European 1: a globally important clonal complex of *Mycobacterium bovis*

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Running Head: *M. bovis* European 1 clonal complex

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ABSTRACT

We have identified a globally important clonal complex of *M. bovis* by deletion analysis of over one thousand strains from over 30 countries. We initially show that over 99% of the strains of *Mycobacterium bovis*, the cause of bovine tuberculosis, isolated from cattle in the Republic of Ireland and the UK are closely related and are members of a single clonal complex marked by the deletion of chromosomal region RDEu,1 and we named this clonal complex European 1 (Eu1). Eu1 strains were present at less than 14% of French, Portuguese and Spanish isolates of *M. bovis* but are rare in other mainland European countries and Iran. However, strains of the Eu1 clonal complex were found at high frequency in former trading partners of the UK (USA, South Africa, New Zealand, Australia and Canada). The Americas, with the exception of Brazil, are dominated by the Eu1 clonal complex which was at high frequency in Argentina, Chile, Ecuador and Mexico as well as North America. Eu1 was rare or absent in the African countries surveyed except South Africa. A small sample of strains from Taiwan were non-Eu1 but, surprisingly, isolates from Korea and Kazakhstan were members of the Eu1 clonal complex. The simplest explanation for much of the current distribution of the Eu1 clonal complex is that it was spread in infected cattle, such as Herefords, from the UK to former trading partners, although there is evidence of secondary dispersion since. This the first identification of a globally dispersed clonal complex *M. bovis* and indicates that much of the current global distribution of this important veterinary pathogen has resulted from relatively recent International trade in cattle.

1. Introduction

The *Mycobacterium tuberculosis* complex comprises many species and sub-species that cause tuberculosis (TB) in a variety of mammalian hosts and includes *Mycobacterium bovis,* the principle cause of tuberculosis in cattle (Smith et al., 2006a). The most notable member of the complex is *M. tuberculosis*, the most important bacterial pathogen of humans; however, the preferred host of *M. bovis* is domesticated cattle, although this pathogen can frequently be isolated from other mammals including man (Smith et al., 2006a). Because of the close genetic similarity of the *M. tuberculosis* complex of bacteria and the similarity of pathology, despite widely different host-adaptation, it has been suggested that different host-adapted forms would better be referred to as 'ecotypes' rather than species (Smith et al., 2006b). Bovine TB has been found in cattle on every continent where cattle are farmed (Amanfu, 2006; Cosivi et al., 1998).

In most of mainland Europe, the United States of America (USA), Canada, Australia, Cuba and some South American countries bovine TB has been reduced or eliminated from domestic cattle by the long term application of a test-and-slaughter policy that removed infected cattle (Amanfu, 2006; Ayele et al., 2004; Cosivi et al., 1998; Cosivi et al., 1995; Smith et al., 2006a; Thoen et al., 2006a; Thoen et al., 2006b). With the exception of Australia and some Caribbean Islands (Tweddle and Livingstone, 1994), many of these countries still have occasional, and sometimes persistent, outbreaks of bovine TB associated with either the import of infected cattle from other countries or the maintenance of the disease in a wildlife host.

In the United Kingdom (UK), test-and-slaughter brought the disease to a very low incidence in the 1970s. Since then the incidence of the disease has inexorably risen (Smith et al., 2006a) and the Republic of Ireland (RoI) and the UK have the highest incidence of bovine TB in the European Union (Reviriego Gordejo and Vermeersch, 2006). For the rest of the European Union bovine TB has mainly been controlled by test-and-slaughter but it continues to be a persistent problem in parts of Spain, Italy and Portugal (Pavlik, 2006).

Bovine TB has been shown to be present in most countries in Africa but in general, due to economic constraints, the true extent of the disease has not been evaluated (Ayele et al., 2004; Cosivi et al., 1995). The exception is South Africa where an extensive test-and-slaughter has reduced the disease in cattle to minimal levels (Michel et al., 2008). In North America bovine TB is endemic in Mexican cattle but has been largely eliminated from cattle in the USA and Canada (Milian-Suazo et al., 2008; Wobeser, 2009). However, in the USA a persistent problem has been reported in white-tailed deer in Michigan, as well as small breakdowns in Minnesota and Molokai Island, Hawaii (associated with feral swine) (Bany and Freier, 2000). The USA also suffers from the occasional import of bovine TB in Mexican cattle (Milian-Suazo et al., 2008; Rodwell et al., 2010). In Canada there are two areas where wildlife populations are still infected with bovine TB; free-ranging populations of wood bison in and around Wood Buffalo National Park, which straddles the provinces of Alberta and the Northwest Territories, and deer and elk (wapiti) in Riding Mountain National Park, Manitoba (Wobeser, 2009). The Bovine Tuberculosis Eradication Program in Mexico has successfully reduced the prevalence of TB in cattle in certain regions. Beef cattle in the northern-most states have

the lowest prevalence, averaging between 0.01 to 0.25% (Ritacco et al., 2006). However, the prevalence of *M. bovis* in Mexican dairy cattle is higher, with an estimated infection rate in this population of 16-17% (Milian-Suazo et al., 2000; Milian et al., 2000).

Bovine TB is endemic in cattle in South America (de Kantor et al., 2008). About 70% of the cattle are found in areas with high disease prevalence although nearly 17% are in areas virtually free from TB (de Kantor and Ritacco, 2006). For the rest of the world, bovine TB is thought to be endemic in cattle and there have been, with notable exceptions, few molecular epidemiological surveys of the strains present in each country (Cosivi et al., 1998; Jeon et al., 2008; Tadayon et al., 2006; Thoen et al., 2006a).

In the North American hotspots bovine TB persistence is associated with maintenance in an alternative wildlife host (white-tailed deer in Michigan, pigs in Hawaii and buffalo in the Canadian National Parks (Rhyan and Spraker, 2010)). In a similar manner the persistence of bovine TB in New Zealand cattle is associated with brush-tailed possums (Tweddle and Livingstone, 1994) and the failure of the test-and-slaughter in the UK is associated with disease maintenance in Eurasian badgers (*Meles meles*) (Gallagher and Clifton-Hadley, 2000; Jenkins et al., 2010). In South Africa, bovine TB is thought to have been transmitted from cattle to buffalo in both the Kruger National Park and Hluhluwe-iMfolozi Park and is affecting several wildlife species in these national parks (Michel et al., 2009). It is becoming a feature of bovine TB control, internationally, that the test-and-slaughter protocol for cattle, that worked so well in mainland Europe, the USA and Australia, is failing in other countries because of a wildlife maintenance host for the disease (Van Campen and Rhyan, 2010).

Spoligotyping, a PCR and hybridisation technique, is a common molecular typing method applied to isolates of the *M. tuberculosis* complex and identifies polymorphism in the presence of spacer units in the direct repeat (DR) region (Kamerbeek et al., 1997; van der Zanden et al., 1998). The DR region is composed of multiple, virtually identical, 36-bp repeats interspersed with unique DNA spacer sequences of a similar size (direct variant repeat or DVR units). The DR region may contain over 60 DVR units, however, 43 of the spacer units were initially selected and are used in the standard spoligotyping method used to type strains of the *M. tuberculosis* complex (Groenen et al., 1993; Kamerbeek et al., 1997; van Embden et al., 2000). The DR region is polymorphic because of the loss (deletion) of single or multiple spacers, and each spoligotype pattern from strains of the animal-adapted lineage of the *M. tuberculosis* complex is given a unique identifier by www.Mbovis.org.

The population structure of the *M. tuberculosis* complex of bacteria is apparently highly clonal and no cases of transfer and recombination of house-keeping genes between strains have been identified (Cole et al., 1998; Gutacker et al., 2002; Hershberg et al., 2009; Smith et al., 2003; Smith et al., 2006a). However, there have been reports of between-strain recombination in close proximity to the hypervariable and immunogenic *PE* and *PPE* genes (McEvoy et al., 2009) In a strictly clonal population the loss by deletion of unique chromosomal DNA cannot be repaired by recombination from another strain and the deleted region will act as a molecular marker for the strain and all its descendants. Deletions of specific chromosomal regions (Regions of Difference – RDs or Large Sequence Polymorphisms - LSPs) have been very successful at identifying phylogenetic relationships in the *M. tuberculosis* complex (Brosch et al., 2002; Gagneux

et al., 2006; Gagneux and Small, 2007; Huard et al., 2006; Mostowy et al., 2005; Narayanan et al., 2008; Smith et al., 2006a; Smith et al., 2006b; Tsolaki et al., 2005). Deletions of spoligotype spacers generate novel spoligotype patterns, however, the loss of spacers is so frequent that identical spoligotype patterns can occur independently in unrelated lineages (homoplasy) and therefore a spoligotype pattern may be an unreliable indicator of phylogenetic relationship (Schurch et al., 2011; Smith et al., 2006a; Warren et al., 2002).

In previous work two other epidemiologically important clonal complexes of *M. bovis*, named African 1 (Af1, dominant in Cameroon, Nigeria, Chad and Mali) and African 2 (Af2, at high frequency in East Africa) have been identified (Berg et al., 2011; Muller et al., 2009). All members of the Af1 clonal complex of *M. bovis* are defined by a specific chromosomal deletion (RDAf1) and lacked spacer 30 in their spoligotype pattern and strains of the Af2 clonal complex are identified by a specific deletion (RDAf2) and are associated with the absence of spoligotype spacers 3 to 7. Here, we show that a third clonal complex, called European 1 (Eu1),is dominant in the Republic of Ireland and the UK, some former British colonies, Korea and the New World (with the exception of Brazil). This is the first identification of a globally important clonal complex of M. bovis that has, apparently, been spread throughout the world by the International trade in cattle.

2. Materials and methods

2.1. Bacterial strains, spoligotyping and sequencing

Details of all strains deletion typed for this manuscript are given in the supplementary data. Strains were spoligotyped according to the method of Kamerbeek et al. (Kamerbeek et al., 1997) with minor modifications (Cadmus et al., 2006). Sequencing across the deletion boundary of RDEu1 was carried out using standard sequencing methods using the RDEu1 deletion primers.

2.2. RDEu1 deletion typing

The status of the RDEu1 region was assessed by a PCR assay using a pair of primers located at a suitable distance flanking the deletion boundary (RDEu1 primer set A). The forward primer was RDEu1_FW (5' CCGATGAACTTGGCCCACAG 3' (position 1767904 to1767923 in H37Rv) and the reverse primer was RDEu1_ Rv (5'-CGTGGTGG TGGGATGTCTTG3' (position 1769110 to 1769091 in H37Rv). Final PCR reactions contained 2µl of heat-killed mycobacterial cell supernatant, 10 uM HotStartTaq Master Mix (Qiagen), 1 µM of primers RDEu1_FW and RDEu1_Rv, and sterile distilled water to a final volume of 20µl. Thermal cycling was performed with an initial denaturation step of 15 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1min at 58°C and 2.5 min at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were visualised after electrophoresis on a 1 % agarose gel. A 1206 bp fragment was generated if the

RDEu1 region was intact and a 400 bp fragment if the region was deleted. Strains CHAD491 (RDEu1 intact) and AF61/2122/97 (RDEu1 deleted) were used as controls.

2.3. Measuring the frequency of Eu1 strains

To determine the maximum frequency of Eu1 clonal complex strains in a population the following algorithm was used. From previously published spoligotype surveys for a country isolates of the most common spoligotype patterns, usually several of each, were deletion typed. We also surveyed as many minor clones with spacer 11 missing as possible. Assuming that spoligotype patterns marked clones this deletion analysis was used to determine, from the spoligotype survey, the basic frequency of Eu1 clonal complex strains in a population. To determine the maximum possible frequency of Eu1 clonal complex strains in the population we then added to this basic frequency the frequency of all strains in the spoligotype population surveys that had spacer 11 missing for which RDEu1 deletion results were unavailable.

3. Results

3.1 Strains with spacer 11 absent

It has previously been shown that many strains of *M. bovis* isolated from cattle in the RoI and the UK have a spoligotype pattern lacking spacer 11 (Smith et al., 2006a). Spacer 11 is missing in over 96% of the 55,000 spoligotyped isolates of *M. bovis* found in Great Britain (GB) [Veterinary Laboratories Agency (VLA), Weybridge, UK, Spoligotype

Database, 1994 – 2009] and is missing in all 16,373 isolates of *M. bovis* from Northern Ireland (NI) (Agri-Food and Biosciences Institute (AFBI), Belfast, UK, Spoligotype Database, 2003-2009]. Furthermore, in an analysis of 452 *M. bovis* isolates from both cattle and other animals in the RoI the total of twenty spoligotype patterns identified were also deleted for spacer 11 (Costello et al., 1999). In total, spacer 11 was missing from the spoligotype pattern of 99% of the *M. bovis* isolates from RoI and UK.

3.2 Identification of a specific deletion – RDEu1

A deletion, RD17 and here called RDEu1, has previously been shown to be phylogenetically informative among strains of *M. bovis* (Gordon et al., 2001). We examined the regions surrounding this deletion and determined that they show no similarity to insertion sequences or repetitive DNA, that there are no direct or inverted repeats in the regions immediately flanking the deletion and that they show the same %GC content as the rest of the *M. bovis* genome. These observations suggest that this region is not prone to independently generating deletions and that deletion RDEu1 may provide a suitable phylogenetic marker for a clonal complex of *M. bovis*.

In an unpublished analysis of approximately 500 randomly selected strains isolated from cattle in each of the three regions (GB, NI and RoI) - we identified the spoligotype patterns that were unique to each region, giving a total of 53 spoligotype patterns. We determined the frequency of the RDEu1 deletion among a sample of strains from this population survey using a simple PCR deletion assay. An isolate of every available spoligotype pattern was assayed for the presence of the RDEu1 deletion (RoI 25 strains,

NI 11 strains, GB 13 strains). For these 49 strains RDEu1 was deleted in all but the strain with spoligotype SB0134 (spacer 11 present) from GB.

The RDEu1 region was deleted in a further 130 strains from GB, 240 strains from NI and 90 strains from RoI chosen to represent the spoligotype diversity in each region. Only strains with spoligotype pattern SB0134 (spacer 11 present), were intact at the RDEu1 region (n = 10). We conclude that a clonal complex of *M. bovis* characterised by the deletion of region RDEu1 was ubiquitous in the RoI and UK. This clonal complex is marked by the loss of spoligotype spacer 11. However, the most common spoligotype pattern associated with this clonal complex was SB0140 which has spacer 6 absent as well as spacers 8 to 12, in addition to spacers 3, 16, and 39-43, which are absent in all *M. bovis* (Smith et al., 2006b) strains. We named this clonal complex of *M. bovis* European 1 (Eu1).

3.3. Eu1 in mainland Europe

To determine the frequency of the Eu1 clonal complex in mainland Europe we have used previously published large surveys of strains of *M. bovis* from countries to identify the common spoligotypes present in the population. A sample of strains, representing the most common spoligotype patterns, were then analysed for the status of the RDEu1 region. From this analysis, and assuming that the spoligotype pattern marks a clone, we determined the maximum percentage of strains in each population that could represent the Eu1 clonal complex. That is, we assumed all strains with spacer 11 missing were potential members of the Eu1 clonal complex and then used the PCR deletion assay to

eliminate some spoligotypes and to test the linkage between the loss of spacer 11 and the deletion of RDEu1.

The results of analysing the population structure of *M. bovis* for the presence of the Eu1 clonal complex in population size samples from Spain, Portugal, Italy, Belgium and France are shown in Table 1 and Figure 1. The most common spoligotype pattern of the Eu1 clonal complex was SB0140 (see above) which was found in every country except Belgium (Allix et al., 2006). Here, and for all other strains analysed in this study, strains with region RDEu1 deleted also had spoligotype spacer 11 deleted. Details of all strains deletion typed in this study can be found in the supplementary data.

To gain insight into the population structure of *M. bovis* in The Netherlands prior to the eradication of bovine TB in cattle in 1990 we have analysed a small set of isolates from elderly Dutch patients (all born prior to 1933). No strains of the Eu1 clonal complex were identified by deletion typing and, assuming that these patients were infected with *M. bovis* prior to the eradication of the disease in cattle, these data suggest that the Eu1 clonal complex may have been rare in The Netherlands (Table 1).

In a similar manner we analysed 20 isolates from humans born in Sweden before 1940; bovine TB was eradicated from cattle in Sweden in 1958 (Szewzyk et al., 1995). The majority of these human isolates had spoligotype patterns identical, or similar to the spoligotype pattern of vaccine strain BCG (SB0120, n = 16, spacer 11 present and RDEu1 intact), however, five strains with spoligotype pattern SB0130 were deleted for spacer 11 and RDEu1 (Table 1). Because we do not know where these patients were infected with bovine TB we can only conclude that the Eu1 clonal complex was probably

at much lower frequency in Sweden, prior to its eradication from cattle, than it currently is the RoI and UK.

From Germany 39 isolates of *M. bovis* representing the most common *M. bovis* spoligotype patterns found in 166 patients diagnosed with TB not caused by *M. tuberculosis* between 1999 and 2001 (Kubica et al., 2003) were tested by RDEu1 deletion analysis. We also tested five strains of *M. bovis* that were isolated from animals during the same period. All but one strain, isolated from a human born in the USA, were intact for RDEu1. These data suggest, assuming that these isolates represent a sample of the bovine TB population present in German cattle prior to its elimination in 1997 (Hartung, 2001), that the RDEu1 clonal complex was rare in Germany.

3.4. Eu1 in Africa

It has previously been shown that the African 1 clonal complex of *M. bovis*, defined by deletion RDAf1 and marked by the loss of spacer 30, is dominant in Nigeria, Chad, Cameroon and Mali (Muller et al., 2009). The RDEu1 region was intact in a group of Af1 strains from these countries (n = 26) showing that the Af1 clonal complex and the Eu1 clonal complex are phylogenetically distinct clonal complexes. In a reciprocal experiment, RDAf1 was intact in a collection of Eu1 strains from GB representing the local spoligotype diversity (n = 21, unpublished data) confirming that Eu1 and Af1 are phylogenetically distinct. Because of the previously documented dominance of Af1 in Nigeria, Chad and Cameroon (over 90% of strains) we can conclude that the Eu1 clonal complex was absent or at low frequency in these three West-central African countries. In Mali 65% of

the isolates are Af1 (Muller et al., 2009) and the presence of the RDEu1 region in the most common non-Af1 strain from Mali (SB0134) suggests that the Eu1 clonal complex is also rare or absent in Mali (Table 2).

Another clonal complex of *M. bovis,* marked by both a deletion (RDAf2) and a specific spoligotype signature, is present at high frequency in Uganda, Ethiopia, Burundi and Tanzania (Berg et al., 2009; Berg et al., 2011). This East African clonal complex of *M. bovis* has been designated African 2 (Af2) and represents over 70% of all cattle isolates from each of these East African countries. Strains of the Af2 clonal complex are intact at the RDEu1 region and spoligotype surveys of these countries showed only very low levels of strains with spacer 11 missing (Berg et al., 2011). We surveyed a sample of available strains (n = 38) from Ethiopia, Burundi and Tanzania for the Eu1 specific deletion including 27 strains of the Af2 clonal complex; no strains deleted for RDEu1 were identified.

A set of twelve strains of *M. bovis* from the Buzi District of Central Mozambique all had spoligotype pattern SB0961 (spacer 11 present) and were shown to be intact at RDEu1. Previously published surveys of *M. bovis* strains from Madagascar, Zambia and Algeria also suggest that spoligotype patterns with spacer 11 missing are rare in these countries (Munyeme et al., 2009; Rasolofo Razanamparany et al., 2006; Sahraoui et al., 2009). We concluded that in the African countries surveyed the Eu1 clonal complex was absent or at very low frequency (Table 2).

3.5. Eu1 in southern Africa

We analysed 35 strains isolated from South African cattle between 1991 and 2008. Many isolates from cattle in South Africa had spoligotype patterns similar to those found in the RoI and UK (Michel et al., 2008). All but eight strains were deleted at the RDEu1 region and we concluded that the Eu1 clonal complex was common in cattle in South Africa. A single isolate from Swaziland was also deleted for RDEu1.

The molecular epidemiology of *M. bovis* isolates from free ranging wildlife in South African game reserves, Kruger National Park (KNP) and Hluhluwe-iMfolozi Park, KwaZulu-Natal (HiP), has been described (Michel et al., 2009). Strains from KNP were characterised by the loss of spacer 21 (SB0121) whereas strains from HiP were characterised by the loss of spacer 11 (SB0130). Twelve strains isolated from various animals from the KNP were intact for RDEu1, however, eight strains of spoligotype pattern SB0130 isolated from buffalo in the HiP were all deleted for RDEu1 and therefore members of the Eu1 clonal complex.

3.6. Eu1 in South America

We tested 77 isolates from Argentina, mainly from cattle, and 43 isolates from swine for the presence of the RDEu1 deletion; all but eight strains were deleted for both spacer 11 and RDEu1. We also analysed a collection of 30 strains from cattle isolated throughout Chile. The commonest spoligotype pattern among Chilean isolates was SB0140 and all but one of the isolates were deleted for RDEu1 and lacked spacer 11. Ten strains from the most important dairy region in Ecuador, all with spoligotype pattern SB0980 (a single spacer loss derivative of SB0140), were analysed; all strains were

deleted for RDEu1 and lacked spacer 11. Finally, a collection of strains from Brazilian cattle (n = 29) and goats (n = 7) were deletion assayed for RDEu1. In contrast to the results for other South American countries only six of the 36 strains (all from cattle) were deleted for RDEu1.

3.7. Eu1 in North America, Australia and New Zealand

A previously reported spoligotype survey of 84 Mexican and American *M. bovis* isolates from cattle, deer, and feral pigs grouped the strains into 27 clusters named A to AA (Milian-Suazo et al., 2008). Strains with spacer 11 present were only found in clusters V, W, X and Y. Thirty-eight isolates representing the commonest clusters identified from both Mexico and the USA were assayed for the status of the RDEu1 region by deletion typing. All strains, except single isolates representative of clusters V, W, X and Y, were deleted for RDEu1. From Canada, a sample of strains (n = 10) from the Riding Mountain Eradication Area, mainly from elk (Lutze-Wallace et al., 2005), were analysed for the RDEu1 deletion. All strains were deleted for the RDEu1 region.

We concluded that the Eu1 clonal complex was common in the USA and Mexico as well as Riding Mountain National Park in Canada. Both the Michigan strains, associated with white-tailed deer, and the Hawaiian strains associated with feral pigs were also members of the Eu1 clonal complex.

We deletion surveyed 34 strains from Australia, mainly isolated prior to 1994; all were deleted for the RDEu1 region and therefore members of the Eu1 clonal complex. Sixteen strains from New Zealand, isolated from cattle between 1989 and 2003, were

deletion typed for RDEu1; all 16 were deleted for RDEu1. We concluded that both Australia and New Zealand were dominated by strains of the Eu1 clonal complex.

3.8. Eu1 in Asia

We analysed 56 *M. bovis* strains isolated from dairy cattle throughout the Gyeonggi-do province of Korea (Jeon et al., 2008); 75% of the strains were of spoligotype SB0140 and all 56 isolates were deleted for RDEu1. We also RDEu1 deletion typed two strains of *M. bovis* isolated from Taiwanese nationals (SB0265, spacer 11 present) representing the two major VNTR types of this spoligotype found in Taiwan (Jou et al., 2008). Both these human isolates were intact at RDEu1. A single, previously unpublished isolate from a human with spoligotype pattern SB1040 (spacer 11 missing) was deleted for RDEu1. Furthermore, no RDEu1 deleted strains were found in a survey of 20 animal isolates suggesting that the Eu1 clonal complex is rare or absent in Taiwan (data not shown).

Spoligotype surveys of *M. bovis* isolates from TB-test reactor cattle in 24 of the 28 Iranian provinces where bovine TB has been reported showed either BCG-like spoligotype patterns (SB0120, spacer 11 present, 41% of isolates) or simple variants of this ancestral pattern (Tadayon et al., 2008). We selected a sample of 47 strains from these surveys for deletion analysis and, as expected, all strains were intact at RDEu1.

In 2006 eight strains of *M. bovis* with an unusual combination of phenotypic and biochemical characteristics were isolated from humans from the oblast of Kostanajskaya in north Kazakhstan (Kubica et al., 2006). Seven of these strains, with spoligotype

pattern SB0131, a single spacer loss derivative of Eu1 type SB0130, were deleted for RDEu1.

3.10. Reference strains of M. bovis

The neotype strain of *M. bovis*, NCTC 10772 (ATCC 19210), was obtained from the National Collection of Type Cultures and spoligotyped as SB0267 (spacer 11 missing) and was deleted for RDEu1. This strain was isolated by W. D. Yoder in Texas from a granulomatous lesion in a lymph node of a 6-month-old heifer in 1965. The strain AN5 that is used worldwide for bovine PPD production was originally isolated in England around 1948 (Paterson, 1948) and has spoligotype pattern SB0268 (missing spacer 11) and is deleted for RDEu1. The *M. bovis* progenitor of the vaccine strain, BCG, was isolated by Nocard in France in 1902 from a cow with tuberculous mastitis. While this *M. bovis* strain was lost, we can infer its spoligotype from the BCG derivative, which has spoligotype pattern SB0120 (spacer 11 present). However, recently a BCG strain with a noncanonical spoligotyping profile has been identified (Mokrousov et al., 2010). Strains BCG Sweden, Danish, Russia, Tice, Frappier and Tokyo (Garcia Pelayo et al., 2009) are intact for RDEu1. Strain ATCC35723 was originally isolated from a cow by A. G. Karlson at the Mayo Clinic, Rochester, Minnesota and he deposited it in the Trudeau Mycobacterial Collection in 1950 where it was designated TMC405. This strain has spoligotype pattern SB1185 (spacer 11 missing) and is deleted for RDEu1. Strains NCTC8438 and NCTC9320 were isolated from English cows in 1945 and 1954, respectively; both strains are of spoligotype SB0140 and deleted for RDEu1. *M. bovis* strain AF 61/2122/97, the first *M. bovis* strain to have its entire genome sequenced

(Garnier et al., 2003), was isolated from an English cow in 1997, has spoligotype pattern SB0140 and is deleted for RDEu1.

To confirm that the RDEu1 deletion was identical by descent we nucleotide sequenced across the deletion boundary in a total of 89 isolates from 10 countries. The RDEu1 deletion boundary was identical in all 89 isolates.

4. Discussion

We have identified a globally important clonal complex of *M. bovis* by a deletion analysis of 1014 strains from over 30 countries and have named this clonal complex European 1 (Eu1). Members of this clonal complex are defined by a previously identified 806 bp deletion (RD17) of chromosomal DNA which we have named Region of Difference Eu1 (RDEu1) (Gordon et al., 2001). Sequencing across the RDEu1 deletion boundaries in many isolates has shown that the deletion boundaries are identical and, in the absence of repetitive elements flanking RDEu1 or other features promoting deletions, and the apparent strict clonality of *M. bovis* (Smith et al., 2006a), we conclude that this deletion is identical by descent in these strains throughout the world. That is, RDEu1 was deleted from the most recent common ancestor of this clonal complex and this region is therefore absent in all descendants of that most recent common ancestor. A definition and summary of the Eu1 clonal complex is shown in Table 3.

Strains of the Eu1 clonal complex can be identified by the loss of spacer 11 in the spoligotype pattern although this characteristic is not necessarily specific for this clonal complex. Because the loss of spacers in spoligotype patterns can be homoplastic (Smith

et al., 2006a; Warren et al., 2002), strains that are not members of the Eu1 clonal complex (RDEu1 region intact) can also lack spacer 11; for example the strains with spoligotype pattern SB1284 from Spain (supplementary data). Furthermore, it is theoretically possible that the most recent common ancestor of the Eu1 clonal complex had RDEu1 deleted and had spacer 11 present; the loss of spacer 11 could have happened later and then become the major sub-clone of the Eu1 clonal complex. However, although all 476 strains that were shown to be deleted for RDEu1 in this study were also deleted for spacer 11 (supplementary data). the spoligotype signature of the Eu1 clonal complex, as well as the spoligotype signatures of the Af1 and Af2 clonal complexes, should be used as a guide to direct deletion analysis. It is the deletions that define membership of these clonal complexes and not spoligotype signature.

The Eu1 clonal complex showed a remarkable difference in frequency throughout Western Europe (Table 1). Strains of this clonal complex were virtually fixed in the RoI and UK (99%), were at less than 14% in the Iberian Peninsula and France and rare or absent in Belgium and Italy. If surveys of strains from elderly human patients can be used to indicate the population structure of bovine TB prior to its elimination from cattle then our data suggest that the Eu1 clonal complex was rare in Germany, Sweden and The Netherlands prior to its eradication from cattle (Figure 1). In more eastern European countries spoligotype surveys suggest that strains of the Eu1 clonal complex are at low frequency and strains of *M. caprae* are more common (Pavlik, 2006).

Throughout most of the African countries surveyed and Iran, the Eu1 clonal complex is apparently at low frequency, with the exception of South Africa, where Eu1 strains represent just over 60% of strains isolated from cattle. We show that Eu1 strains

are common in wildlife in the Hluhluwe-iMfolozi Park, while another clonal complex has been established in Kruger National Park .

However, the Eu1 clonal complex dominates most of the South American countries assayed with the exception of Brazil.

Strains from Brazil had spoligotype patterns similar to the vaccine strain BCG (SB0120) or were lacking spacer 21 and the difference in the population structure of *M. bovis* in Brazil, compared to neighboring South American countries is supported by further spoligotype analyses from that country (Viana-Niero et al., 2006; Zanini et al., 2005; Zumarraga et al., 1999). Although there is an obvious historical difference between Portuguese speaking Brazil and the rest of Spanish speaking South America the current populations of *M. bovis* in Spain and Portugal do not reflect the population structure differences between Brazil and the rest of South America (Eu1). Both Spain and Portugal have similar population structures for *M. bovis*; strains missing spoligotype spacer 21 are common, the Eu1 clonal complex is at low frequency (6%) and the BCG-like spoligotype pattern (SB0120) is rare (Boniotti et al., 2009; Duarte et al., 2008; Rodriguez et al., 2009).

In the USA the Eu1 clonal complex is common and included RDEu1 deleted strains isolated from coyotes, cattle and white-tailed deer in Michigan and from feral pigs in Hawaii. The strains identified as members of the Eu1 clonal complex in Michigan, Hawaii and New Mexico are distinct in lacking spacers 5 to 13 (SB1165) and similar spoligotypes are found in Mexico where Eu1 is also common (Milian-Suazo et al., 2008). Strains from the southern states of the USA are, in general, more similar to SB0140, lacking spacer 8 to 12 and strains of this type are common in Mexico (Cobos-Marin et al., 2005; Cousins and Roberts, 2001; Milian-Suazo et al., 2008).

Our Canadian sample of Eu1 strains was isolated from deer (elk) at the Riding Mountain National Park (RMNP) however this may not reflect the bovine TB that was present in Canadian cattle prior to its general elimination from cattle in 2005 (Wobeser, 2009). The origin of bovine TB in the RMNP may have involved introduced bison whose ultimate origin was the USA (Wobeser, 2009). However, the spoligotype patterns of strains from the RMNP are distinctly different from strains currently found in the USA.

The RDEu1 region was deleted in all strains from Australia and New Zealand and the fixation of the Eu1 clonal complex in Australia prior to its elimination in 1997 is supported by the previously recorded absence of spacer 11 in the spoligotype pattern of 211 Australian *M. bovis* isolates surveyed in 1998; the most common pattern in this survey was SB0140 (72%) (Cousins et al., 1998). However, the populations of *M. bovis* in these two English speaking nations are not identical. The small survey of New Zealand strains presented here suggests that strains with spoligotype pattern SB0130, the presumptive ancestral spoligotype pattern of the Eu1 clonal complex (Table 3), are more common in New Zealand (9 of 16 strains) than Australia [not seen in a spoligotype survey of 211 strains (Cousins et al., 1998)]. It has been pointed out before that New Zealand is an isolated island nation and possibly only a limited group of *M. bovis* was introduced (Collins et al., 1993).

In most of Asia, both the population structure and prevalence of bovine TB is unknown, however, this study gives a first indication to where the Eu1 clonal complex is distributed. We did not identify any strains of the Eu1 clonal complex in Iran, however, in the Republic of Korea, where bovine TB affects more than 500 dairy cattle each year and causes major economic losses in spite of a continued test-and-slaughter (Jeon et al.,

2008; Wee et al., 2009), isolates from dairy cattle in Gyeonggi-do province of the Republic of Korea (Jeon et al., 2008) were deleted for Eu1 and the spoligotype patterns were of two main types SB0140 (over 75%) or SB1040, a spacer deletion derivative of SB0140. Finally, Eu1 strains were identified in humans from a rural area of northern Kazakhstan (Kubica et al., 2006).

The RDEu1 deletion. The RDEu1 deletion is 806 bp long and is located entirely within the gene for malto-oligosyltrehalose synthase (*treY*) which encodes an enzyme in the biosynthesis of the disaccharide trehalose (De Smet et al., 2000). The deletion truncates the protein and causes a frameshift which presumably affects the catalytic function of the enzyme. Three biosynthetic pathways for the production of trehalose have been identified in bacteria (Kaasen et al., 1992; Maruta et al., 1996; Tsusaki et al., 1997) and screening of the *M. tuberculosis* genome shows that homologs of all three biosynthetic pathways are present (De Smet et al., 2000). Furthermore, cell-free extracts from *M. bovis* BCG, which is intact at RDEu1, were also observed to catalyze the production of trehalose from a variety of substrates (De Smet et al., 2000) suggesting that the ancestral *M. bovis* strain (RDEu1 intact) could synthesise trehalose via each of these three biosynthetic pathways. The existence of multiple biosynthetic pathways and the resulting redundancy in trehalose synthesis, suggests that the loss of one pathway, caused by deletion RDEu1, may be selectively neutral.

Diaspora from the UK? The presence of the Eu1 clonal complex of *M. bovis* in so many trading partners and English speaking former colonies of the UK (Figure 2) does offer a

simple explanation for the global distribution of this clonal complex (Cataldi, 2002). The suggestion that the UK was the epicenter for the distribution of the Eu1 clonal complex can be supported by the large number of modern cattle types that were originally bred there (Decker et al., 2009). For example, Hereford beef cattle, bred in Herefordshire, UK in the 18th century, have since been exported and re-exported to become the most numerous and widely distributed beef breed in the world (Porter, 1991). Herefords have been exported since 1817, first to North America from where they spread to Mexico and South America. This breed and its crosses still dominate the beef herds of North and South America, Australia, and New Zealand. Furthermore, the Hereford has contributed to the formation and improvement of at least two dozen breeds across the world (Porter, 1991). For example, the Kazakh White-headed cattle breed was developed by crossing local cattle from Kazakhstan with Hereford cattle, imported from England and Uruguay between 1928 and 1932 (Porter, 1991). If Eu1 was distributed in Hereford beef cattle it has not remained within this breed; isolates from Korea and many of the isolates from the GB were from dairy cattle.

Secondary dispersal. The dispersal of Eu1 strains may be more complicated than a simple bovine diaspora from the UK. For example, in the Republic of Korea Eu1 strains were identified and Holstein cattle were imported to Korea from France in 1902; the first report of bovine TB in Korea was in 1913 (Wee et al., 2009). However, the most likely source of the Eu1 strains in Korea identified here are the many Holstein dairy cattle imported in the 1960s from USA, Canada, New Zealand and Australia, all countries where strains of the Eu1 clonal complex have been identified at high frequency (Bae, 1997; Wee

et al., 2009). The Eu1 strains currently found in the Republic of Korea may represent the introduction of the disease from a source other than the UK and it is interesting to note the dominance of the SB0140 spoligotype pattern in both Australian and Korean isolates.

A complex history of cattle importation may even apply to the English speaking former British colonies. South Africa imported cattle not just from Europe but also from Argentina and Australia (Huchzermeyer et al., 1994). For both Australia and New Zealand, again, the introduction of the Eu1 clonal complex may not have been directly from the UK. The import of cattle to Australia has been recorded since the 1790's, however, these cattle were not primarily imported from the UK (Pierce, 1975). Between 1788 and 1825 cattle were imported from the, then, British colonies of India and South Africa. The initial import of cattle into New Zealand were from Australia in 1814 (Pierce, 1975). It was not until 1871 that Australia introduced a quarantine act to provide protection from various cattle diseases. However, the Custom Act of 1879 banned the import of cattle and sheep from all countries except GB and the RoI.

Why is Eu1 so common? The simplest explanation for the global dominance of the Eu1 clonal complex is demography. Perhaps the Eu1 clonal complex was the lucky group of strains that happened to be distributed throughout the world as specialized breeds of cattle were exported from a single source and then re-exported between other countries.

However, an obvious explanation for the dominance of the Eu1 clonal complex over other strains of *M. bovis* is increased fitness such as reduced virulence (asymptomatic disease) or increase transmissibility. It is not clear to us that the RDEu1 deletion does convey such a fitness advantage; as discussed above the loss of *treY*

function may be selectively neutral. Furthermore, just because a clonal complex is common does not necessarily imply that it has a fitness advantage. We note that strains of the Eu1 clonal complex have frequently become established in wildlife species: brush-tailed possums in New Zealand; white-tailed deer in Michigan, USA; wild boar in Hawaii, USA; badgers in GB and buffalo in South Africa. However, the ability to 'jump host' is not a unique characteristic for Eu1 strains; another clonal complex of *M. bovis* has established itself in wildlife in the Kruger National Park. It is more likely that the frequency of Eu1 strains in wildlife reflects the global prevalence of these strains worldwide, and thus an increased chance of spill over, rather than a specific attribute of this clonal complex.

In conclusion. The Eu1 clonal complex of *M. bovis* is common in many countries throughout the world (Figure 2). Although the number of strains sampled was small for many countries we were, nonetheless, able to demonstrate the presence of Eu1 clonal complex strains. We do not have enough data to measure the ultimate importance of this clonal complex but it must constitute a significant proportion of the total bovine TB in the world. We are not convinced that Eu1 has a selective advantage over other clones of *M. bovis* and we suggest that simple demography might better explain the global distribution of Eu1; it was the lucky clone in the right place at the right time.

We note the association of the Eu1 clonal complex with countries that were formally part of the British Empire, yet, this is not a simple relationship. The Eu1 clonal complex is not at high frequency in the former British colonies of Nigeria, Uganda, and Tanzania (Berg et al., 2011; Muller et al., 2009) and we suspect that the global

distribution of this clonal complex may be more complex than a simple dispersal from one country. Furthermore, it is entirely possible that the Eu1 clonal complex did not evolve in the UK but was imported into the UK from another country; in which case the UK may have merely been a distribution center for a clonal complex of bovine TB that evolved elsewhere. We note that Hereford beef cattle, bred in and distributed from the UK since the 19th century, would have provided a good vehicle for the global distribution of this clonal complex.

For the molecular epidemiologist the identification of clonal complexes provides a new tool in the analysis of otherwise large and intractable genotype datasets. In combination with geographical localization of genotype, which is becoming an important observation for genotypes of *M. bovis*, the analysis of clonal complexes can be used to attribute imported strains to their International source. This has been done successfully with strains of the Af1 and Af2 clonal complexes isolated from humans in the UK and France and, in unpublished data, Eu1 strains in Italy were found in cattle recently imported from the British isles and thus given unequivocal attribution to source. However, and perhaps more important , the identification of clonal complexes is generating testable hypotheses that are a first step in understanding the phylogeography, demography and global distribution of this important veterinary pathogen

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TABLES

Table 1. The frequency of the Eu1 clonal complex of *M. bovis* in the European nations surveyed by deletion typing and spoligotyping.

RDEu1 deletion surveys of European strains.						
Country	Reference	Number of isolates	Number of spoligotype patterns	Percentage of strains with spacer 11 missing	Number of strains deletion typed for RDEu1	Maximum % of Eu1 strains *
Great Britain	This study	490	13	97.3	13	97.3
Northern Ireland	This study	528	11	100.0	11	100.0
Republic of Ireland	This study	503	29	100.0	25	100.0
France	Hadad et al.,2001	1349	153	14.2	95	13.8
Portugal	Duarte et al., 2008	283	29	11.8	58	7.6
Spain	Rodriguez et al., 2009	6215	252	10.7	45	6.1
Italy	Boniotti et al., 2009	1503	76	1.0	40	<1.0
Belgium	Allix et al., 2006	127	17	49.6	8	<1.0
The Netherlands	This study	41 (humans)	18	17.0	15	0.0
Sweden	This study	20 (humans)	5	25	20	25.0
Germany	Kubica et al. 2003	176 (human, animal)	59	36.9	44	<1.0

* The percentage of strains with spacer 11 missing was taken as the starting point for calculating the maximum percentage of the Eu1 clonal complex in each population. Then strains with the commonest spoligotype patterns were assayed for the RDEu1 deletion. Strain with spacer 11 deleted that were not members of the Eu1 clonal complex reduced the maximum possible percentage of the Eu1 clonal complex in each population.

Table 2. The frequency of the *M. bovis* Eu1 clonal complex in the African nations surveyed by both deletion typing and previously published spoligotype surveys.

		Surveys of African strains					
Country	Reference	Type of survey	Number of strains	Number of spoligotype patterns	Percentage of strains with spacer 11 missing	Number of strains deletion typed for RDEu1	Maximum % of Eu1 strains
Nigeria	(Muller et al. 2009)	Abattoir	178	34	4.5	3	0.0
Cameroon	(Muller et al. 2009)	National	75	10	13.0	16	0.0
Mali	(Muller et al. 2009)	Abattoir	20	7	0.0	3	0.0
Chad	(Muller et al. 2009)	Abattoir	65	13	1.5	5	0.0
Ethiopia	Berg et al., 2009	National	58	7	0.0	15	0.0
Burundi	(Rigouts, Maregeya et al. 1996)	National	10	3	0.0	10	0.0
Tanzania	(Muller et al. 2009)	Abattoir	14	3	36.0	13	7.0
Mozambique	unpublished	Localised	12	1	0.0	12	0.0
South Africa	(Michel et al. 2008)	National	50	12	62.0	35	62.0
Algeria	(Sahraoui et al. 2009)	Abattoir	88	22	1.0	Spoligotype survey*	1.0
Uganda	(Oloya et al. 2007)	humans	19	10	0.0	Spoligotype survey	0.0
Uganda	(Asiimwe et al. 2009)	Abattoir	11	6	0.0	Spoligotype survey	0.0
Madagascar	(Rasolofo Razanamparany et al. 2006)	National	100	12	2.0	Spoligotype survey	2.0
Zambia	(Munyeme et al. 2009)	Localised	31	10	6.4	Spoligotype survey	6.4

*Previously published surveys by spoligotype only.

Table 3. Definition and summary of the European 1 (Eu1) clonal complex of *M. bovis.*

European 1 (Eu1) clonal complex of *M. bovis*	
Definition	Presence of deletion RDEu1 (806 bp in *TreY*)
Spoligotype marker	Absence of spacer 11
Spoligotype signature[a]	11011111010111101111111111111111111111100000 (SB0130)
Most common	11011010000011101111111111111111111111100000 (SB0140)
Distribution	At high frequency in: The British isles, South Africa, Australia, New Zealand, The New World (except Brazil), Korea. At low frequency (<10%) in: Brazil, Spain, Portugal Found in many other countries
IS*6110* copy number	1 or 2 copies (infrequently 3 or 4)

[a] The spoligotype signature represents the assumed spoligotype pattern in the progenitor strain of this clonal complex and is shown as a series of 1s and 0s with 1 representing hybridisation to the spacer and 0 representing absence of hybridisation. International names for these spoligotype patterns were assigned by www.Mbovis.org

FIGURES

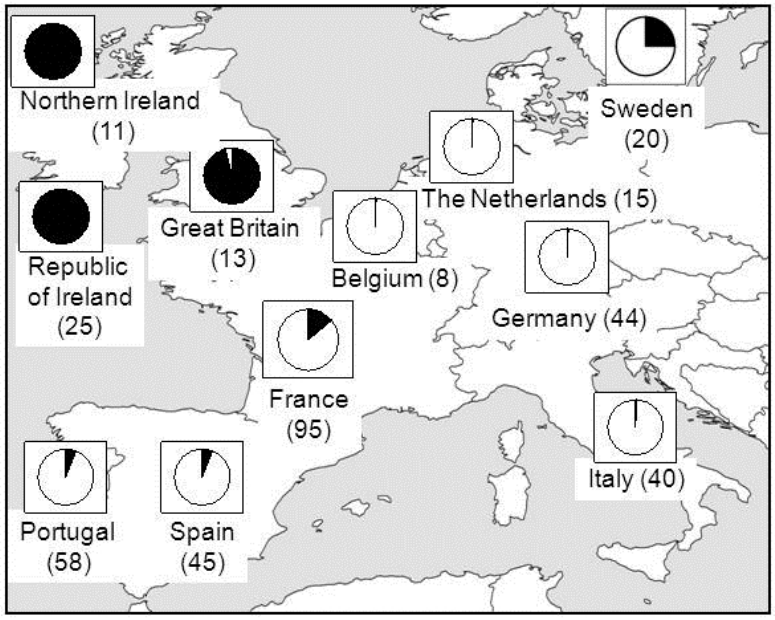

FIG. 1. Distribution of the Eu1 clonal complex of *M. bovis* throughout Europe. The pie charts show the proportion of isolates that are members of the Eu1 clonal complex; black = Eu1, white = other clonal complexes. The number of strains deletion typed for RDEu1 in each region are shown in parentheses. Strains of Eu1 are dominant in the RoI and the UK, at less than 14% frequency in France and the Iberian Peninsula and rare in the other countries surveyed. The proportion of Eu1 strains in Sweden, The Netherlands and Germany was determined from human samples assuming they reflect the population structure of bovine TB prior to its elimination from cattle

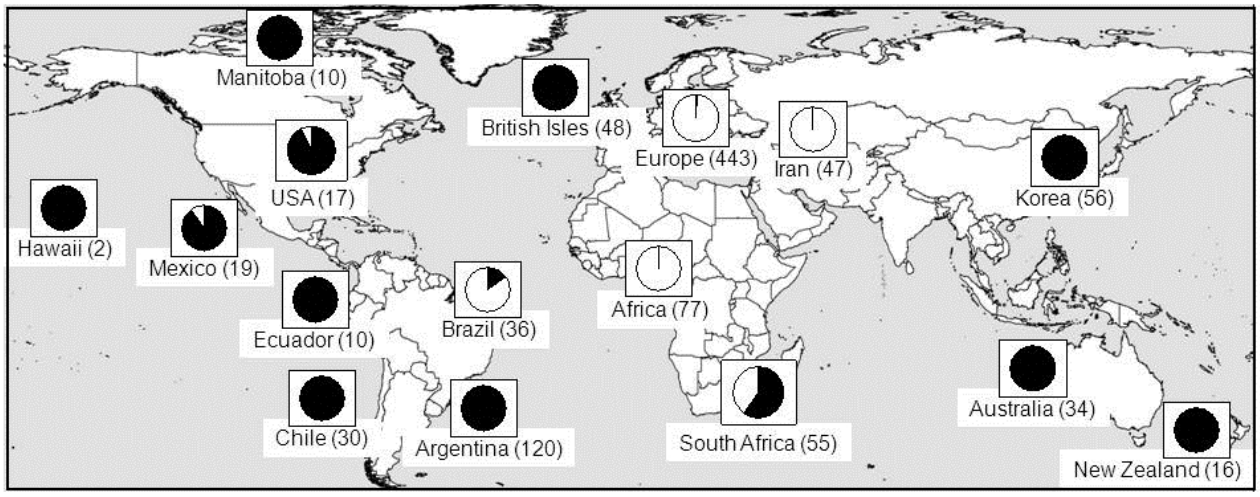

FIG. 2. Distribution of the Eu1 clonal complex of *M. bovis* in the countries surveyed. The pie charts show the proportion of isolates that are members of the Eu1 clonal complex; black = Eu1, white = other clonal complexes. The number of strains deletion typed for RDEu1 in each region are shown in parentheses. Eu1 strains have also been found in humans in Kazakhstan and Taiwan.