Whole-genome sequencing investigation of animal-skin-drum-associated UK anthrax cases reveals evidence of mixed populations and relatedness to a US case

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There have been two anthrax cases affecting people that played and/or made animal-skin drums in the UK during the last 10 years, with single fatal occurrences in Scotland in 2006 and London in 2008. Investigations by the Health Protection Agency (now Public Health England) employing multi-locus-variable number tandem repeat analysis had previously linked the clinical cases to spores associated with animal skins and drums the patients had been in contact with. In this study, whole-genome sequencing of 23 Bacillus anthracis isolates harvested during the investigations was performed. High-quality draft assemblies of these genomes provided greater characterization of the B. anthracis strains present and placed them all upon a new branch of the global phylogeny. Although closely related, the clinical isolates from the two events, and another isolated from a drum-skin-associated case in New York in 2006, were distinct from each other. Multiple distinct genotypes were found during both investigations, implying either multiple contamination events or a single heterogeneous contamination. One environmental isolate from the Scottish incident was more closely related to London isolates than to the other Scottish isolates. As B. anthracis of this subgroup was present at both geographically and temporally distinct events, it may be more widespread at the source of contamination. All isolates were distinct from currently characterized West African strains, despite this being the likely origin of the drums and hides, therefore adding to our knowledge of B. anthracis diversity in the region.

Keywords: animal-hide; anthrax; phylogeography; UK.

Abbreviations: canSNP, canonical SNP; MLVA, multi-locus variable number tandem repeat analysis; WGS, whole-genome sequencing.

Data statement: Table S4 has been deposited in Figshare.

All supporting data, code and protocols have been provided within the article or through supplementary data files.

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Introduction

Human cases of anthrax, caused by the Gram-positive endospore-forming bacterium *Bacillus anthracis*, have historically often been associated with the industrial processing of animal hides (CDC, 2006; Osborn, 1920). However, there have been a number of anthrax cases in the USA and UK associated with the recreational use of drums that were skinned using hides imported from West Africa. In the USA, anthrax cases occurred in New York in 2006 (CDC, 2006; Nguyen et al., 2010), Connecticut in 2007 (Guh et al., 2010) and New Hampshire in 2009 (CDC, 2010). Four individuals were infected: two were drum makers, one a relative of the drum maker and one simply played drums. Genotyping (Marston et al., 2011) determined that all isolates belonged to the A.Br.011/009 canonical SNP (canSNP) lineage (Van Ert et al., 2007). In the UK, there have been two cases of inhalational anthrax associated with West African drum skins. In Scotland, in 2006, a 50-year-old man died from a non-specific flu-like illness that was retrospectively diagnosed as anthrax (Riley, 2007). In London, in 2008, a 34-year-old man also suffered a fatal case of anthrax (Anaraki et al., 2008). In both cases the victims were known to have interests in the playing and making of Djembe drums, and had access to hides and drums thought to have been imported from West Africa [Guinea in the 2006 case (Riley, 2007); multiple locations, including The Gambia in the 2008 case (Anaraki et al., 2008)]. Contemporaneous investigations isolated *B. anthracis* from drums, hides and storage areas that matched the genotype of the clinical isolates, as determined by multi-locus variable number tandem repeat analysis (MLVA-8) (Keim et al., 2000; Public Health England, internal communications). In neither case was an individual drum or skin identified as the source, as *B. anthracis* was harvested from multiple items. In addition, in each case an environmental isolate was found with an alternative MLVA-8 profile to that of the clinical isolates, demonstrating that a diverse genotypic population of spores was present at each site. The aim of this study was to employ whole-genome sequencing (WGS), which has revolutionized the study of disease outbreaks in the intervening years since the events described for a number of pathogens (Eyre et al., 2013; Gardy et al., 2011; Köser et al., 2012) including *B. anthracis* (Derzelle et al., 2015; Price et al., 2012), to further characterize all isolates collected from both UK events, to place them in the global anthrax phylogeography and to better estimate the level of population variation present.

Methods

*B. anthracis* isolates. Isolates were recultured from the Public Health England anthrax strain collection glycerol stocks. A 10 μl loop of glycerol suspension was streaked onto Columbia agar plus 5% horse blood (bioMérieux) and incubated aerobically for 16–24 h at 37 °C.

Impact Statement

This paper describes whole-genome sequencing (WGS) of 23 *Bacillus anthracis* isolates collected during public health investigations into two fatal anthrax cases involving animal-skin-drum enthusiasts in 2006 and 2008 in the UK. The data confirm, at a level of resolution far greater than previously achieved, the link between the clinical cases and the contaminated drums and skins that the deceased individuals had been in contact with. WGS revealed that the incidents involved related but distinct strains and allowed us to phylogenetically place the two sets of strains together with an isolate from a similar US case, on a new branch of the global *B. anthracis* phylogeny that is distinct from existing characterized West African isolates, despite this being the source of the skins in question. This information adds to the fundamental understanding of *B. anthracis* phylogeography and is potentially of value in the source attribution of anthrax outbreaks which may occur from one of an increasing number of routes of exposure and infection observed in the last decade.

DNA extraction. DNA was harvested from a single colony suspended in 500 μl ATL buffer using a QIAamp UCP Pathogen Mini kit (Qiagen) according to manufacturer’s instructions and including the mechanical pre-lysis protocol.

MLVA-8. MLVA-8 (Keim et al., 2000) using the modified HCvrrC1 and HCvrrC2 loci primers (Stratilo & Bader, 2007) was used. Briefly, 23 μl molecular-grade water, 1 μl each primer (10 μM) and 1 μl genomic DNA (50–100 ng) were added to PCR beads (GE Healthcare). Samples were heated to 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, with a final extension at 72 °C for 7 min. PCR products were purified using a DyeEx 2.0 spin kit (Qiagen) and concentrated (Concentrator Plus; Eppendorf) following the manufacturer’s protocols. Samples were resuspended in 0.5 μl GeneScan-500 ROX size standard (Applied Biosystems) with the addition of 19.5 μl Hi-Di Formamide. Product length was determined using a 3130xl genetic analyser and analysed with GeneMapper software (Applied Biosystems). Estimated fragment sizes were corrected to the nearest exact actual size reported (Keim et al., 2000; Stratilo & Bader, 2007).

CanSNP typing. CanSNP analysis was performed as described previously (Van Ert et al., 2007). A 10 μl total reaction volume comprised of 1× TaqMan Universal PCR Mastermix (Applied Biosystems), 250 nM each probe, 600 nM each primer, 1 μl DNA template (50–100 ng) and 3 μl molecular-grade water. Samples were heated in a thermocycler at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C.
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for 1 min. End-point fluorescence data were measured using an ABI 7900 real-time platform (Applied Biosystems).

WGS. Purified genomic DNA was sequenced by Public Health England Genomic Services and Development Unit. DNA was tagged and multiplexed with a Nextera XT DNA kit (Illumina) and sequenced on an Illumina Hi-Seq 2500 platform with paired-end read lengths of 150 bp. A minimum 150 Mb of Q30-quality data was obtained for each isolate.

Data analysis. Sequencing reads were trimmed to remove adaptors and low-quality bases, with quality checked using Trimmomatic (Bolger et al., 2014) and FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Reads were then mapped to the ‘Ames Ancestor’ chromosomal sequence (GenBank accession number NC_007530.2), as well as pX01 and pX02 sequences (GenBank accession numbers NC_007322.2 and NC_007323.3) using bwa 0.7.5 (Li & Durbin, 2009). The SAM output file was converted to BAM format using SAMtools (Li et al., 2009). Unified Genotyper 0.7.0 from gatk2 (DePristo et al., 2011) was used to call bases and create a VCF file for each sample, which was further filtered to extract core genome SNPs that passed the filtering criteria of mapping quality >30, variant ratio >0.9, read depth >5 and ratio of reads with MQ0 to total number of reads <0.05. Polymorphic positions were included in a multiple sequence alignment of all samples. All of the above were performed on the Public Health England Galaxy instance (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010). SNP filtering criteria were validated using simulated data generated using ArtificialFastQGenerator (Frampton & Houlston, 2012). Maximum-parsimony phylogeny was inferred in MEGA 6 (Tamura, 2012). FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) was used to produce all final figures. SPAdes 3.1.1 (Bankevich et al., 2012) was used to produce draft assemblies for each isolate. Harvest suite (Treangen et al., 2014) was used to generate a core genome alignment including publicly available sequence data listed in Table S3 (available in the online Supplementary Material) and export core genome SNPs that passed quality filters to a multiFASTA file used as input for MEGA. FASTQ data were deposited in the Short Read Archive under accession PRJNA287512 [Data Citation 1].

Results and Discussion

MLVA-8

MLVA-8 analysis performed during the Health Protection Agency (now Public Health England) investigation into the cases, at the times they occurred (Table S1, available in the online Supplementary Material), suggested that there were three distinct profiles discovered in isolates collected during the Scottish investigation. A common profile was shared by the clinical samples and all but three of the environmental samples (profile M1). The three other environmental samples consisted of one isolate, Scotland_474, with a profile that differed at the VrrA locus (profile M2), and two isolates with genotypic profiles that differed from the majority profile at the pX01 locus. Caution was taken in the interpretation of the latter, as the repeat size was small and the product size estimation method used was susceptible to inaccuracy in this range. The WGS performed in this study allowed the confirmation of repeat size at this short locus. Extraction of reads spanning across the region demonstrated that in fact all isolates had an equal repeat length at the loci, therefore reducing the number for MLVA-8 genotypes present to two.

The London isolates all shared a common MLVA-8 profile (profile M3), with the exception of London_496, which had alternative lengths at the vrrA and vrrC1 loci (profile M4). Both were distinct from Scottish isolate profiles.

In both cases, the clinical isolates matched the most common environmental profile and these results were used to conclude in both investigations that the anthrax cases were contracted from association with animal skins or drums.

CanSNP profiling

Van Ert et al. (2007) identified 13 canSNPs that describe the three major lineages and 12 clonal sublineages of B. anthracis. All animal-skin-drum-associated strains isolated in the USA belong to the A.Br.008/009 group, as defined by the 13 canSNPs, and have been further classified with the addition of novel SNP assays as belonging to the A.Br.011/009 subgroup (Marston et al., 2011).

CanSNP analysis of the UK isolates showed that all have the A.Br.008/009 group canSNP profile identical to the USA isolates. In silico extraction of the A.Br.011 and A.Br.011/009_3692595 SNP loci from the WGS also showed agreement with the USA isolates and placed all UK isolates into the A.Br.011/009 group data (in agreement when measured by either mapping to the Ames Ancestor reference or through de novo assembly and alignment). In both the USA and UK cases, the skins involved were thought to have been imported from West Africa. Agreement in their canSNP profiles reflects their likely shared geographical origin.

WGS

WGS was performed to further characterize the diversity present within the UK isolates. Sequence reads were mapped to the Ames Ancestor genomic sequence, as well as to the pX01 and pX02 plasmid sequences. All samples provided at least 97 % chromosomal sequence coverage at a read depth of ≥10 (mean coverage varying between 39 × and 96 ×; Table S3).

All 15 Scottish isolates and six of the nine London isolates mapped to both plasmid sequences with >99 % coverage at a read depth of ≥10, confirming them as true B. anthracis isolates (mean coverage varying
between 98 × and 535 ×), but three of the nine London samples showed insignificant mapping to pX02. Contemporaneous MLVA-8 analysis of the outbreak had recorded a positive signal for the pX02 locus, as had the pX02-targeted capsule PCR test (Public Health England, result not shown). It is therefore likely that the pX02 plasmid has been cured during storage in glycerol or during the latest culture of these isolates. For comparison, Centers for Disease Control and Prevention sequence data from an isolate from the 2006 New York case (CDC, 2006; Nguyen et al., 2010) was also included in the analysis.

**Whole-genome SNP analysis**

SNPs meeting strict mapping and quality criteria (see Methods for details) were identified in each sample using the Ames Ancestor sequence as reference. In total, 572 SNPs were found within the isolates and these positions were used to infer a maximum-parsimony phylogeny, shown in Fig. 1. The UK strains fall into two groups: Group A consists solely of strains isolated during the Scottish case, whilst Group B contains all London case strains and a single Scottish strain. The two separate clades are well supported; with 38 synapomorphic SNPs separating Group A and 25 Group B from their most recent common ancestor (a synapomorphic SNP is a genome position that has mutated such that the new nucleotide is shared with all descendants). The phylogeny is robust and contains no homoplasies. Group A is the less diverse of the two groups, containing just three genotypes; a genotypic group of 10 strains, a genotypic pair defined by a single synapomorphic SNP and a unique genotype defined by a single autapomorphic SNP (an autapomorphic SNP is

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**Fig. 1.** Maximum-parsimony phylogeny of the drum-skin-associated isolates. Reconstructed in MEGA6 (Tamura et al., 2013) using the SPR algorithm, with 572 SNPs identified by WGS of the 24 isolates using Ames Ancestor as reference. Bootstrap values (1000 replicates) are shown, but likely underestimate confidence in clonal populations with little homoplasy (Pearson et al., 2009). Branch length in units of number of changes over the whole sequence. There are no homoplasies in the topology. Location and sample source are shown for each isolate, MLVA-8 profiles are indicated in parentheses.
a genome position that has mutated but is found only in a single descendant). Group B is more diverse, containing eight genotypes, with 84 SNPs represented within the group, with only two genotypic pairs within the 10 strains. The subgroup of Group B, containing the London_496 and Scotland_474 strains, is defined by 22 synapomorphic SNPs.

**Scottish isolates**

MLVA-8 analysis performed at the time of the 2006 case indicated that three genotypes were present amongst the isolated strains. Correction of the pX01 locus lengths using the WGS data reduced this to just two: a predominant profile (M1) describing the clinical isolates and the majority of environmental isolates, whilst a second profile (M2) was found in a single strain (Scotland_474) that differed only at the vrrA locus (Table S1). The WGS data presented here clearly support the distinct genotype attributed to Scotland_474, as it is the only Scottish isolate to fall outside of Group A in the phylogeny (Fig. 1). All other isolates that shared the M1 profile are closely related, within a maximum distance of two SNPs. The clinical isolates Scotland_472 and Scotland_473 differ from all other Group A members by a single shared SNP, located within the GBAA_5087 gene, encoding an OmpR family DNA-binding response regulator. The presence of this SNP within duplicate patient isolates makes it unlikely to have arisen during culture for analysis. Selection of this mutation within the host, or presence of this variant within the population prior to infection are more likely. This SNP is not found in any other publicly available genomes (listed in Table S2). The level of relatedness suggests that all Group A strains had a common source. As they were isolated from several different drums, as well as multiple areas in which the drums had been stored or played, it is most likely that cross-contamination from a single initial contaminated item occurred. The presence of the phylogenetically distinct Scotland_474 strain, on a hide that did not test positive for any other strains, implies that a separate contamination event introduced this strain to the environment.

**London isolates**

The London isolates shared a common MLVA-8 profile (M3) with the exception of London_496, which had alternative lengths at the vrrA and vrrC1 loci (M4) (Table S1). The whole-genome SNP-based phylogeny also places this isolate as an outlier, falling within Group B, but within a subgroup along with Scotland_474. The presence of these two closely related strains during the geographically distinct cases implies that strains belonging to this subgroup are a more common contaminant of hides. The clinical isolate London_491 has an MLVA-8 profile identical to all environmental isolates other than London_496. However, WGS reveals a wider range of diversity within the isolates and places London_491 on its own branch, defined by 20 autapomorphic SNPs. The level of diversity seen within the London isolates is much greater than that seen in the Group A isolates. It is far beyond what is credible to have arisen during culture for analysis (Vogler et al., 2002; Ågren et al., 2014). It is more likely to represent contamination by a heterogeneous population through multiple contamination events.

This increased analytical resolution highlights the discriminatory potential of the whole-genome approach when compared to traditional molecular analysis alone. In addition, prior to the application of WGS, the level of relatedness of Scotland_474 and London_496 and their link to Group B was unclear, as their MLVA-8 shares more in common with Group A.

**New York isolate**

The isolate taken from the New York 2006 case shares a more recent common ancestor to the Group A strains, but is differentiated from them by 26 synapomorphic SNPs.

**Phylogeography**

In all of the UK and USA drum-related infections, the skins involved were thought to have been imported from West Africa (Anaraki et al., 2008; Marston et al., 2011; Riley, 2007). Recently, the genome sequences of three West African strains, i.e. two from Senegal and one from Gambia, have been reported (Rouli et al., 2014). These, along with *de novo* assemblies of the drum strains and the entire collection of *B. anthracis* assemblies present in GenBank (listed in Table S1) were aligned and core genome SNPs identified using Harvest suite tools (Treangen et al., 2014). The exported SNPs were then used to infer a maximum-parsimony phylogeny in MEGA6 (Tamura et al., 2013). The full tree is shown in Fig. S1. The drum-skin-related isolates form a novel clade, nested within a larger clade containing the West African strains as well as Canadian and US strains; Fig. 2 shows this subtree. The drum-skin-specific clade is defined by 101 shared SNPs, specific to this branch, and the West African strains clade defined by 222. The branch leading to the putative ancestor of both the West Africa and western North America clades is defined by four SNPs, which are not present in any other of the 124 analysed sequences (positions 1 776 654, 2 917 555, 4 544 130 and 4 544 131; full SNP data for all strains can be found in supplementary files). The consistency index for the full phylogeny is 97.5 %. There is only one homoplastic SNP between the existing West African strains clade and the new Drum isolates, and this is only present in a single drum strain of 23 and a single West African isolate. There are 17 homoplasious sites shared by Ames Ancestor and at least one of the existing West African strains, but not by any of the A.Br.008/009 lineage strains placed between them,
none of which affect the placement of the novel drum isolates branch. There is strong support therefore for the isolates forming a novel branch, distinct from the existing West African strains.

The strains associated with the two UK and one New York cases are more closely related to each other than they are to any previously published sequences. Furthermore, no epidemiologically unrelated samples in >1300 genotyped geographically diverse isolates from the Northern Arizona University B. anthracis collection fall into this novel West African clade (P. Keim, D. Birdsell and T. R. Pearson unpublished data). This reflects their likely shared geographical origins as all hides were thought to have been imported from West Africa. The fact that they are distinct from the West African strains sequenced to date is also not surprising as the number of sequences available is low and thus not likely to be representative for such a large geographical region. The skins and hides present in the New York incident were known to have been imported from Coˆte d’Ivoire (CDC, 2006) and those from the Scottish incident were thought to have been imported from Guinea (Riley, 2007) which are not represented amongst currently available sequences. The multiple skins associated with the London incident were thought to be of mixed origin including The Gambia (Anaraki et al., 2008), but show no significantly closer relationship to the published Gambian sequence. These strains therefore increase our knowledge of global B. anthracis phylogeography.

Conclusions
The two UK animal-skin-drum-related anthrax cases were caused by independent importation of animal hides contaminated with B. anthracis strains belonging to the same lineage as that responsible for drum-skin-related anthrax cases in New York. They also belong to the same canSNP group as other US cases for which whole-genome data are unavailable. The two fatalities were caused by distinct strains, but a pair of closely related isolates was found between the two sites, implicating them as a common presence in contaminated hides. The use of WGS has increased our understanding of the populations of anthrax spores present at each scene and given us new insight into the relationship between the two events as well as their relatedness to a US case. The strains isolated and sequenced have added to the global B. anthracis phylogeographic profile.

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