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

Noel H. Smith, Stefan Berg, James Dale, Adrian Allen, Sabrina Rodriguez, Beatriz Romero, Filipa Matos, Solomon Ghebremichael, Claudine Karoui, Chiara Donati, Adelina da Conceicao Machado, Custodia Mucavele, Rudovick R. Kazwala, Markus Hilty, Simeon Cadmus, Bongo Naré Richard Ngandolo , Meseret Habtamu, James Oloya, Annéle Muller, Feliciano Milian-Suazo, Olga Andrievskaia, Michaela Projahn, Soledad Barandiarán, Analía Macías, Borna Müller, Marcos Santos Zanini, Cassia Yumi Ikuta, Cesar Alejandro Rosales Rodriguez, Sônia Regina Pinheiro, Alvaro Figueroa, Sang-Nae Cho, Nader Mosavari, Pei-Chun Chuang, Ruwen Jou, Jakob Zinsstag, Dick van Soolingen, Eamonn Costello, Abraham Aseffa, Freddy Proaño-Perez, Françoise Portaels, Leen Rigouts, Angel Adrián Cataldi, Desmond M. Collins, María Laura Boschioli, R. Glyn Hewinson, José Soares Ferreira Neto, Om Surujballi, Keyvan Tadyon, Ana Botelho, Ana María Zárraga, Nicky Buller, Robin Skuce, Anita Michel, Alicia Aranaz, Stephen V. Gordon, Bo-Young Jeon, Gunilla Källenius, Stefan Niemann, M. Beatrice Boniotti, Paul D. van Helden, Beth Harris, Martín José Zumárraga and Kristin Kremer.

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

*Corresponding author: Dr. Noel H. Smith, VLA Weybridge, New Haw, Surrey KT15 3NB, UK; email:Noel@Sussex.ac.uk

Key words: World; bovine tuberculosis; clonal complex; localisation; cattle; phylogeography; *Mycobacterium bovis*

Dr. Noel H. Smith
Email: Noel@Sussex.ac.uk
Tel: +44 1932 341111
Centre for the Study of Evolution, University of Sussex and Veterinary Laboratories Agency, Weybridge,
New Haw, Surrey KT15 3NB, UK.

Dr. Stefan Berg
Email: s.berg@vla.defra.gsi.gov.uk
Tel: +44 1932 341111
Veterinary Laboratories Agency, Weybridge, New Haw, Surrey KT15 3NB, UK.

Mr. James Dale
Email: j.dale@vla.defra.gsi.gov.uk
Tel: +44 1932 341111
Veterinary Laboratories Agency, Weybridge, New Haw, Surrey KT15 3NB, UK.

Dr. Adrian Allen
Email: Adrian.Allen@afbini.gov.uk
Tel: +44 (0)28 90 255689
Agri Food and Biosciences Institute, AFBI Stormont, Stoney Road, Belfast, BT4 3SD, UK

Ms. Sabrina Rodriguez
Email: sabrina.rodriguez@visavet.ucm.es
Tel: +34 91 3944089
Dept. de Sanidad Animal, Facultad de Veterinaria, and Centro Vigilancia Sanitaria Veterinaria
(VISAVET), Universidad Complutense, Avenida, Puerta de Hierro s/n, 28040 Madrid, Spain

Ms. Beatriz Romero
Email: bromerom@visavet.ucm.es
Tel: +34 91 3944096
Dept. de Sanidad Animal, Facultad de Veterinaria, and Centro Vigilancia Sanitaria Veterinaria
(VISAVET), Universidad Complutense, Avenida, Puerta de Hierro s/n, 28040 Madrid, Spain

Ms. Filipa Matos
Email: filipadematos@gmail.com
Tel: +351 217115340
Laboratório Nacional de Investigação Veterinária (INRB, IP-LNIV) Estrada de Benfica, 701, 1549-011,
Lisboa, Portugal

Mr. Solomon Ghebremichael
Email: solomon.ghebremichael@smi.se
Tel: +46 8 4572300
Department of Bacteriology, Swedish Institute for Infectious Disease Control, S-17182 Solna, Sweden

Ms. Claudine Karoui
Email: c.karoui@afssa.fr
Tel: 33 1 49 77 13 00
Unité de Zoonoses Bactériennes, AFSSA-LERPAZ, 23, avenue du Général de Gaulle, 94706, Maisons-
Alfort, France

Dr. Chiara Donati
Email: chdonati@libero.it
Tel: 0039 030 2290 273
Reparto Genomica, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia - Via Bianchi n. 9 -
25124 Brescia - Italia

Ms. Adelina da Conceicao Machado
Email: adelm1966@hotmail.com
Tel: (+258) 21 475155
Faculdade de Veterinaria, Universidade Eduardo Mondlane, CP 257 Maputo, Mocambique.

Ms. Custodia Mucavele
Email: custodiamucavele@hotmail.com
Tel: +258 21 475155
Faculdade de Veterinaria, Universidade Eduardo Mondlane, CP 257 Maputo, Mocambique.

Prof. Rudovick R. Kazwala
Email: kazwala@gmail.com
Tel: +255 23 2604542
Sokoine University of Agriculture, Morogoro, Tanzania

Dr. Markus Hilty
Email: Markus.Hilty@ifik.unibe.ch
Tel: 41 31 632 49 83
Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, CH-3010 Bern, Switzerland

Dr. Simeon Cadmus
Email: sibcadmus@yahoo.com
Tel: 234 80 237 51093
Department of Veterinary Public Health & Preventive Medicine. University of Ibadan, Ibadan, Nigeria.

Mr. Bongo Naré Richard Ngandolo
Email: bongo_nar@yahoo.fr
Tel: +235 66 23 05 24
Laboratoire de Recherches Vétérinaire et Zootechnique de Farcha, BP 433, N'Djaména, Chad

Ms. Meseret Habtamu
Email: mekonnen.meseret@gmail.com
Tel: 251 113 211334
Armauer Hansen Research Institute, P.O. Box 1005, Addis Ababa, Ethiopia.

Dr. James Oloya
Email: joloya2001@yahoo.co.uk
Tel: +1 706 583 0918
Department of Epidemiology & Biostatistics/Population Health, College of Public Health, 132 Coverdell Centre, University of Georgia, Athens, GA, 30602-7396, USA

Ms. Annélie Muller
Email: annelle_m@sun.ac.za
Tel: +27-21-9389401
Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg, South Africa, 7505

Dr. Feliciano Milian-Suazo
Email: miliansf@yahoo.es
Tel: 52 4192920036
Centro Nacional de Investigación Disciplinaria en Fisiología y Mejoramiento Animal-INIFAP, Km 1 Carretera a Colón, Ajuchitlán, Querétaro. México. C.P. 76280

Dr. Olga Andrievskaia
Email: Olga.Andrievskaia@inspection.gc.ca
Tel: +1-613-228-6698
Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield Ottawa, 3851 Fallowfield Rd., Ottawa, Ontario, K2H 8P9, Canada

Ms. Michaela Projahn
Email: mprojahn@fz-borstel.de
Tel: 0049-4537188274
Molecular Mycobacteriology, Research Center Borstel, Parkallee 1, 23845 Borstel, Germany

Ms. Soledad Barandiarán
Email: SBaran@fvvet.uba.ar
Tel: +54-11-524-8407

School of Veterinary Medicine of Buenos Aires University, Argentina.

Ms. Analía Macías

Email: amacias@ayv.unrc.edu.ar

Tel: +54-03584676510

School of Veterinary Medicine of Rio IV, University, Córdoba, Argentina.

Dr. Borna Müller

Email: bmuller@sun.ac.za

Tel: +27-21-9389482

Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg, South Africa, 7505

Dr. Marcos Santos Zanini

Email: zanini@cca.ufes.br

Tel: 55-28-3552.8916

Dept. Medicina Veterinária, Centro de Ciencias Agrárias, Universidade Federal do Espírito Santo, Brasil

Ms. Cassia Yumi Ikuta

Email: cassiayi@yahoo.com.br

Tel: 55 11-30917927

Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária – SP/SP, CEP 05508-270, Brasil

Dr. Cesar Alejandro Rosales Rodriguez

Email: pancho@usp.br

Tel: 55 11-30917927

Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária – SP/SP, CEP 05508-270, Brasil

Prof. Sônia Regina Pinheiro

Email: soniapi@usp.br

Tel: 55 11-3091.1383

Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine and Zootecnic, University of São Paulo. Av. Prof. Dr. Orlando Marques de Paiva n.87 Cidade Universitária - São Paulo (SP), CEP 05508-270, Brasil

Mr. Alvaro Figueroa

Email: alvaroalejandrofigueroa@gmail.com

Tel: 56-63-221907

Instituto de Bioquímica, Universidad Austral de Chile, Valdivia. Chile

Prof. Sang-Nae Cho

Email: raycho@yuhs.ac

Tel: +822-2228-1819

Department of Microbiology, Yonsei University College of Medicine, 250 Seongsanno, Seodaemun-gu, Seoul, 120-752, Republic of Korea

Dr. Nader Mosavari

Email: n.mosavari@rsvi.ir & n.mosavari@gmail.com

Tel: +98-261-4502895

PPD Tuberculin Department, Razi Vaccine & Serum Research Institute, Karaj 3197619751, Iran

Dr. Pei-Chun Chuang

Email: peichun@cdc.gov.tw

Tel: (+886)2-26531369

Reference Laboratory of Mycobacteriology, Research and Diagnostic Center, Centers for Disease Control, Department of Health, 161 Kun-Yang Street, Nan-Kang, Taipei, 115, Taiwan, Republic of China.

Prof. Ruwen Jou
Email: rwj@cdc.gov.tw
Tel: (+886)2-26531370
Reference Laboratory of Mycobacteriology, Research and Diagnostic Center, Centers for Disease Control, Department of Health, 161 Kun-Yang Street, Nan-Kang, Taipei, 115, Taiwan, Republic of China.

Dr. Jakob Zinsstag
Email: Jakob.Zinsstag@unibas.ch
Tel: +41612848139
Swiss Tropical and Public Health Institute, Socinstrasse 57, 4002 Basel, Switzerland.

Dr. Dick van Soolingen
Email: dick.van.soolingen@rivm.nl
Tel: +31-30-2742363
Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control (CIb/LIS), P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Mr. Eamonn Costello
Email: eamonn.costello@agriculture.gov.ie
Tel: +353 1 6157145
Central Veterinary Research Laboratory, Backweston Laboratory Complex, Celbridge, Co. Kildare, Republic of Ireland.

Dr. Abraham Aseffa
Email: aseffaa@gmail.com
Tel: +251911247525
Armauer Hansen Research Institute, P.O. Box 1005, Addis Ababa, Ethiopia.

Mr. Freddy Proaño-Perez
Email: freddyproanoperez@yahoo.com
Tel: +593 2 2904801
Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium

Prof. Françoise Portaels
Email: portaels@itg.be
Tel: 32 3 2476317
Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium

Dr. Leen Rigouts
Email: lrigouts@itg.be
Tel: 32 3 2476317
Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerpen, Belgium

Prof. Angel Adrián Cataldi
Email: acataldi@cni.inta.gov.ar
Tel: +54 11 4621 1447 ext. 109
Biotechnology Institute of INTA, CICVyA, Castelar. N. Repetto y Los Reseros s/n 1686-, Hurlingham, Buenos Aires, Argentina

Dr. Desmond M. Collins
Email: desmond.collins@agresearch.co.nz
Tel: +64 4 529 0310
AgResearch, National Centre for Biosecurity and Infectious Disease, Wallaceville, P. O. Box 40063, Upper Hutt, New Zealand

Dr. María Laura Boschioli
Email: ml.boschioli@AFSSA.FR
Tel: +33 (0)1 49 77 13 21
Unité de Zoonoses Bactériennes, AFSSA-LERPAZ, 23, avenue du Général de Gaulle, 94706, Maisons-

Alfort, France

Prof. R. Glyn Hewinson
Email: r.g.hewinson@vla.defra.gsi.gov.uk
Tel: +44 1932 341111
Veterinary Laboratories Agency Weybridge, New Haw, Surrey KT15 3NB, UK.

Prof. José Soares Ferreira Neto
Email: jsoares@vps.fmvz.usp.br
Tel: 55 11-30917927
Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária – SP/SP, CEP 05508-270, Brasil

Dr. Om Surujballi
Email: Om.Surujballi@inspection.gc.ca
Tel: +1-613-228-6698
Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield Ottawa, 3851 Fallowfield Rd., Ottawa, Ontario, K2H 8P9, Canada

Dr. Keyvan Tadyon
Email: mmb093@googlemail.com & k.tadayon@rvsri.ir
Tel: +98-261-4502892
Department of Veterinary Aerobic Bacterial Research & Vaccine Production, Razi Vaccine and Serum Research Institute, Karaj 3197619751, Iran

Dr. Ana Botelho
Email: ANA.BOTELHO@LNIV.MIN-Agricultura.pt
Tel: +351 217115339
Laboratório Nacional de Investigação Veterinária (INRB, IP-LNIV) Estrada de Benfica, 701, 1549-011, Lisboa, Portugal

Dr. Ana María Zárraga
Email: anamaria.zarraga@gmail.com
Tel: 56-63-221907
Instituto de Bioquímica, Universidad Austral de Chile, Valdivia. Chile.

Dr. Nicky Buller
Email: nicky.buller@agric.wa.gov.au
Tel: 08-93683425
Australian Reference Laboratory for Bovine Tuberculosis, Animal Health Laboratories, Department of Agriculture and Food Western Australia, 3 Baron-Hay Court, South Perth WA 6151, Australia

Dr. Robin Skuce
Email: Robin.Skuce@afbini.gov.uk
Tel: +44 (0)28 90 525771
Agri Food and Biosciences Institute, AFBI Stormont, Stoney Road, Belfast, BT4 3SD, UK

Dr. Anita Michel
Email: Anita.Michel@up.ac.za
Tel: +27 12 5299 384
Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110 and ARC-Onderstepoort Veterinary Institute, Private Bag x05, Onderstepoort 0110, South Africa

Dr. Alicia Aranaz
Email: alaranaz@vet.ucm.es
Tel: +34 91 3943721
Dept. Sanidad Animal, Facultad de Veterinaria and Centro Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Avenida Puerta de Hierro s/n, 28040 Madrid, Spain

Dr. Stephen V. Gordon
Email: stephen.gordon@ucd.ie

Tel: 353 (0)1 7166181
Schools of Agriculture, Food Science and Veterinary Medicine, Medicine and Medical Science,
Biomolecular and Biomedical Science, College of Life Sciences, and UCD Conway Institute, University
College Dublin, Dublin 4, Ireland.

Dr. Bo-Young Jeon
Email: bojeon87@gmail.com
Tel: +822-2228-2548
Department of Microbiology, Yonsei University College of Medicine, 250 Seongsanno, Seodaemun-gu,
Seoul, 120-752, Republic of Korea

Prof. Gunilla Källénus
Email: Gunilla.kallenius@ki.se
Tel: +46 70 6741517
Department of Clinical Science and Education, Karolinska Institutet, Södersjukhuset, 118 83 Stockholm

Dr. Stefan Niemann
Email: sniemann@fz-borstel.de
Tel: 0049-4537188762
Mycobacteriology, Research Center Borstel, Parkallee 1, 23845 Borstel, Germany

Dr. M. Beatrice Boniotti
Email: mariabeatrice.boniotti@izsler.it
Tel: 0039 030 2290 273
Reparto Genomica, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia - Via Bianchi n. 9 -
25124 Brescia - Italia

Prof. Paul D. van Helden
Email: PVH@sun.ac.za
Tel: 27-21-9389401
Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, Stellenbosch University,
PO Box 19063, Tygerberg, South Africa, 7505

Dr. Beth Harris
Email: Beth.N.Harris@aphis.usda.gov
Tel: 1-515-663-7362
U.S. Dept. of Agriculture, Animal and Plant Health Inspection Services, National Veterinary Services
Laboratories, Mycobacteria and Brucella Section 1920 Dayton Ave. Ames, IA 50010

Dr. Martín José Zumárraga
Email: mzumarraga@cni.inta.gov.ar
Tel: +54 11 4621 1447 ext. 145
Biotechnology Institute of INTA, CICVyA, Castelar. N. Repetto y Los Reseros s/n 1686-, Hurlingham,
Buenos Aires, Argentina

Dr. Kristin Kremer
Email: Kristin.Kremer@rivm.nl
Tel: +31-30-2742720
Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment (RIVM),
Centre for Infectious Disease Control (CIb/LIS), P.O. Box 1, 3720 BA Bilthoven, The Netherlands

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 is 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 subspecies 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' CCGATGAACTTGGCCACAG 3' (position 1767904 to 1767923 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 Rol 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

ACKNOWLEDGEMENTS

We thank M. Okker and K. Gover from the VLA, and R. de Zwaan from the RIVM for excellent technical help. This work was funded by: TBadapt project (LSHp-CT-2007-

037919); BM received financial support from the Swiss National Science Foundation; Swedish Research Council, Swedish Heart-Lung foundation, Swedish International Development Agency; Department of Agriculture and Rural Development Northern Ireland (project DARD0407); EU project TB-STEP (KBBE-2007-1-3-04, no. 212414); Swiss National Science Foundation (Grant No. 107559); Damien Foundation, Belgium; Commission Universitaire pour le Développement (CUD), University of Liege (Project PIC); The Wellcome Trust Livestock for Life and Animal Health in the Developing World initiatives (075833/A/04/Z); Chilean National Livestock Service -FONDOSAGC5-100-10-23 and CONICYT-FIC-R-EQU18 and by the Department of Environment, Food and Rural Affairs, UK (project SB4020).

References

- Allix, C., Walravens, K., Saegerman, C., Godfroid, J., Supply, P., Fauville-Dufaux, M., 2006. Evaluation of the epidemiological relevance of variable-number tandem-repeat genotyping of *Mycobacterium bovis* and comparison of the method with IS6110 restriction fragment length polymorphism analysis and spoligotyping. *J Clin Microbiol* 44, 1951-1962.
- Amanfu, W., 2006. The situation of tuberculosis and tuberculosis control in animals of economic interest. *Tuberculosis (Edinb)* 86, 330-335.
- Ayele, W.Y., Neill, S.D., Zinsstag, J., Weiss, M.G., Pavlik, I., 2004. Bovine tuberculosis: an old disease but a new threat to Africa. *Int J Tuberc Lung Dis* 8, 924-937.
- Bae, D.-H., 1997. *Dairy Science : The principle and application*. Sunjin Press, Seoul.
- Bany, S.A., Freier, J.E., 2000. Use of GIS to evaluate livestock-wildlife interactions relative to tuberculosis spread on Molokai Island, Hawaii. U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Centers for Epidemiology and Animal Health, Fort Collins, CO.
- Berg, S., Firdessa, R., Habtamu, M., Gadisa, E., Mengistu, A., Yamuah, L., Ameni, G., Vordermeier, M., Robertson, B.D., Smith, N.H., Engers, H., Young, D., Hewinson, R.G., Aseffa, A., Gordon, S.V., 2009. The burden of mycobacterial disease in ethiopian cattle: implications for public health. *PLoS One* 4, e5068.
- Berg, S., Garcia-Pelayo, M.C., Muller, B., Hailu, E., Asimwe, B., Kremer, K., Dale, J., Boniotti, M.B., Rodriguez, S., Hilty, M., Rigouts, L., Firdessa, R., Machado, A., Mucavele, C., Ngandolo, B.N., Bruchfeld, J., Boschioli, L., Muller, A., Sahraoui, N., Pacciarini, M., Cadmus, S., Joloba, M., van Soelingen, D., Michel, A.L., Djonne, B., Aranaz, A., Zinsstag, J., van Helden, P., Portaels, F., Kazwala, R., Kallenius, G., Hewinson, R.G., Aseffa, A., Gordon, S.V., Smith, N.H., 2011. African 2, a Clonal Complex of *Mycobacterium bovis* Epidemiologically Important in East Africa. *J Bacteriol* 193, 670-678.
- Boniotti, M.B., Gorla, M., Loda, D., Garrone, A., Benedetto, A., Mondo, A., Tisato, E., Zanoni, M., Zoppi, S., Dondo, A., Tagliabue, S., Bonora, S., Zanardi, G., Pacciarini, M.L., 2009. Molecular typing of *Mycobacterium bovis* strains isolated in Italy from 2000 to 2006 and evaluation of

variable-number tandem repeats for geographically optimized genotyping. *J Clin Microbiol* 47, 636-644.

-Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D., Cole, S.T., 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci U S A* 99, 3684-3689.

-Cadmus, S., Palmer, S., Okker, M., Dale, J., Gover, K., Smith, N., Jahans, K., Hewinson, R.G., Gordon, S.V., 2006. Molecular analysis of human and bovine tubercle bacilli from a local setting in Nigeria. *J Clin Microbiol* 44, 29-34.

-Cataldi, A.A., Goffre, A., Santtangelo, M.P., Alito, A., Caimi, K., Bigi, F., Romano, M.I., Zumarraga, M., 2002. The genotype of the principal *Mycobacterium bovis* in Argentina is also that of the British Isles: Did bovine tuberculosis come from Great Britain? *Rev. Argent. Microbiol.* 34, 1-6.

-Cobos-Marin, L., Montes-Vargas, J., Zumarraga, M., Cataldi, A., Romano, M.I., Estrada-Garcia, I., Gonzalez-y-Merchand, J.A., 2005. Spoligotype analysis of *Mycobacterium bovis* isolates from Northern Mexico. *Can J Microbiol* 51, 996-1000.

-Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Barrell, B.G., et al., 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537-544.

-Collins, D.M., Erasmuson, S.K., Stephens, D.M., Yates, G.F., De Lisle, G.W., 1993. DNA fingerprinting of *Mycobacterium bovis* strains by restriction fragment analysis and hybridization with insertion elements IS1081 and IS6110. *J Clin Microbiol* 31, 1143-1147.

-Cosivi, O., Grange, J.M., Daborn, C.J., Raviglione, M.C., Fujikura, T., Cousins, D., Robinson, R.A., Huchzermeyer, H.F., de Kantor, I., Meslin, F.X., 1998. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerg Infect Dis* 4, 59-70.

-Cosivi, O., Meslin, F.X., Daborn, C.J., Grange, J.M., 1995. Epidemiology of *Mycobacterium bovis* infection in animals and humans, with particular reference to Africa. *Rev Sci Tech* 14, 733-746.

-Costello, E., O'Grady, D., Flynn, O., O'Brien, R., Rogers, M., Quigley, F., Egan, J., Griffin, J., 1999. Study of restriction fragment length polymorphism analysis and spoligotyping for epidemiological investigation of *Mycobacterium bovis* infection. *J Clin Microbiol* 37, 3217-3222.

-Cousins, D., Williams, S., Liãebana, E., Aranaz, A., Bunschoten, A., Van Embden, J., Ellis, T., 1998. Evaluation of four DNA typing techniques in epidemiological investigations of bovine tuberculosis. *J Clin Microbiol* 36, 168-178.

-Cousins, D.V., Roberts, J.L., 2001. Australia's campaign to eradicate bovine tuberculosis: the battle for freedom and beyond. *Tuberculosis (Edinb)* 81, 5-15.

-de Kantor, I.N., Ambroggi, M., Poggi, S., Morcillo, N., Da Silva Telles, M.A., Osorio Ribeiro, M., Garzon Torres, M.C., Llerena Polo, C., Ribon, W., Garcia, V., Kuffo, D., Asencios, L., Vasquez Campos, L.M., Rivas, C., de Waard, J.H., 2008. Human *Mycobacterium bovis* infection in ten Latin American countries. *Tuberculosis (Edinb)* 88, 358-365.

de Kantor, I.N., Ritacco, V., 2006. An update on bovine tuberculosis programmes in Latin American and Caribbean countries. *Vet Microbiol* 112, 111-118.

-De Smet, K.A., Weston, A., Brown, I.N., Young, D.B., Robertson, B.D., 2000. Three pathways for trehalose biosynthesis in mycobacteria. *Microbiology* 146 (Pt 1), 199-208.

-Decker, J.E., Pires, J.C., Conant, G.C., McKay, S.D., Heaton, M.P., Chen, K., Cooper, A., Vilkki, J., Seabury, C.M., Caetano, A.R., Johnson, G.S., Brenneman, R.A., Hanotte, O., Eggert, L.S., Wiener, P., Kim, J.J., Kim, K.S., Sonstegard, T.S., Van Tassell, C.P., Neiberghs, H.L., McEwan, J.C., Brauning, R., Coutinho, L.L., Babar, M.E., Wilson, G.A., McClure, M.C., Rolf, M.M., Kim, J., Schnabel, R.D., Taylor, J.F., 2009. Resolving the evolution of extant and extinct ruminants with high-throughput phylogenomics. *Proc Natl Acad Sci U S A* 106, 18644-18649.

- Duarte, E.L., Domingos, M., Amado, A., Botelho, A., 2008. Spoligotype diversity of *Mycobacterium bovis* and *Mycobacterium caprae* animal isolates. *Vet Microbiol* 130, 415-421.
- Fang, Z., Morrison, N., Watt, B., Doig, C., Forbes, K.J., 1998. IS6110 transposition and evolutionary scenario of the direct repeat locus in a group of closely related *Mycobacterium tuberculosis* strains. *J Bacteriol* 180, 2102-2109.
- Gagneux, S., Deriemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S., Nicol, M., Niemann, S., Kremer, K., Gutierrez, M.C., Hilty, M., Hopewell, P.C., Small, P.M., 2006. Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*.
- Gagneux, S., Small, P.M., 2007. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. *Lancet Infect Dis* 7, 328-337.
- Gallagher, J., Clifton-Hadley, R.S., 2000. Tuberculosis in badgers; a review of the disease and its significance for other animals. *Res Vet Sci* 69, 203-217.
- Garcia Pelayo, M.C., Uplekar, S., Keniry, A., Mendoza Lopez, P., Garnier, T., Nunez Garcia, J., Boschioli, L., Zhou, X., Parkhill, J., Smith, N., Hewinson, R.G., Cole, S.T., Gordon, S.V., 2009. A comprehensive survey of single nucleotide polymorphisms (SNPs) across *Mycobacterium bovis* strains and *M. bovis* BCG vaccine strains refines the genealogy and defines a minimal set of SNPs that separate virulent *M. bovis* strains and *M. bovis* BCG strains. *Infect Immun* 77, 2230-2238.
- Garnier, T., Eiglmeier, K., Camus, J.C., Medina, N., Mansoor, H., Pryor, M., Duthoy, S., Grondin, S., Lacroix, C., Monsempe, C., Simon, S., Harris, B., Atkin, R., Doggett, J., Mayes, R., Keating, L., Wheeler, P.R., Parkhill, J., Barrell, B.G., Cole, S.T., Gordon, S.V., Hewinson, R.G., 2003. The complete genome sequence of *Mycobacterium bovis*. *Proc Natl Acad Sci U S A* 100, 7877-7882.
- Gordon, S.V., Eiglmeier, K., Garnier, T., Brosch, R., Parkhill, J., Barrell, B., Cole, S.T., Hewinson, R.G., 2001. Genomics of *Mycobacterium bovis*. *Tuberculosis (Edinb)* 81, 157-163.
- Groenen, P.M., Bunschoten, A.E., van Soolingen, D., van Embden, J.D., 1993. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Mol Microbiol* 10, 1057-1065.
- Gutacker, M.M., Smoot, J.C., Migliaccio, C.A., Ricklefs, S.M., Hua, S., Cousins, D.V., Graviss, E.A., Shashkina, E., Kreiswirth, B.N., Musser, J.M., 2002. Genome-wide analysis of synonymous single nucleotide polymorphisms in *Mycobacterium tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics* 162, 1533-1543.
- Hartung, M., 2001. Bericht u"ber die epidemiologische Situation der Zoonosen in Deutschland fur 2000–Ubersicht uber die Meldungen der Bundeslander. RKI-Hausdruckerei, Berlin.
- Hershberg, R., Lipatov, M., Small, P.M., Sheffer, H., Niemann, S., Homolka, S., Roach, J.C., Kremer, K., Petrov, D.A., Feldman, M.W., Gagneux, S., 2009. High functional diversity in *M. tuberculosis* driven by genetic drift and human demography. *PLoS Biology* 6, 12.
- Huard, R.C., Fabre, M., de Haas, P., Lazzarini, L.C., van Soolingen, D., Cousins, D., Ho, J.L., 2006. Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. *J Bacteriol* 188, 4271-4287.
- Huchzermeyer, H., Brueckner, G., van Heerden, A., Kleeberg, H., van Rensburg, I., Koen, P., Loveday, R., 1994. Tuberculosis, in: Coetzer, J., Thomson, G., Tustin, R. (Eds.), *Infectious Diseases of Livestock with special reference to Southern Africa*. Oxford University Press, Oxford, pp. 1973-1992.
- Jenkins, H.E., Woodroffe, R., Donnelly, C.A., 2010. The duration of the effects of repeated widespread badger culling on cattle tuberculosis following the cessation of culling. *PLoS One* 5, e9090.
- Jeon, B., Je, S., Park, J., Kim, Y., Lee, E.G., Lee, H., Seo, S., Cho, S.N., 2008. Variable number tandem repeat analysis of *Mycobacterium bovis* isolates from Gyeonggi-do, Korea. *J Vet Sci* 9, 145-153.
- Jou, R., Huang, W.L., Chiang, C.Y., 2008. Human tuberculosis caused by *Mycobacterium bovis*, Taiwan. *Emerg Infect Dis* 14, 515-517.

- Kaasen, I., Falkenberg, P., Styrvoid, O.B., Strom, A.R., 1992. Molecular cloning and physical mapping of the *otsBA* genes, which encode the osmoregulatory trehalose pathway of *Escherichia coli*: evidence that transcription is activated by *katF* (AppR). *J Bacteriol* 174, 889-898.
- Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J., 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 35, 907-914.
- Kubica, T., Agzamova, R., Wright, A., Rakishev, G., Rusch-Gerdes, S., Niemann, S., 2006. *Mycobacterium bovis* isolates with *M. tuberculosis* specific characteristics. *Emerg Infect Dis* 12, 763-765.
- Kubica, T., Rusch-Gerdes, S., Niemann, S., 2003. *Mycobacterium bovis* subsp. *caprae* caused one-third of human *M. bovis*-associated tuberculosis cases reported in Germany between 1999 and 2001. *J Clin Microbiol* 41, 3070-3077.
- Lutze-Wallace, C., Turcotte, C., Sabourin, M., Berlie-Surujballi, G., Barbeau, Y., Watchorn, D., Bell, J., 2005. Spoligotyping of *Mycobacterium bovis* isolates found in Manitoba. *Can J Vet Res* 69, 143-145.
- Marraffini, L.A., Sontheimer, E.J., 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* 11, 181-190.
- Maruta, K., Hattori, K., Nakada, T., Kubota, M., Sugimoto, T., Kurimoto, M., 1996. Cloning and sequencing of trehalose biosynthesis genes from *Rhizobium* sp. M-11. *Biosci Biotechnol Biochem* 60, 717-720.
- McEvoy, C.R., van Helden, P.D., Warren, R.M., Gey van Pittius, N.C., 2009. Evidence for a rapid rate of molecular evolution at the hypervariable and immunogenic *Mycobacterium tuberculosis* PPE38 gene region. *BMC Evol Biol* 9, 237.
- Michel, A.L., Coetzee, M.L., Keet, D.F., Mare, L., Warren, R., Cooper, D., Bengis, R.G., Kremer, K., van Helden, P., 2009. Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves. *Vet Microbiol* 133, 335-343.
- Michel, A.L., Hlokw, T.M., Coetzee, M.L., Mare, L., Connaway, L., Rutten, V.P., Kremer, K., 2008. High *Mycobacterium bovis* genetic diversity in a low prevalence setting. *Vet Microbiol* 126, 151-159.
- Milian-Suazo, F., Harris, B., Diaz, C.A., Romero Torres, C., Stuber, T., Ojeda, G.A., Lored, A.M., Soria, M.P., Payeur, J.B., 2008. Molecular epidemiology of *Mycobacterium bovis*: Usefulness in international trade. *Prev Vet Med*.
- Milian-Suazo, F., Salman, M.D., Ramirez, C., Payeur, J.B., Rhy, J.C., Santillan, M., 2000. Identification of tuberculosis in cattle slaughtered in Mexico. *Am J Vet Res* 61, 86-89.
- Milian, F., Sanchez, L.M., Toledo, P., Ramirez, C., Santillan, M.A., 2000. Descriptive study of human and bovine tuberculosis in Queretaro, Mexico. *Rev Latinoam Microbiol* 42, 13-19.
- Mokrousov, I., Vyazovaya, A., Potapova, Y., Vishnevsky, B., Otten, T., Narvskaya, O., 2010. *Mycobacterium bovis* BCG-Russia clinical isolate with noncanonical spoligotyping profile. *J Clin Microbiol* 48, 4686-4687.
- Mostowy, S., Inwald, J., Gordon, S., Martin, C., Warren, R., Kremer, K., Cousins, D., Behr, M.A., 2005. Revisiting the evolution of *Mycobacterium bovis*. *J Bacteriol* 187, 6386-6395.
- Muller, B., Hilty, M., Berg, S., Garcia-Pelayo, M.C., Dale, J., Boschioli, M.L., Cadmus, S., Ngandolo, B.N., Godreuil, S., Diguimbaye-Djaibe, C., Kazwala, R., Bonfoh, B., Njanpop-Lafourcade, B.M., Sahraoui, N., Guetarni, D., Aseffa, A., Mekonnen, M.H., Razanamparany, V.R., Ramarokoto, H., Djonne, B., Oloya, J., Machado, A., Mucavele, C., Skjerve, E., Portaels, F., Rigouts, L., Michel, A., Muller, A., Kallenius, G., van Helden, P.D., Hewinson, R.G., Zinsstag, J., Gordon, S.V., Smith, N.H., 2009. African 1, an epidemiologically important clonal complex of *Mycobacterium bovis* dominant in Mali, Nigeria, Cameroon, and Chad. *J Bacteriol* 191, 1951-1960.

- Munyeme, M., Rigouts, L., Shamputa, I.C., Muma, J.B., Tryland, M., Skjerve, E., Djonje, B., 2009. Isolation and characterization of *Mycobacterium bovis* strains from indigenous Zambian cattle using Spacer oligonucleotide typing technique. *BMC Microbiol* 9, 144.
- Narayanan, S., Gagneux, S., Hari, L., Tsolaki, A.G., Rajasekhar, S., Narayanan, P.R., Small, P.M., Holmes, S., Deriemer, K., 2008. Genomic interrogation of ancestral *Mycobacterium tuberculosis* from south India. *Infect Genet Evol* 8, 474-483.
- Paterson, A.B., 1948. The production of bovine tuberculo-protein. *J Comp Pathol Ther* 58, 302-313.
- Pavlik, I., 2006. The experience of new European Union Member States concerning the control of bovine tuberculosis. *Vet Microbiol* 112, 221-230.
- Pierce, A.E., 1975. An historical review of animal movement, exotic disease and quarantine in New Zealand and Australia. *New Zealand Veterinary Journal* 23, 125-136.
- Porter, V., 1991. *Cattle - a handbook to the breeds of the world*. A & C Black Ltd, London.
- Rasolofo Razanamparany, V., Quirin, R., Rapaliarijaona, A., Rakotoaritahina, H., Vololonirina, E.J., Rasolonavalona, T., Ferdinand, S., Sola, C., Rastogi, N., Ramarokoto, H., Chanteau, S., 2006. Usefulness of restriction fragment length polymorphism and spoligotyping for epidemiological studies of *Mycobacterium bovis* in Madagascar: description of new genotypes. *Vet Microbiol* 114, 115-122.
- Reviriego Gordejo, F.J., Vermeersch, J.P., 2006. Towards eradication of bovine tuberculosis in the European Union. *Vet Microbiol* 112, 101-109.
- Rhyan, J.C., Spraker, T.R., 2010. Emergence of diseases from wildlife reservoirs. *Vet Pathol* 47, 34-39.
- Ritacco, V., Torres, P., Sequeira, M.D., Reniero, A., de Kantor, I., 2006. Bovine tuberculosis in Latin America and the Caribbean, in: Thoen, C., Steele, J.H., Gilsdorf, M.J. (Eds.). Blackwell Publishing, Ames, IA, pp. 149-160.
- Rodriguez, S., Romero, B., Bezos, J., de Juan, L., Alvarez, J., Castellanos, E., Moya, N., Lozano, F., Gonzalez, S., Saez-Llorente, J.L., Mateos, A., Dominguez, L., Aranaz, A., 2009. High spoligotype diversity within a *Mycobacterium bovis* population: Clues to understanding the demography of the pathogen in Europe. *Vet Microbiol*.
- Rodwell, T.C., Kapasi, A.J., Moore, M., Milian-Suazo, F., Harris, B., Guerrero, L.P., Moser, K., Stratthdee, S.A., Garfein, R.S., 2010. Tracing the origins of *Mycobacterium bovis* tuberculosis in humans in the USA to cattle in Mexico using spoligotyping. *Int J Infect Dis*.
- Sahraoui, N., Muller, B., Guetarni, D., Boulahbal, F., Yala, D., Ouzrout, R., Berg, S., Smith, N.H., Zinsstag, J., 2009. Molecular characterization of *Mycobacterium bovis* strains isolated from cattle slaughtered at two abattoirs in Algeria. *BMC Vet Res* 5, 4.
- Schurch, A.C., Kremer, K., Kiers, A., Boeree, M.J., Siezen, R.J., Soolingen, D., 2011. Preferential Deletion Events in the Direct Repeat Locus of *Mycobacterium tuberculosis*. *J Clin Microbiol* 49, 1318-1322.
- Smith, N.H., Dale, J., Inwald, J., Palmer, S., Gordon, S.V., Hewinson, R.G., Smith, J.M., 2003. The population structure of *Mycobacterium bovis* in Great Britain: clonal expansion. *Proc Natl Acad Sci U S A* 100, 15271-15275.
- Smith, N.H., Gordon, S.V., de la Rua-Domenech, R., Clifton-Hadley, R.S., Hewinson, R.G., 2006a. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. *Nat Rev Microbiol* 4, 670-681.
- Smith, N.H., Kremer, K., Inwald, J., Dale, J., Driscoll, J.R., Gordon, S.V., van Soolingen, D., Hewinson, R.G., Smith, J.M., 2006b. Ecotypes of the *Mycobacterium tuberculosis* complex. *J Theor Biol* 239, 220-225.
- Szewzyk, R., Svenson, S.B., Hoffner, S.E., Bolske, G., Wahlstrom, H., Englund, L., Engvall, A., Kallenius, G., 1995. Molecular epidemiological studies of *Mycobacterium bovis* infections in humans and animals in Sweden. *J Clin Microbiol* 33, 3183-3185.

- Tadayon, K., Mosavari, N., Sadeghi, F., Forbes, K.J., 2008. *Mycobacterium bovis* infection in Holstein Friesian cattle, Iran. *Emerg Infect Dis* 14, 1919-1921.
- Tadayon, K., Mosavari, N., Shahmoradi, A.H., Sadeghi, F., Azarvandi, A., Forbes, K., 2006. The Epidemiology of *Mycobacterium bovis* in Buffalo in Iran. *J Vet Med B Infect Dis Vet Public Health* 53 Suppl 1, 41-42.
- Thoen, C., Lobue, P., de Kantor, I., 2006a. The importance of *Mycobacterium bovis* as a zoonosis. *Vet Microbiol* 112, 339-345.
- Thoen, C.O., Steele, J., Gilsdorf, M.J., 2006b. *Mycobacterium bovis* Infection in Animals and Humans, Second Edition ed. Blackwell Publishing.
- Tsolaki, A.G., Gagneux, S., Pym, A.S., Goguet de la Salmoniere, Y.O., Kreiswirth, B.N., Van Soolingen, D., Small, P.M., 2005. Genomic deletions classify the Beijing/W strains as a distinct genetic lineage of *Mycobacterium tuberculosis*. *J Clin Microbiol* 43, 3185-3191.
- Tsusaki, K., Nishimoto, T., Nakada, T., Kubota, M., Chaen, H., Fukuda, S., Sugimoto, T., Kurimoto, M., 1997. Cloning and sequencing of trehalose synthase gene from *Thermus aquaticus* ATCC33923. *Biochim Biophys Acta* 1334, 28-32.
- Tweddle, N.E., Livingstone, P., 1994. Bovine tuberculosis control and eradication programs in Australia and New Zealand. *Vet Microbiol* 40, 23-39.
- Van Campen, H., Rhyan, J., 2010. The Role of Wildlife in Diseases of Cattle. *Veterinary Clinics of North America: Food Animal Practice* 26, 147-161.
- Van der Zanden, A.G., Hoentjen, A.H., Heilmann, F.G., Weltevreden, E.F., Schouls, L.M., van Embden, J.D., 1998. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin wax embedded tissues and in stained microscopic preparations. *Mol Pathol* 51, 209-214.
- Van Embden, J.D., van Gorkom, T., Kremer, K., Jansen, R., van Der Zeijst, B.A., Schouls, L.M., 2000. Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. *J Bacteriol* 182, 2393-2401.
- Viana-Niero, C., Rodriguez, C.A., Bigi, F., Zanini, M.S., Ferreira-Neto, J.S., Cataldi, A., Leao, S.C., 2006. Identification of an IS6110 insertion site in *plcD*, the unique phospholipase C gene of *Mycobacterium bovis*. *J Med Microbiol* 55, 451-457.
- Warren, R.M., Streicher, E.M., Sampson, S.L., van der Spuy, G.D., Richardson, M., Nguyen, D., Behr, M.A., Victor, T.C., van Helden, P.D., 2002. Microevolution of the direct repeat region of *Mycobacterium tuberculosis*: implications for interpretation of spoligotyping data. *J Clin Microbiol* 40, 4457-4465.
- Wee, S.H., Kim, C.H., More, S.J., Nam, H.M., 2009. *Mycobacterium bovis* in Korea: An update. *Vet J*.
- Wobeser, G., 2009. Bovine tuberculosis in Canadian wildlife: an updated history. *Can Vet J* 50, 1169-1176.
- Zanini, M.S., Moreira, E.C., Salas, C.E., Lopes, M.T., Barouni, A.S., Roxo, E., Telles, M.A., -----
- Zumarraga, M.J., 2005. Molecular typing of *Mycobacterium bovis* isolates from south-east Brazil by spoligotyping and RFLP. *J Vet Med B Infect Dis Vet Public Health* 52, 129-133.
- Zumarraga, M.J., Martin, C., Samper, S., Alito, A., Latini, O., Bigi, F., Roxo, E., Cicuta, M.E., Errico, F., Ramos, M.C., Cataldi, A., van Soolingen, D., Romano, M.I., 1999. Usefulness of spoligotyping in molecular epidemiology of *Mycobacterium bovis*-related infections in South America. *J Clin Microbiol* 37, 296-303.

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 <i>M. bovis</i>	
Definition	Presence of deletion RDEu1 (806 bp in <i>TreY</i>)
Spoligotype marker	Absence of spacer 11
Spoligotype signature ^a	110111110101111011111111111111111111100000 (SB0130)
Most common	110110100000111011111111111111111111100000 (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
IS6110 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.