not allow for the monitoring of USA300. Antimicrobial susceptibility profiles have been used as a

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surrogate marker of community lineages on the basis of greater susceptibility overall compared with previous hospital-adapted lineages. This is prone to increasing inaccuracy over time since lineages may accumulate drug resistance. For example, in the past, ciprofloxacin susceptibility was used as a marker of UK community lineages,[42] but a substantial amount of resistance to this drug is seen within USA300 (Fig. 2). The molecular identification of USA300 remains challenging since putative gene markers such as the ACME element may be lost, and the four CAP5 mutations recently described are also seen in the USA300 progenitor, USA500.[27] The use of whole-genome sequencing for the genetic characterisation of USA300 overcomes these barriers. The national Staphylococcus Reference Unit at Public Health England provides a reference service for microbiological characterisation of invasive *S. aureus* isolates, which includes *spa*-typing and toxin gene detection. However, isolate submission is currently voluntary, highly selective, and in practice tends to only be used to test isolates associated with life-threatening infection.[43] Review of the local laboratory records showed that only three of the 56 USA300 isolates were documented as having been submitted to the national reference laboratory and subsequently identified as consistent with potential USA300 strains.

We acknowledge a number of limitations in our study. Without undertaking prevalence studies of all residents within a defined geographical area, studies are biased towards the healthcare-seeking population. We tried to reduce this by including all disease and carriage isolates over a year-long period. Secondly, there is likely to be UK-wide geographic variation in USA300 distribution. Thirdly, ethical constraints mean that epidemiological links between patients have been deduced using electronic medical records, which do not capture all epidemiological links. Despite these limitations, our study has identified the covert presence of USA300 in the East of England. More broadly, it shows that systematic WGS within a sentinel centre could function as

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an effective surveillance mechanism to monitor MRSA lineages. If undertaken systematically, WGS-based sentinel surveillance within a co-ordinated network could be used to provide an early-warning of strain emergence and dissemination; such epidemic intelligence would allow appropriate targeting of resources towards interventions to limit further spread.

Conflict of Interest:

The authors declare no conflicts of interest.

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Authors' contributions:

BB conducted the laboratory work. MST undertook the analysis and wrote the first draft of the manuscript. SR provided guidance throughout this process. EH, FC, SJP and SR assisted in further drafting the manuscript. MET and SJP designed the study, and obtained ethical and R&D approvals for the study. NB provided clinical laboratory support. MET, JP and SJP supervised the study. All authors had access to the data and read, contributed and approved the final manuscript.

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Figure legends

Figure 1A: Phylogenetic, midpoint-rooted tree of study CC8 isolates, with USA300 isolates highlighted. 56 isolates residing in the sub-clade within the grey box were phylogenetically identified as USA300 isolates. Figure 1B: Detailed USA300 phylogenetic tree rooted on the isolate from P01 with summary of metadata for each isolate. Person (P) numbers represent the study ID of each individual from which the sample was from, with grey boxes indicating pairs or clusters with presumptive epidemiological links.

± Red: Resistant; yellow: intermediate; blue: susceptible.

* Ciprofloxacin: Red: S80Y, S84L; blue: S80F only; white: nil identified; grey: not done.

SCC*mec* IVa, PVL, ACME, SAPI5: Red: present; black: absent.

^ Red: 4 mutations associated with CAP5 locus identified.

Figure 2: Comparison of the first USA300 isolate from each study case (n=24, circles) relative to previously published USA300 isolates from the US (n=348).[6] Mid-point rooted maximum likelihood tree based on SNPs in the core genome of MRSA with branch colours representing fluoroquinolone genotypes. Red branches: S80F/Y and S84A/L; yellow: S80F only; blue branches: nil; black branch: reference genome FPR3757. Letters alongside circles indicate epidemiologically linked pairs or clusters.

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Figure 1A

~ 10 SNPs

Figure 1B

~ 1 SNP

A

B

C

P01
P02
P02
P03
P03
P04
P04
P05
P06
P07
P07
P07
P08
P08
P09
P09
P09
P09
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P11
P12
P13
P13
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P14
P14
P15
REF
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Clinical (red) vs. screen

Fusidic Acid
Mupirocin
Rifampicin
Erythromycin
Ciprofloxacin

Phenotype±

Ciprofloxacin

Genotype*

SCCmecIVa
PVL
ACME
SAPI5
CAP5 mutations^

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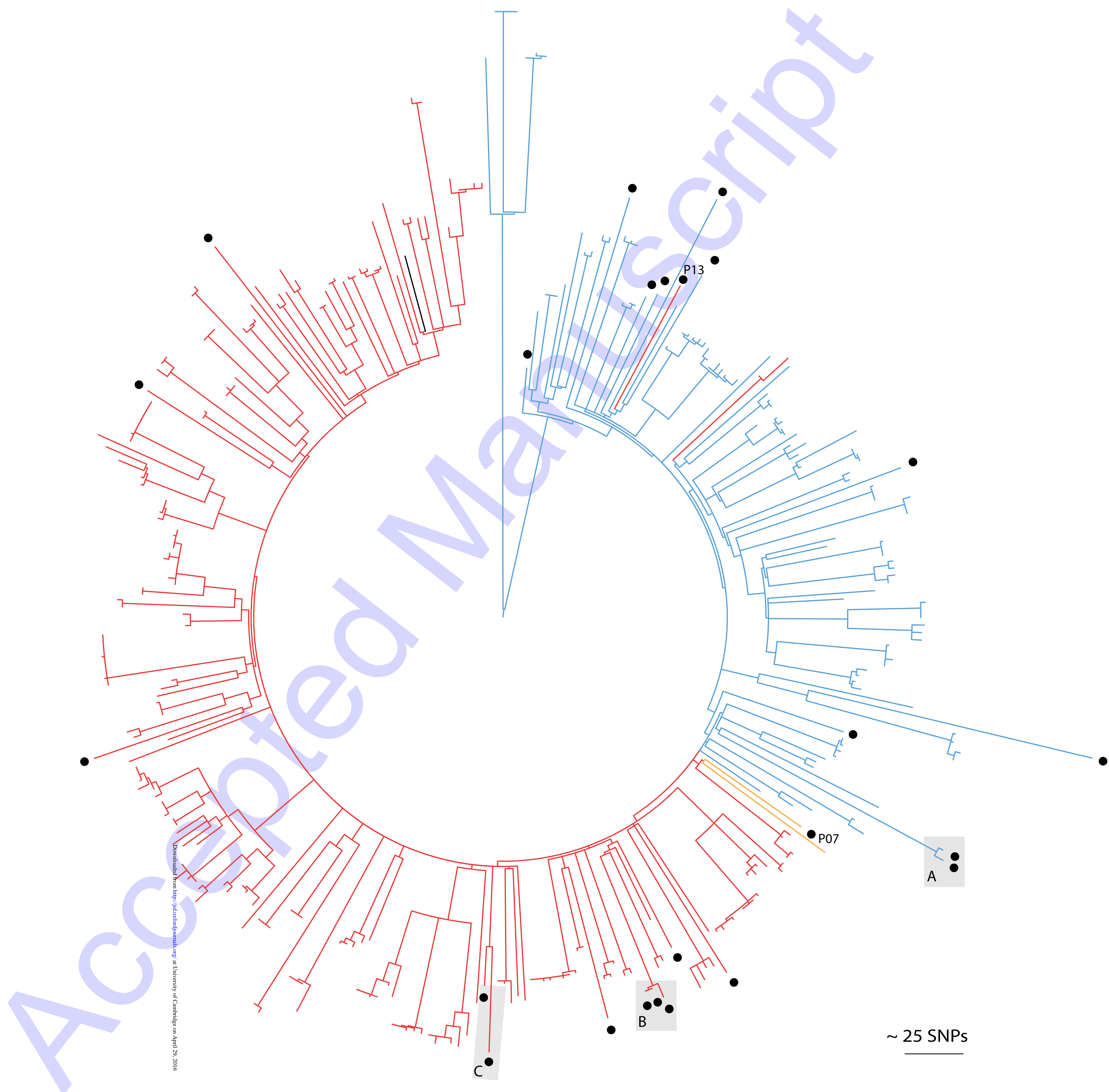

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